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TITLE: FIFTH SYMPOSIUM ON SUBTYPES OF MUSCARINIC RECEPTORS

SUBTITLE: Subtypes of Muscarinic Receptors V

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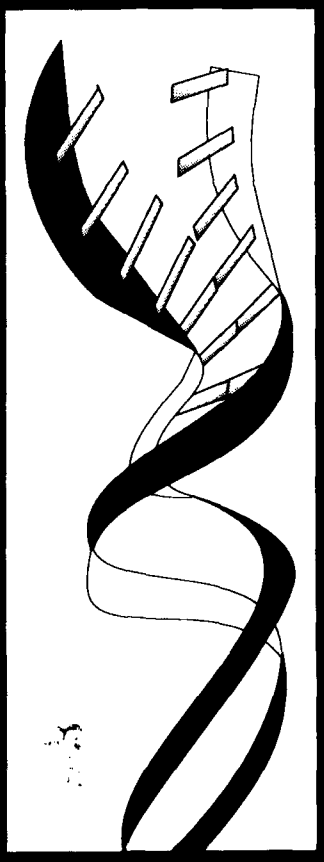

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13. ABSTRACT *(Maximum 200 words)*

Twenty-one papers of 20 minute duration were presented during the 3-day period of the Symposium. Eighteen of these papers and the abstracts of 94 posters have been published in the Proceedings (attached) which appeared as Volume 52, Numbers 5/6, 1993, of Life Sciences. The Fifth Symposium was a pre-meeting satellite of the Society of Neuroscience Meeting.

A total of 200 invited scientists registered and attended the symposium (names and addressees attached). The attendance at each of the sessions was excellent and appeared to include all registrants. The 10 minute discussion period between papers was lively, provocative and informative. The authors of posters manned their posters for their assigned hour on each of two days, but many spent more time at their posters because there was so much enthusiasm for the work being presented. Posters were accessible for the entire period of the Symposium.

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Ten predoctoral and postdoctoral students received support to attend the Symposium; eight of them presented posters.

The Proceedings of the Symposium was sent to all subscribers to Life Sciences and to all active participants in the symposium.

Grant support from the U.S. Army Medical Research and Development Command has been acknowledged in the program distributed to all registrants and in the published Proceedings.

The comments of participants at the Symposium and those received in response to the Proceedings indicate great interest in the program in general and in the specific topics and papers of the Symposium. It appears quite evident that the Fifth Symposium on Subtypes of Muscarinic Receptors has stimulated and is stimulating additional research which should, indeed, lead to the development of new and better therapeutic agents as well as agents useful to the U.S. Army Research and Development Command.

As another mark of the success of the Fifth Symposium, the attendees requested a Sixth Symposium to be held in two years. Planning for another symposium was, therefore, initiated at the meeting and an appropriate site and time will be determined shortly: the site and date selected will again be coordinated with a larger national or international scientific meeting.

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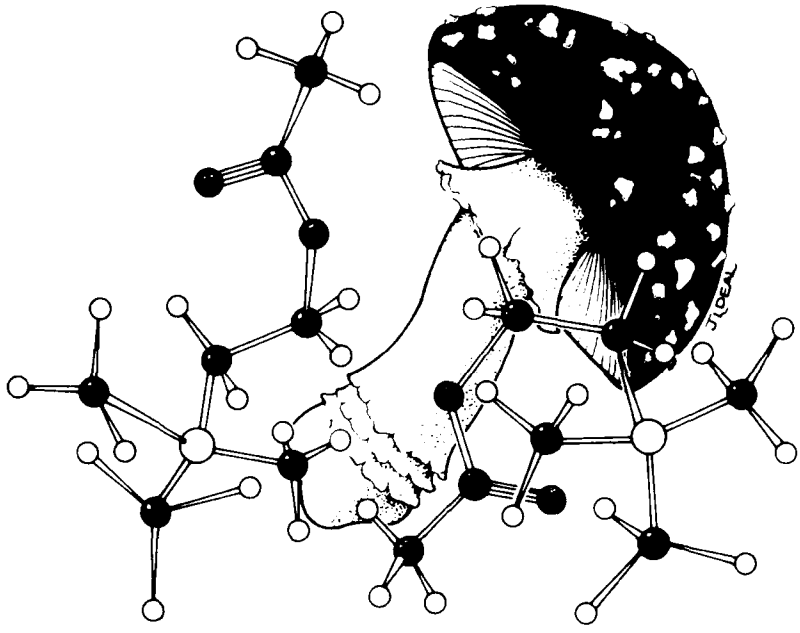
SUBTYPES OF MUSCARINIC RECEPTORS V

Proceedings of the Fifth International Symposium on Subtypes of Muscarinic Receptors



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STRUCTURE/FUNCTION RELATIONSHIPS OF MUSCARINIC ACETYLCHOLINE RECEPTORS

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Summary

The regions of muscarinic receptors that specify G-protein-coupling and ligand-binding have been defined in several recent studies. Overall, these studies have shown that amino acids within the third cytoplasmic loop of the receptors define their selectivity for different G-proteins, and that multiple, discontinuous epitopes contribute to their selectivities for different ligands. In fact, several competitive and allosteric antagonists can be classified into groups based on which of these epitopes contribute to their subtype selectivity. Site-directed mutagenesis, combined with covalent-labeling studies have suggested that ligands bind to a hydrophobic core of the receptors that is formed by multiple transmembrane (TM) domains. An aspartic acid located in TM3 is likely to bind to the ammonium headgroup of muscarinic ligands, and multiple hydroxyl-containing amino acids contribute to agonist but not antagonist binding. These data are discussed in the context of a computational model of a muscarinic receptor. Our model is based on a sequence alignment with bacteriorhodopsin, a seven TM protein for which a higher resolution structure is available. Most of the mutagenic data can be rationalized in the context of this model, and predict testable hypotheses concerning the mechanism by which ligands control the activity of muscarinic receptors.

In the early 1980's muscarinic receptors were purified to apparent homogeneity from both porcine brain (1) and heart (2). Sufficient peptide sequence was obtained from these preparations to allow the cloning of the m1 (3) and m2 (4,5) muscarinic receptor subtypes from these tissues. Using homology cloning, the human and rat forms of these receptors as well as three additional subtypes (m3-m5) were identified (6,7). The sequences of these receptor subtypes were subsequently confirmed in the human (8,9) and defined in several other species (10,11). Comparison of the amino acid sequences of the five muscarinic receptor subtypes indicates that they are derived from a highly conserved gene family. Maximum sequence homology among the members occurs in the seven hydrophobic regions that are predicted to be TM domains. These are the regions where muscarinic receptors have the most sequence homology with other receptors that mediate signal transduction by coupling with G-proteins (12).

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Knowledge of the primary sequences of the muscarinic receptors, together with the availability of receptor clones has inspired many studies attempting to define functional regions within the receptor subtypes. In this paper we discuss data collected using several approaches: (a) the use of chimeric receptors for the identification of the regions of the receptors that are involved in defining their selectivity for G-proteins and ligands. (b) identification of amino acids that are involved in ligand/receptor interactions by use of point mutations and labeling with covalent ligands. (c) computational molecular modeling of the receptors using alignments with bacteriorhodopsin.

Localization of functional epitopes.

Individual muscarinic receptor subtypes can be differentiated based on their selectivities for G-proteins and functional responses. For example, the m1, m3 and m5 receptors selectively couple with a pertussis toxin (PTX) insensitive G-protein to stimulate phospholipase C, while m2 and m4 selectively couple with a PTX-sensitive G-protein that inhibits adenylyl cyclase. The m2 and m4 receptors also weakly stimulate phospholipase C via a PTX sensitive G-protein. These receptors can also be classified based on which ion channels they modulate. Briefly m1, m3 and m5 open Ca^{++}-dependent potassium channels via PTX insensitive G-proteins, while m2 and m4 open nonspecific cation conductances and inwardly rectifying potassium conductances via PTX sensitive G-proteins (see Jones chapter for primary references and 13,14 for reviews).

Chimeric muscarinic receptors have been constructed where individual epitopes have been exchanged between subtypes that differ in their functional selectivities. Chimeras between m1 and m2 receptors were constructed and expressed in oocytes where selective coupling to electrophysiological responses were examined. These studies demonstrated that the third cytoplasmic loop (i3) was sufficient to define functional selectivity for ion channels, while the C-terminus of the receptor did not qualitatively influence electrophysiological responses. In contrast to the i3 loop defining functional selectivity, this region had no effect on the selectivity of these receptors for ligands (15).

Chimeras between m2 and m3 receptors were constructed and expressed in mammalian cells where selective coupling to G-proteins and biochemical responses were examined. These studies confirmed a critical role of the i3 loop in defining functional responses, and the lack of influence of the C-terminus. These studies also demonstrated that within the i3 loop the N-terminal region proximal to TM5 was critical in defining coupling selectivity (16,17,18). The latter results are consistent with data from experiments with deletion mutants. These experiments have shown that only regions proximal to the TM domains are involved in coupling to G-proteins (19). A critical role for the N-terminus of the i3 loop has recently been extended to electrophysiological (20) and calcium responses (21) by expressing very similar m2/m3 chimeras in oocytes. In studies of chimeric beta adrenergic/muscarinic receptors, it was observed that both the N-terminal region of i3 and the i2 loop must be exchanged to reverse the functional phenotype (22).

Muscarinic receptors can be classified based on their differential sensitivity to various ligands. In fact, the first compelling evidence for the existence of multiple muscarinic receptor subtypes came from the demonstration that muscarinic receptors expressed by brain have higher affinity for the antagonist pirenzepine than those expressed by heart (23). Analysis of the affinity profiles of many muscarinic antagonists indicates that they can be divided into families based on their selectivity among the subtypes. For example, trihexyphenidyl, pirenzepine and derivatives such as UH-AH 37 have higher affinity for the m1 and m4 receptor than for the other subtypes.

On the other hand, himbacine, methoctramine, and derivatives of AF-DX 116 have much higher affinity for m2 and m4 than for m5 receptors (24). A similar profile of relative selectivity for the individual subtypes has been observed for the allosteric antagonist gallamine (25). While much less is known about agonist interaction with the receptors, many agonists have higher potency and efficacy at the m2 and m4 receptors (for a review see 14).

To investigate which epitopes within the receptor contribute to the subtype selectivity of ligands, the binding properties of several chimeric m2/m3 and m2/m5 receptors have been investigated. First as indicated earlier, exchange of the i3 loop between m2 and m3 receptors does not influence antagonist affinity, but does reverse the relative affinity for some agonists (18). Analysis of the series of chimeric m2/m5 and m2/m3 receptors has demonstrated that multiple regions contribute the subtype selectivity of several antagonist ligands, and these ligands can be classified based on which regions contribute to binding selectivity (17,26). For example, himbacine and AQ-RA 741 have a very similar binding profile among the chimeras, suggesting that they recognize similar structural epitopes. The high affinity of both of these drugs for m2 receptors is highly dependent on the N-and C-terminal regions. On the other hand, the higher affinity of UH-AH 37 for m5 receptors is largely defined by differences within the TM6 and/or third outer loop, a region that does not influence the binding of the former compounds (27). This same region defines the subtype selectivity of the allosteric antagonist gallamine, in spite of the fact that gallamine and UH-AH 37 have divergent selectivities among the wild-type subtypes. The relative contributions of various domains of the receptors to the selectivity of ligands are illustrated in figure 1.

Amino acids involved in ligand/receptor interactions.

Labeling with covalent ligands and site-directed mutagenesis have been used to identify amino acids within the muscarinic receptors that contribute to ligand binding. ^{3}H-propylbenzilylcholine mustard (PBCM) covalently attaches to muscarinic receptors through its ammonium headgroup. By sequencing fragments of purified muscarinic receptors that have been labeled with this ligand, its primary attachment site has been shown to be an aspartic acid in TM3 (28). Using similar methods, a muscarinic agonist has also been shown to attach to the same amino acid (29). A critical role of this amino acid in ligand binding has been confirmed using site-directed mutagenesis, as substitutions at this position disrupt binding (30). The observation that an ammonium headgroup is a critical feature of most muscarinic ligands (31), suggests that formation of an ionic bond between the ammonium headgroup and the aspartic acid of TM3 may be a general feature of ligand binding to muscarinic receptors.

Examination of the TM domains of the five muscarinic receptors indicates a series of threonine and tyrosine residues which are identical for the five muscarinic receptor subtypes, but are not conserved in the other G-protein coupled receptor sequences. The hypothesis that these amino acids may contribute to binding of muscarinic ligands was tested by substituting phenylalanine and alanine for the tyrosine and threonine residues, respectively. The working hypothesis was that one or more of the hydroxyl groups contributed by one or more of these amino acids would form a hydrogen bond with the ester group of acetylcholine. A tyrosine and a threonine located in TM5 and 6, respectively (the TM's that bound the i3 loop) had the most dramatic effects on decreasing agonist binding and activation of PI metabolism. None of these substitutions had any effect on antagonist binding (32). However, too many of the substitutions selectivly reduced agonist affinity for the results to be interpreted as being due solely to direct interactions between the ligand and the individual residues (eg. hydrogen bonding to the ester region).

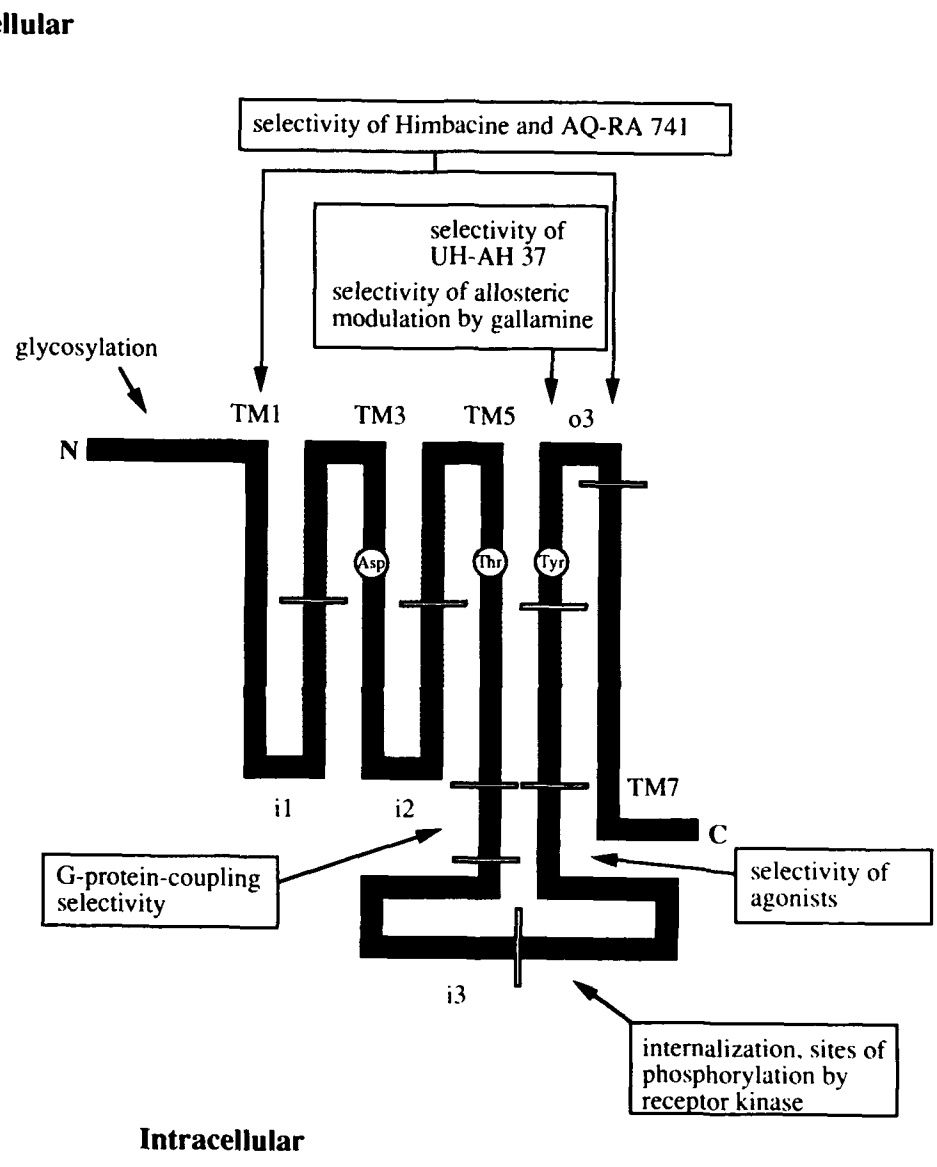

Figure 1. Functional domains of muscarinic acetylcholine receptor subtypes. The amino acids that strongly influence agonist binding are marked: Asp of TM3, Thr of TM5, and Tyr of TM6. Hollow lines indicate the approximate positions of the junctions between the chimeric m2/m3 and m2/m5 receptors used in these studies. Shaded box indicates the relative position of the membrane.

Computational molecular models.

Since both the ligand-binding and G-protein-coupling regions of muscarinic receptors involve TM domains and regions proximal to them, molecular models must be based on proteins for which the structures of TM domains have been resolved at high resolution. Unfortunately, there are no such structures available for any of the G-protein-coupled receptors, in fact very few TM-containing proteins have been resolved with the requisite molecular resolution.. Fortunately, one of the TM-containing proteins that has been resolved at high resolution, bacteriorhodopsin, has many structural and functional features that make it a promising model for the G-protein-coupled receptors (34). While bacteriorhodopsin is not a G-protein-coupled receptor, it has is an integral membrane bound protein that has seven TM domains. Also, like eukoryotic opsins and most of the G-protein-coupled receptors, bacteriorhodopsin binds a small organic molecule (11-cis-retinal for eukaryotic opsins and all-trans-retinal for bacteriorhodopsin) to its hydrophobic core. For all of the opsins absorption of light leads to isomerization of the bound retinal, which in turn leads to a conformational change in the structure of the protein (34, 35).

In spite of the obvious functional and structural similarities between eukaryotic opsins and bacteriorhododopsin, there is very little primary sequence homology between these proteins (34,35). In all cases, the retinal molecule covalently attaches to a alanine-lysine motif present in TM7. Also, eukaryotic opsins and sensory rhodopsin 1 of halobium have aromatic amino acids in TM3 and TM6. The latter protein functions as a light sensor in halobium, has extensive sequence homology with bacteriorhodopsin, but is more like eukaryotic opsins in that it functions as a light sensor (the function of bacteriorhodopsin in halobium is to convert light into energy by pumping protons) and mediates signal transduction by coupling with cytoplasmic proteins (36). Overall, these data strongly suggest that the eukaryotic- and bacterio-opsins are highly homologous regarding their structure/function relationships.

We have used a sequence alignment between bacteriorhodopsin and the m1 muscarinic receptor that was based on sequence homologies and a hydrophobic analysis of the G-protein-coupled receptor family (37). The computational model was constructed within the context of the molecular mechanics force field CHARMm (38,39), using the protein homology modeling facilities in version 3.2 of the modeling program QUANTA (39). The alignment and the available coordinates for bacteriorhodopsin (34) was used to construct the seven TM helices and to provide an initial packing arrangement. With the positions of the backbone atoms of the TM helices fixed in space to maintain their original packing, the side chain (and loop region) positions were refined using a series of energy minimization and molecular dynamics simulations. Further energy minimization was used to relax the whole complex. All energy minimization and molecular dynamics simulations were performed using version 21.3 of the program CHARMm running on the Eli Lilly Cray-2/2S128 supercomputer. S-aceclidine, a structurally rigid acetylcholine analog, was docked into the interior of the structure. It was placed such that the interaction of the cationic amino group with the carboxylic acid group of the TM3 Asp was optimal (31) and the subsequent complex was further energy minimized.

As illustrated in figure 2, most of the point mutations that have been tested for the muscarinic receptors can be rationalized in the context of this model. For example, the amino acid substitutions which did not affect agonist binding (TM2 serine, TM6 threonine and TM7 threonine) are all predicted to be distant from the proposed ligand position. All of the tyrosine residues that affected binding are clustered around the TM3 aspartate to potentially provide hydrogen bonding

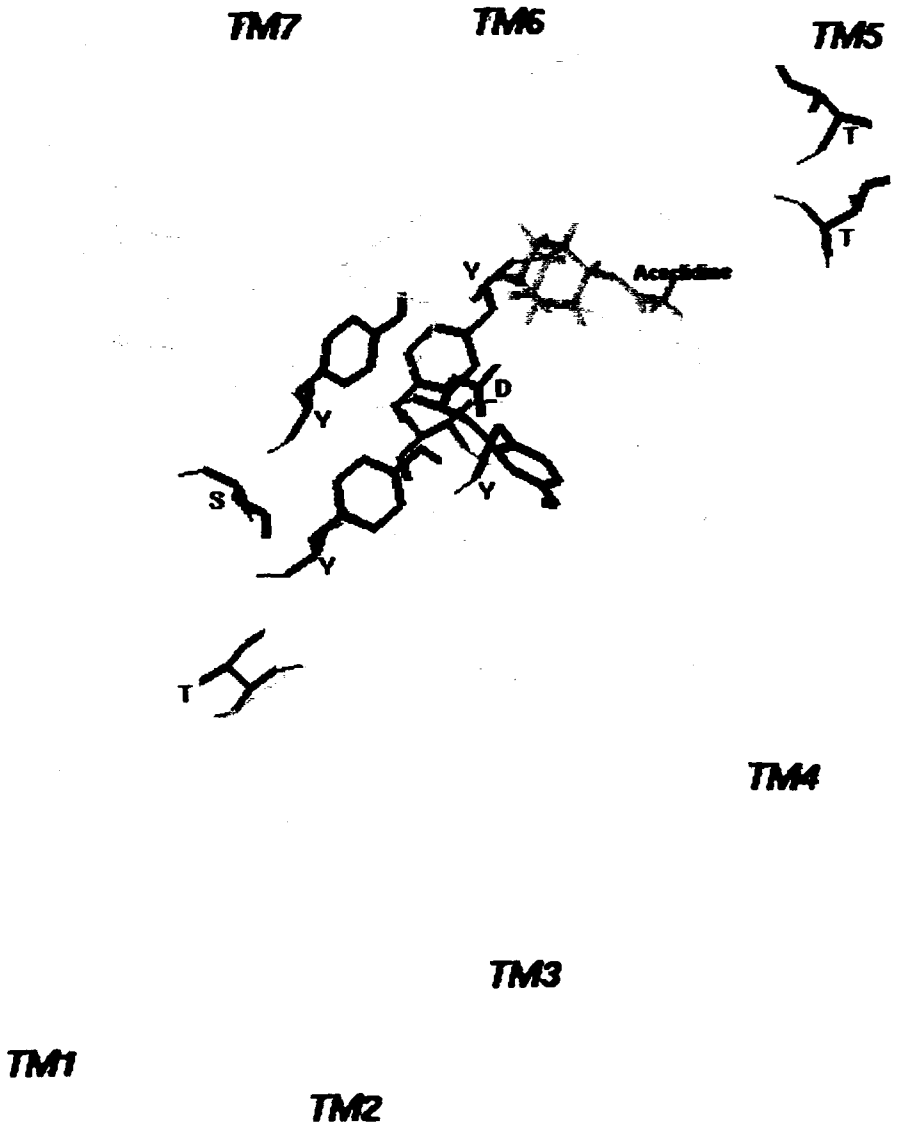

Figure 2. Computational model of a muscarinic acetylcholine receptor. The illustrated model is based on the structure of bacteriorhodopsin. The alpha-carbon trace is illustrated for the TM domains. The muscarinic agonist aceclidine is docked within the structure for optimal interaction with the Asp (D) of TM3. The Tyr's (Y) that influence agonist binding are shown to be clustered around the Asp (D) of TM3. The Ser (S) of TM 2 and the Thr (T) of TM6 (these residues do not influence agonist binding) are shown to be distal from the predicted binding site. The two Thr's (T) of TM5 that influence agonist binding are in the predicted plane of ligand binding, but face away from the predicted binding site.

stabilization for the interacting carboxylic acid group. They also should provide a hydrophobic barrier to isolate the ionic interaction taking place with the ligand. The tyrosine of TM3 extends into the lipid region. This could mean that further positioning of TM3 (and possibly TM4) is needed to either bring this residue into the interior of the protein or to allow it to hydrogen bond with some site on TM4. The threonines of TM5 are near the ester end of the ligand, but their position in the model is not at all optimal for interacting with the ester portion of the ligand, and point to the need for further refining the structure and possibly also the sequence alignment. Since they appear very near the extracellular side, they could alternatively be involved in guiding the ligand to the binding site. On the other hand, it has been noted (37) an asparagine in TM6 is ideally located for interaction with the ester region of the ligand. Overall, the ability to rationalize the phenotypes of the majority of tested mutations, provides a compelling case for the structural similarities between bacteriorhodopsin and the m1 muscarinic receptor, and reinforces the potential utility of these models in rational drug design.

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References.

1. K. HAGA, T. HAGA, J. Biol. Chem. 258 13575-13579 (1983).
2. G.L. PETERSON, G.S. HERRON, M. YAMAKI, D.S. FULLERTOON, M.J. SCHIMERLIK, Proc. Natl. Acad. Sci. USA 81 4993-4997 (1984).
3. T. KUBO, K. FUKUDA, A. MIKAMI, A. MAEDA, H. TAKAHASHI, T. MISHINA, K. HAGA, A. HAGA, A. ICHIYAMA, K. KANAGAWA, M. KOJIMA, H. MATSUO, T. HIROSE, S. NUMA, Nature 323 411-416 (1986).
4. T. KUBO, A. MAEDA, K. SUGIMOTO, I. AKIBA, A. MIKAMI, T. TAKAHASI, K. HAGA,A. HAGA, A. ICHIYAMA, K. KANAGAWA, H. MATSUO, T. HIROSE, S. NUMA, FEBS Lett. 209 367-372 (1986).
5. E.G. PERALTA, A. ASHKENAZI, J.W. WINSLOW, J. RAMACHANDRAN, D.J. CAPON, Science 236 600-605 (1987).
6. T.I. BONNER, N.J. BUCKLEY, A.C. YOUNG, M.R. BRANN, Science 237 527-532 (1987).
7. T.I. BONNER, A. YOUNG, M.R. BRANN, N.J. BUCKLEY, Neuron 1 403-410 (1988).
8. E.G. PERALTA, J.W. WINSLOW, G.L. PETERSON, D.H. SMITH, A. ASHKENAZI, J. RAMACHANDRAN, M.I.. SCHIMERLIK, D.J. CAPON, EMBO J. 6 3923-3929 (1987).
9. C-F. LIAO, A.P.N. THEMMEN, R. JOHO, C. BARBERIS, M. BIRNBAUMER, L. BIRNBAUMER, J. Biol. Chem. 264 7328-7337 (1989).
10. R.A. SHAPIRO, B.T. WAKIMOR, E.M. SUBERS, N.M. NATHANSON, Proc. Natl. Acad. Sci. USA 86 9039-9043 (1989).
11. K.M. TIETJE, P.S. GOLDMAN, N.M. NATHANSON, J. Biol. Chem. 265 2828-2834 (1990).
12. Molecular Biology of G-Protein-Coupled Receptors, M.R. Brann (ed), Birkhauser, Boston (1992).
13. E.C. HULME, N.J.M. BIRDSALL, N.J. BUCKLEY, Annu. Rev. Pharmacol. Toxicol. 30 633-673 (1990).

14. S.V.P. JONES, A.I. LEVEY, D.M. WEINER, J. ELLIS, E. NOVOTNY, S-H. YU, F. DORJE, J. WESS, M.R. BRANN, Molecular Biology of G-protein-coupled receptors, M.R. Brann (ed),170-197 Birkhauser, Boston (1992).
15. T. KUBO, H. BUJO, I. AKIBA, J. NAKAI, M. MISHINA, S. NUMA, FEBS Lett 241 119-125 (1988).
16. J. WESS, M.R. BRANN, T.I. BONNER, FEBS Lett 258 133-136 (1989).
17. J. WESS, T.I. BONNER, M.R. BRANN, Mol. Pharm. 38 872-877 (1990).
18. J. WESS, T.I. BONNER, F. DORJE, M.R. BRANN, Mol. Pharm. 38 517-523 (1990).
19. R.A. SHAPIRO, N.M. NATHANSON, Biochemistry 28 8946-8950 (1989).
20. J. LECHLEITER, S. GIRARD, D. CLAPMAN, E. PERALTA, Nature 350 505-508 (1991).
21. J. LECHLEITER, R. HELLMISS, K. DUERSON, D. ENNULAT, N. D., CLAPHAM, E. PERALTA, EMBO J. 9 4381-4390 (1991).
22. S.K.F. WONG, E.M. PARKER, E.M. ROSS, J. Biol. Chem. 265 6219-6224 (1990).
23. R. HAMMER, C.P. BERRIE, N.J.M. BIRDSALL, A.S.V. BURGEN, E.C. HULME, Nature 283 90-92 (1980).
24. R. DORJE, J. WESS, G. LAMBRECHT, E. MUTSCHLER, M.R. BRANN, J. Pharmacol. Exp. Ther 256 727-733 (1991).
25. J. ELLIS, J. HUYLER, M.R. BRANN, Biochem. Pharmacol. 42 1927-1932 (1991).
26. J. WESS, D. GDULA, M.R. BRANN, Mol. Pharm. 41 369-374 (1991).
27. J. ELLIS, M. SEIDENBERG, J. WESS, M.R. BRANN, Soc. Neurosci. Abs. (1992).
28. C.A.M. CURTIS, M. WHEATLEY, S. BANSAL, N.J.M. BIRDSALL, P. EVELEIGH, E.K. PEDDER, D. POYNER, E.C. HULME, J. Biol. Chem. 264 489-495 (1989).
29. N.J.M. BIRDSALL, T.A. SPALDING, J.E.T. CORRE, C.A.M. CURTIS, E.C. HULME. This meeting.
30. C.M. FRASER, C.D. WANG, D.A. ROBINSON, J.D. GOCAYNE, J.C. VENTER, Mol. Pharm. 36 840-847 (1989).
31. J.M. SCHULMAN, M.L. SABIO, R.L. DISCH, J. Med. Chem. 26 817-823 (1983).
32. J. WESS, D. GDULA, M.R. BRANN, EMBO J. 10 3729-3734 (1991).
34. R. HENDERSON, J.M. BALDWIN, T.A. CESKA, F. ZEMLIN, E. BECKMANN, K.H. DOWNING, Mol. Biol. 213 899-493 (1990).
35. N.J.P. RYBA, M.D. HALL, J.B.C. FINDLAY, Molecular Biology of G-protein-coupled receptors, M.R. Brann (ed),1-30, Birkhauser, Boston (1992).
36. A. BLANCK, D. OESTERHELT, E. FERRANDO, E.S. SCHEGK, F. LOTTSPEICH, EMBO J. 8 3963-3971 (1989).
37. M.F. HIBERT, S. TRUMPP-KALLMEYER, A. BRUINVELS, J. HOFLACK, Mol. Pharm. 40 8-15 (1991).
38. B.R. BROOKS, R.D. BRUCCOLERI, B.O. OLAFSON, D.J. STATES, S. SWAMINATHAN, M. KARPLUS, J. Comp. Chem. 4 187-217 (1983).
39. Molecular Simulations Incorporated (formerly Polygen corporation), 200 Fifth Avenue, Waltham, MA, 02154.

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REGULATION OF THE M1 MUSCARINIC RECEPTOR-G_q-PHOSPHOLIPASE C-β PATHWAY BY NUCLEOTIDE EXCHANGE AND GTP HYDROLYSIS

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Summary

M1 muscarinic cholinergic receptors, G_q and G_{11} ($G_{q/11}$), and phospholipase C-β1 were highly purified from both natural sources and cells that express the appropriate cDNA's. When the proteins were co-reconstituted into phospholipid vesicles, the receptor efficiently and selectively promoted the activation of $G_{q/11}$, leading to marked stimulation of PLC activity in the presence of GTPγS. No stimulation was observed in the presence of GTP, however, which led to the finding that PLC-β1 stimulates the hydrolysis of $G_{q/11}$-bound GTP at least 50-fold. Thus, PLC-β1 is a GTPase activating protein, a GAP, for its physiologic regulator $G_{q/11}$. We discuss the implications of PLC-β1's GAP activity on the M1 muscarinic cholinergic signaling pathway.

Muscarinic cholinergic receptors regulate the function of target cells through a multitude of second messenger pathways: release of IP_3 to elevate Ca^{2+} levels, activation of K^+ channels, inhibition and (probably indirect) activation of adenylyl cyclase, etc. All effects of muscarinic receptors are presumably modulated through the action of a family of GTP-binding regulatory proteins known as G proteins. The diversity of these signals derives from the receptors' selective use of different G proteins to regulate distinct cytoplasmic effectors, enzymes or channels that synthesize, degrade or release second messengers. This pattern of specificity was best clarified for the M1 and M2 receptors by the work of Ashkenazi and Peralta (1,2), who demonstrated that M1 receptors utilize both a pertussis toxin-insensitive G protein and a toxin-sensitive G protein, probably a G_i, to activate a phosphatidylinositol-diphosphate-specific phospholipase C (PLC). In contrast, they showed that M2 receptors act through at least two toxin-sensitive G proteins, probably both G_i's, to activate PLC and to inhibit adenylyl cyclase. Confirmatory studies are reviewed in ref. 3 and discussed elsewhere in this issue.

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When we initially expressed and purified recombinant M1 and M2 muscarinic receptors using baculovirus vectors, we found the M2 receptor efficiently activates the G_i's, G_o and G_z (4). Activation of G_z was unexpected, but may explain certain toxin-insensitive effects of the M2 receptor. The M1 receptor, in contrast, regulated none of these three groups of G proteins, nor did it regulate G_s. It should be remembered that coupling of a receptor to a G protein that it would not normally regulate in vivo can be driven either by reconstituting the G protein with high concentrations of the receptor or by vast overexpression of the receptor in transfected cells. This potential derives from the homology shared within the receptor and G protein families. However, the results referred to here clearly showed that the M2 receptors display a marked selectivity for G_i's, G_o, and G_z and that the M1 receptors, where effective at all, produced trivially small effects on these G proteins even at high concentrations.

The G_q family, initially identified by cDNA cloning in Simon's laboratory (5,6), were likely candidates for mediators of the M1 signaling pathway. They are pertussis toxin-insensitive, and both G_q and G_{11} were shown by the groups of Sternweis and Exton to stimulate PLC-β1 (7,8). Consequently, we initiated collaborative studies with these groups and with Sue Goo Rhee and co-workers to test the ability of the M1 muscarinic receptor to regulate PLC-β1 through the G_q family of G proteins. Our findings indicated that G_q can mediate M1 stimulation of PLC-β's and led to the unexpected finding that PLC-β substantially increases the GTPase activity of G_q. Details of these studies are provided elsewhere (9,10); some of their implications are discussed below.

Methods

The basic experimental system consists of reconstituted lipid vesicles that contain human M1 muscarinic receptor purified from baculovirus-infected Sf9 cells (4) and $G_{q/11}$, an approximately equimolar mixture of G_q and G_{11} purified from either bovine brain (7) or liver (11). Key results have now been confirmed with recombinant G_q purified from Sf9 cells, a gift of Dr. John Hepler in this department. PLC-β1, purified either from bovine brain (12,13) or from HeLa cells infected with recombinant vaccinia virus (14), was added to the vesicles after reconstitution. All relevant procedures have been described in detail elsewhere (9,10).

Results and Discussion

G proteins are activated by the binding of GTP such that they can in turn activate cellular effector proteins such as PLC. Deactivation of the G protein and consequent termination of signaling occurs when bound GTP is hydrolyzed to GDP. Receptors act by catalyzing the exchange of bound GDP for GTP (see Fig. 1, ref. 15). Purified G_q is unusual because its functional activation by GTP or GTPγS is difficult to demonstrate. For unknown reasons, functional activation by GTPγS is significant only

after prolonged incubation with unusually high GTPγS concentrations and the binding of radiolabeled GTP or GTPγS to isolated G_q has not been quantitated. In fact, initial identification of G_q as a PLC activator depended on its activation by F^- plus Al^{+3} (7,8).

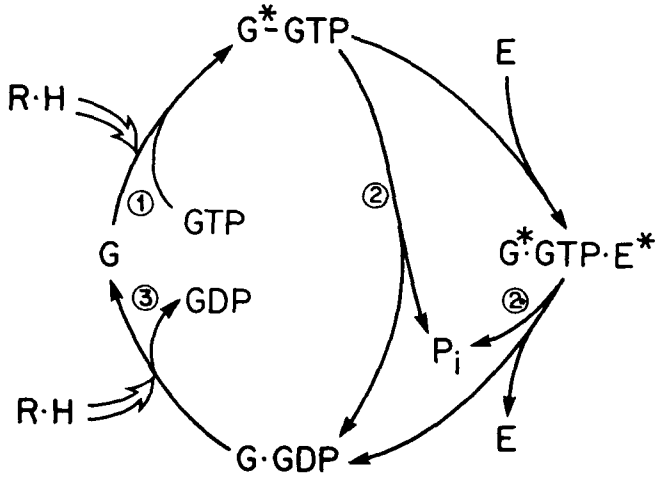

FIG. 1
A G protein (G) is activated by GTP binding (reaction 1) such that it can bind and activate an effector (E). Activation is terminated by GTP hydrolysis (reaction 2). For G_q bound to PLC-β1, hydrolysis is accelerated at least 50-fold (reaction 2•). Reactivation, the release of bound GDP and binding of GTP (reactions 3 and 1), is catalyzed by receptors. From ref. 15.

In initial experiments using reconstituted M1 receptor-G_q vesicles, we found that agonist-liganded M1 muscarinic receptor efficiently catalyzed the activation of G_q by GTPγS. When we compared activation rates in vesicles that contained no receptor, M1 receptor, or M2 receptor, we found that even the unliganded M1 receptor significantly accelerates G_q activation (Fig. 2). As expected, GTPγS bound G_q stimulated PLC-β1 activity at least 40-fold. The agonist-liganded M2 receptor stimulated GTPγS binding to G_q only slightly, at about the same rate as did the unliganded or antagonist-liganded M1 receptor. This finding is consistent with the predominately pertussis toxin-sensitive action of M2 receptors in cells (see ref. 3 or other articles in this issue).

G protein-mediated signaling <u>in vivo</u> is determined both by the rate of receptor-catalyzed GDP/GTP exchange and by the rate of hydrolysis of bound GTP. The M1 receptor efficiently catalyzed both the binding of GTP and the dissociation of GDP by G_q, which resulted in substantial (greater than 10-fold) stimulation of the steady-state GTPase activity. Because G_q hydrolyzes bound GTP slowly, with a $t_{1/2}$ of about 1 min, receptor-catalyzed

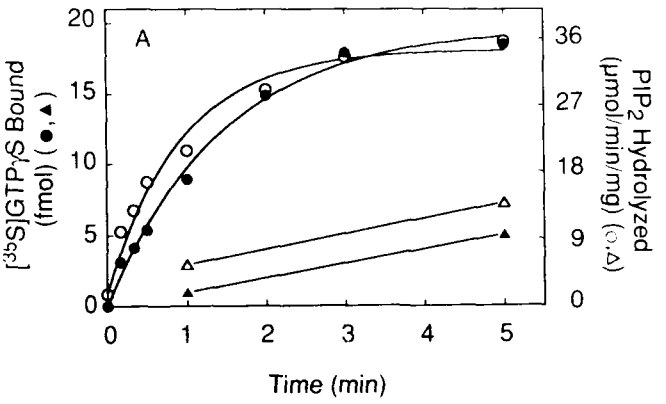

FIG. 2
Binding of GTPγS to $G_{q/11}$ and functional activation of $G_{q/11}$ is stimulated by the M1 muscarinic receptor. The binding of GTPγS to vesicles that contain purified receptor and $G_{q/11}$ was measured in the presence of carbachol (●,○) or atropine (▲,△). Binding was measured either directly using [^{35}S]GTPγS (●,▲) or functionally according to the ability of GTPγS-liganded $G_{q/11}$ to activate PLC-β1 (○,△). From ref. 9.

GTP/GDP exchange results in the steady-state accumulation of a relatively large amount of the activated species, G_q-GTP. We then used GTPγS-activated $G_{q,11}$ to estimate how much GTP-liganded G_q should be necessary to observe a given level of PLC-β1 activation in vesicles that contain receptor, G_q and PLC. Our calculations suggested that carbachol should substantially activate PLC-β1 in the presence of GTP. However, we were unable to demonstrate GTP-supported PLC stimulation by carbachol in the receptor-G_q-PLC vesicles. Failure to obtain an expected result in a complex reconstituted system is always treacherous to interpret, but a possible explanation was that PLC was stimulating the rate of hydrolysis of G_q-bound GTP, such that our initial estimates of its steady-state accumulation would be in error. This provocative explanation turned out to be correct.

As shown in Fig. 3, PLC-β1 stimulates the steady-state GTPase activity of M1 receptor-G_q vesicles up to 20-fold. Numerous control experiments indicate that PLC-β1 regulates G_q directly, including antibody blockade experiments in which monoclonal antibodies against PLC-β1 blocked both its GAP activity and its stimulation by activated G_q with the same rank order of potency. Thus, as documented more fully elsewhere (10), PLC-β1 acts as a GTPase activating protein, or GAP, for its physiologic regulator G_q. The EC_{50} for PLC-β1 as a G_q GAP is 1-2 nM, about equal to the EC_{50} with which GTPγS-activated G_q stimulates PLC-β1. This value may thus be the K_d for the binding of the two proteins to each other. The GAP activity of PLC-β1 is specific for G_q; it had no effect on G_s, G_i, or G_o.

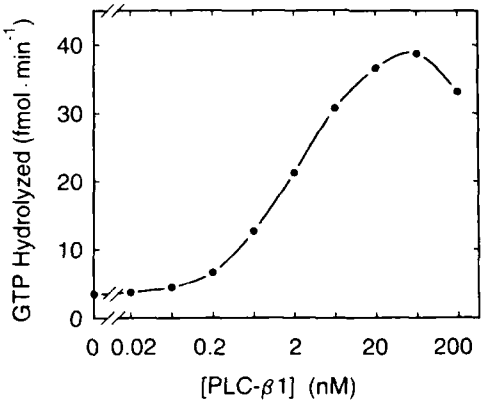

FIG. 3
PLC-β1 stimulates the GTPase activity of $G_{q/11}$. The steady-state GTPase activity of M1 receptor-$G_{q/11}$ vesicles was measured in the presence of 1 mM carbachol and increasing concentrations of purified, recombinant PLC-β1. From ref. 10.

The finding that PLC is a GAP for G_q (10) and the simultaneous finding that the retinal cyclic GMP phosphodiesterase is a GAP for transducin (16) are the first demonstrations of the existence of GAP's for trimeric G proteins. At least in the case of the G_q GAP activity of PLC-β1, the effect appears to be primarily a stimulation of the rate of hydrolysis of bound GTP. Thus, the stimulation of steady-state GTPase activity of PLC-β1 is dependent on receptor-catalyzed GTP/GDP exchange reaction because basal exchange rates are quite low. In addition, when we attempted to measure the rate of hydrolysis of bound GTP directly, we found that a substantial (20-40%) fraction of bound GTP was hydrolyzed extremely rapidly, at least 50-fold faster than the rate observed in the absence of PLC. The G_q GAP activity thus appears similar to the activity expressed by GAPs for the small, monomeric GTP-binding proteins such as ras, rho and rac.

It is likely that many G protein-regulated effectors are also GAP's for their respective G protein activators. The retinal phosphodiesterase displays weak transducin GAP activity (16), and comparison of the in vivo deactivation rates of G protein-gated channels with in vitro GTP hydrolysis rates suggests that the channels will also be GAPs (17). In contrast, no G_s GAP activity was observed for adenylyl cyclase in early reconstitution experiments (18), but G_s and cyclase were reconstituted at a molar ratio that probably would have masked any GAP activity. It is of course possible that adenylyl cyclase, or some cyclase isoforms, have no G_s GAP activity. The rate of hydrolysis of G_s-bound GTP is roughly equivalent to the rate of deactivation of adenylyl cyclase upon removal of agonist (see 19 and refs. therein). In contrast, we predict that the G_s-gated Ca^{2+} channel will be an excellent G_s GAP because it is very rapidly deactivated.

Using G protein-regulated effectors as GAP's for their specific G protein regulators is an attractive regulatory mechanism. It allows different effectors to display distinctive temporal patterns of signaling. Because GTP hydrolysis is a major determinant of a signal's amplitude, it also allows effectors to display diversity in the intensity of their responses to stimulation by a single class of G protein. It assigns a unique quantal output to each effector. Thus, a single G protein could activate multiple effectors with uniquely appropriate time constants and/or efficiencies. Such individuality of signaling can be enhanced further if allosteric or covalent modification of the effector alters its GAP activity.

The GAP activity of effectors may also enhance the specificity with which a single receptor directs its signals to different G protein-effector pathways. Preliminary kinetic studies of the M1 muscarinic receptor-G_q-PLC-β1 system suggests that the relevant signal processor may be a receptor-G_q-effector complex. This complex should be stable only in the presence of agonist and GTP and probably has a lifetime of perhaps 10-50 catalytic cycles. Formation of this complex has the capacity fine-tune the ability of a receptor to recognize different, closely related G proteins according to its abilities to form the complex. This idea would also explain why receptors that appear to be quite selective for a specific signaling pathway in cells are far less specific for their relevant G proteins <u>in vitro</u>. Preliminary observations indicate that different receptors that seem to stimulate the activation of G_q with nearly identical efficiencies can differ dramatically when PLC-β1 is present.

Last, the finding that effectors are GAPs for their G protein regulators prompts a reevaluation of the role of previously discovered GAP's for small GTP-binding proteins, such as $p21^{ras}$. When McCormick and co-workers discovered ras GAP, they proposed that it may be the effector protein through which ras acts (see 20). This proposal has remained controversial. However, the effectors through which the small GTP-binding proteins act are not known. Analogy with trimeric G proteins argues that the GAP's, once they are assigned to their appropriate G proteins, may be excellent candidates for the still undiscovered effectors in the signaling pathways.

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References

1. A. ASHKENAZI, E.G. PERALTA, J.W. WINSLOW, J. RAMACHANDRAN and D. J. CAPON, Cell 56 487-493 (1989).
2. A. ASHKENAZI, J.W. WINSLOW, E.G. PERALTA, G.L. PETERSON, M.I. SCHIMERLIK, D.J. CAPON and J. RAMACHANDRAN, Science 238 672-675. (1987).
3. E.C. HULME, N.J.M. BIRDSALL and N.J. BUCKLEY, Annu. Rev. Pharmacol. Toxicol. 30 633-673 (1991).
4. E.M. PARKER, K. KAMEYAMA, T. HIGASHIJIMA and E.M. ROSS, J. Biol. Chem. 266 519-527 (1991).
5. M. STRATHMAN and M.I. SIMON, Proc. Natl. Acad. Sci.USA 87 9113-9117 (1990).
6. M. STRATHMAN, T.M. WILKIE and M.I. SIMON, Proc. Natl. Acad. Sci. USA 86 7407-7409 (1989).
7. A.V. SMRCKA, J.R. HEPLER, K.O. BROWN and P.C. STERNWEIS, Science 251 804-807 (1991).
8. S.J. TAYLOR, H.Z. CHAE, S.G. RHEE and J.H. EXTON, Nature 350 516-518 (1991).
9. G. BERSTEIN, J.L. BLANK, A.V. SMRCKA, T. HIGASHIJIMA, P.C. STERNWEIS, J.H. EXTON and E.M. ROSS, J. Biol. Chem. 267 8081-8088. (1992).
10. G. BERSTEIN, J.L. BLANK, D-Y. JHON, J.H. EXTON, S.G. RHEE and E.M. ROSS, Cell 70 411-418 (1992).
11. J.L. BLANK, A. H. ROSS and J. H. EXTON, J. Biol. Chem. 266 18206-18216 (1991).
12. S.H. RYU, K.S. CHO, K-Y. LEE, P-G. SUH and S.G. RHEE, J. Biol. Chem. 262 12511-12518 (1987).
13. S.H. RYU, P-G. SUH, K.S. CHO, K-Y. LEE and S.G. RHEE, Proc. Natl. Acad. Sci. USA 84 6649-6653 (1987).
14. D. PARK, D-Y. JHON, R. KRIZ, J. KNOPF and S.G. RHEE, J. Biol. Chem. 267 16048-16055 (1992).
15. E.M. ROSS, Neuron 3 141-152 (1989).
16. V.Y. ARSHAVSKY and M.D. BOWNDS, Nature 357 416-417 (1992).
17. G.E. BREITWIESER and G. SZABO, Nature 317 538-540 (1985).
18. D.C. MAY, E.M. ROSS, A.G. GILMAN and M.D. SMIGEL, J. Biol. Chem. 260 15829-15833 (1985).
19. D.R. BRANDT, T. ASANO, S.E. PEDERSEN and E.M. ROSS, Biochemistry 22 4357-4362 (1983).
20. MCCORMICK, F., Cell 56 5-8 (1989).

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PHOSPHORYLATION OF MUSCARINIC RECEPTORS: REGULATION BY G PROTEINS

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Summary

Effects of G proteins on the phosphorylation of muscarinic receptors (mAChRs) have been examined. Cerebral but not atrial mAChRs were phosphorylated by any one of three types of protein kinase C and 4-6 mol of phosphate were incorporated per mol of mAChR, mostly in the 12-14 kDa from the carboxyterminus. Atrial mAChRs were better substrates of cAMP-dependent protein kinase than cerebral mAChRs. Phosphorylation of mAChRs by protein kinase C or cAMP-dependent protein kinase was not dependent on the presence of agonists and G proteins except that a slight inhibition by G proteins was observed probably because G proteins were also substrates of the two kinases. Agonist-dependent phosphorylation of atrial mAChRs or recombinant human mAChRs (m2 subtype) by a kinase (mAChR kinase), which is the same or very similar to β adrenergic receptor kinase (βARK), was found to be regulated by the G proteins in a dual manner; stimulation by G protein βγ subunits and inhibition by G protein αβγ trimer. The inhibition by the G protein trimer is restored by addition of guanine nucleotides and is considered to be due to the formation of a ternary complex of agonist, mAChR and guanine nucleotide free G proteins. The stimulation by G protein βγ subunits was also observed for the light- or agonist-dependent phosphorylation of rhodopsin and βAR by the mAChR kinase but not for the light-dependent phosphorylation of rhodopsin by rhodopsin kinase. The phosphorylation by βARK i was also found to be stimulated by G protein βγ subunits. The βγ subunit is considered to interact with the extra 130 amino acid residue carboxyterminal tail of βARK, which does not exist in rhodopsin kinase, and the interaction results in the activation of the kinase. We may assume that the G protein coupled receptor kinase is an effector of G protein βγ subunits and that one of the functions of βγ subunits is to stimulate the phosphorylation of G protein coupled receptors thereby facilitating their desensitization.

Muscarinic receptors (mAChRs) as well as the other G protein coupled receptors are known to be desensitized following prolonged exposure to agonists. Phosphorylation of receptors is generally believed to be involved in the desensitization, although the detailed molecular mechanism of the desensitization still remains to be elucidated (1-3). We have examined the phosphorylation of purified mAChRs by several different protein kinases, particularly the effect of G proteins on the phosphorylation.

Phosphorylation of mAChRs by protein kinase C and cAMP-dependent protein kinase

We have examined the phosphorylation of mAChRs purified from porcine cerebrum or atrium. Cerebral mAChRs have been shown to be phosphorylated by protein kinase C, which was partially purified from porcine brain (4). Furthermore, cerebral mAChRs were found to be phosphorylated by any one of three types of protein kinase C, types I, II and III, which correspond to the γ, β, and α

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species respectively. The extent of phosphorylation was similar among the three types and 3.8-5.8 mol of phosphate were incorporated per mol of cerebral mAChRs.

mAChRs purified from porcine atrium were not phosphorylated by any of the three types of protein kinase C under exactly the same conditions, consistent with the previous results obtained by using a mixture of different types of protein kinase C (4,5). This indicates that the m2 subtype is not a good substrate of protein kinase C because the atrial mAChR is considered to be composed of only the m2 subtype. mAChRs purified from porcine cerebrum may contain multiple subtypes: among five major peptides obtained by partial hydrolysis of purified mAChRs, three peptides were found to be derived from m1 and two from m2, the yields of m1 peptides being greater than those of the m2 peptides. In addition, approximately 80% of mAChRs purified from cerebrum showed a 30- to 40-fold higher affinity for pirenzepine than the rest of mAChRs when the purified mAChRs were reinserted into membranes. The atrial mAChRs treated in the same way showed only a low affinity for pirenzepine. These results suggest that a major component of purified cerebral mAChRs is the m1 subtype and the m1 subtype is a substrate of protein kinase C. In fact, the human m1 subtype expressed in Sf9 cells using the baculovirus system was found to be phosphorylated by protein kinase C (Kameyama et al., unpublished results). Cerebral mAChRs may contain m3, m4 and m5, although we could not detect any peptides derived from these subtypes as one of major five peptides obtained by partial hydrolysis of purified mAChRs. These subtypes, particularly m3 and m5, may be substrates of the protein kinase C because a major phosphorylation site of cerebral mAChRs has been located in the 12-14 kDa peptide of the carboxyterminus (6) and the amino acid sequences of these segments are similar for m1, m3 and m5 and for m2 and m4 but not between the two groups. Richardson et al. reported the phosphorylation of chick cardiac mAChRs (m2, m4 or both) by protein kinase C (7,8). It is not known if this is due to differences in the species or in the experimental conditions.

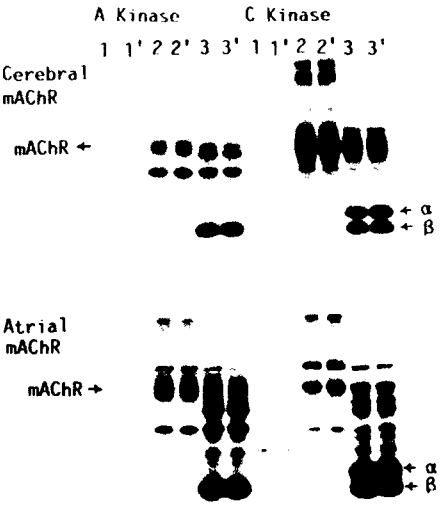

Fig. 1

Phosphorylation of cerebral and atrial mAChRs by cAMP-dependent protein kinase (A Kinase) and protein kinase C (C Kinase). mAChRs purified from porcine cerebrum or atrium were subjected to phosphorylation by A kinase or C Kinase in a soluble state (1,1'), in phospholipid vesicles (2,2'), or in phospholipid vesicles with G proteins (Go)(3,3'), in the presence (1,2,3) or absence (1',2',3') of acetylcholine.

Both the rate and extent of phosphorylation by protein kinase C were not affected by the presence or absence of muscarinic agonists for both porcine cerebral (4) and chick cardiac mAChRs (7). The extent of phosphorylation of porcine cerebral mAChRs was slightly decreased by reconstituting mAChRs with G proteins in phospholipid vesicles (Fig. 1). The extent of decrease was not appreciably affected by addition of guanine nucleotides, which are known to dissociate the receptor-G protein complex. Thus this decrease is not likely to be related to the formation of receptor-G protein complex but is likely to be due to the competitive inhibition of the kinase. The protein kinase C phosphorylates α and β subunits of Go, Gi (Gi1), and Gn (Gi2) and γ subunits of Gi and Go (9). The phosphorylation of G proteins was also not affected by addition of GTPγS. The phosphorylation of chick cardiac mAChRs, on the other hand, is reported to be inhibited by G proteins (Go) in a GTPγS-dependent manner (8).

Porcine atrial (5) and cerebral (10) mAChRs are known to be phosphorylated by a catalytic subunit of cAMP-dependent protein kinase. In parallel experiments, the extent of phosphorylation was greater for atrial mAChRs than for cerebral mAChRs (Fig. 1). The phosphorylation by cAMP-dependent protein kinase was independent of the presence of agonists, irrespective of the presence or absence of G proteins. Rosenbaum et al. (5) reported a stimulatory effect of agonist in the presence of G proteins. The phosphorylation of mAChRs was slightly inhibited by reconstituting mAChRs with G proteins, and this effect was not affected by the presence of GTPγS or other guanine nucleotides. G protein β subunits, but not α subunits, were found to be phosphorylated by cAMP-dependent protein kinase (Fig. 1). The slight inhibition by G proteins is likely to be due to the competitive inhibition.

Agonist-dependent phosphorylation of mAChRs

Rhodopsin and β adrenergic receptors (βARs) are known to be phosphorylated in a light- or agonist-dependent manner by rhodopsin kinase and βARK, respectively, and the phosphorylation is considered to be involved in the homologous desensitization (3). Rhodopsin kinase is reported to be capable of phosphorylating βAR in an agonist-dependent manner, and βARK is also capable of phosphorylating rhodopsin in a light dependent manner, although the Km value of rhodopsin is lower for rhodopsin kinase than for βARK and Km values of βAR are the reverse (11). Chick cardiac mAChRs (12) and α2 adrenergic receptors (13) are reported to be phosphorylated in an agonist-dependent manner by the βARK.

We have partially purified a kinase which phosphorylates mAChRs in an agonist-dependent manner by a method modified from that used for purification of βARK: the kinase is designated temporalily as mAChR kinase. The mAChR kinase phosphorylated both cerebral and atrial mAChRs in an agonist-dependent manner (14). Thus it is apparent that the m2 subtype is a substrate of the mAChR kinase, and we could detect the phosphorylation by mAChR kinase of human m2 subtype expressed in and purified from Sf9 cells. On the other hand, we have not yet identified which subtypes in cerebral mAChR preparations are substrates of the mAChR kinase. We suggested in the original report that the m1 subtype was phosphorylated by the mAChR kinase, but in our preliminary studies we could not detect an agonist-dependent phosphorylation by the mAChR kinase of human m1 subtype expressed in and purified from Sf9 cells (Kameyama et al. unpublished data). Thus it remains to be elucidated if mAChR subtypes other than m2 are substrates of the mAChR kinase.

As βARK and mAChR kinase preparations may include several kinases which have similar characteristics but different substrate specificities, it was not clear whether the βAR and mAChR were phosphorylated by the same kinase or different kinases. In fact, two kinds of cDNAs encoding similar but distinct proteins with βARK activity have been identified and the two proteins were designated as βARK1 and βARK2 (15,16). Recently we have found that the βARK1 expressed in COS-7 cells phosphorylates mAChR m2 subtype in an agonist-dependent manner and rhodopsin in a light-dependent manner (Kameyama et al. submitted). Thus a single kind of kinase, βARK1, is capable of phosphorylating not only βAR but also mAChR and rhodopsin at least *in vitro*. The question raised is what is (are) the substrate(s) of the βARK *in vivo* and whether the βARK phosphorylates multiple kinds of receptors. The name of βARK or mAChR kinase may not be appropriate and receptor kinase or G protein coupled receptor kinase might be better.

The sites of phosphorylation by rhodopsin kinase are located in the carboxyterminal tail of rhodopsin and approximately 7 moles of serine and threonine are phosphorylated. βARK also phosphorylates 8-11 moles of serine and threonine in the carboxyterminal tail of βAR. On the other hand, the carboxyterminal tails of the m2 mAChR subtypes and α2 adrenergic receptors are shorter than those of rhodopsin and βAR, and contain only 1-2 serine and threonine residues. In contrast, both mAChR and α2 adrenergic receptors have very long third internal loops. We have tentatively localized the phosphorylation sites in the human m2 subtype (10-12 serine and threonine residues) in the central part of the third internal loop (Nakata et al. unpublished results). The phosphorylation site of α2 adrenergic receptors by βARK was also suggested to be in the central part of the third internal loop (17).

Inhibition by G proteins of the agonist-dependent phosphorylation of mAChRs

The agonist-dependent phosphorylation of mAChRs purified from porcine atrium was found to be regulated by G proteins in a dual way, stimulation at a lower concentration of G proteins and inhibition at the higher concentration (Fig. 2) (18). This is not likely to be due to competitive inhibition, because G proteins are not phosphorylated by the mAChR kinase, in contrast to the phosphorylation of G proteins by protein kinase C and cAMP-dependent protein kinase. The inhibition was restored by addition of GTPγS. Either GDP or GTP could restore the inhibition and a little higher concentration of GDP than GTP was necessary (14,18). This inhibition occurs in parallel with the formation of guanine nucleotide-sensitive high affinity agonist binding with regard to the effects and effective concentrations of G proteins and guanine nucleotides. In addition, both phenomena are observed with three different G proteins, Gi($\alpha_{41}\beta\gamma$), Go($\alpha_{39}\beta\gamma$).

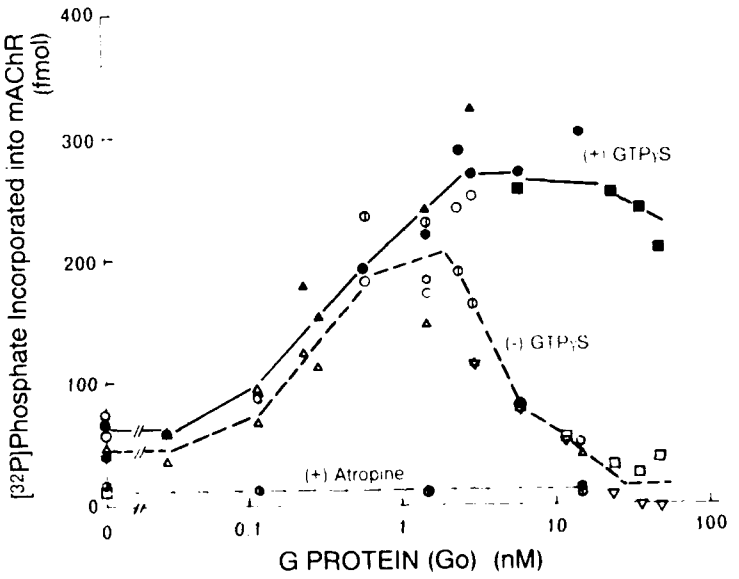

Fig. 2

Effects of concentrations of G proteins (Go) on the agonist-dependent phosphorylation by mAChR kinase of mAChRs purified from porcine atrium. Atrial mAChRs (1.1 nM) reconstituted with different concentrations of Go were phosphorylated with mAChR kinase in the presence (closed symbols) or absence (open symbols) of 10 μM GTPγS in the presence of 1 mM carbamylcholine or 10 μM atropine (broken line). Data were taken from Ref. 18.

and Gn($\alpha_{i0}\beta\gamma$), and require the presence of both α and $\beta\gamma$ subunits of G proteins. It is most likely that the two phenomena are based on the same molecular event, that is the formation of a ternary complex of agonist, receptor and nucleotide-free G protein. A similar inhibition by transducin of the light-dependent phosphorylation of rhodopsin by rhodopsin kinase has been reported (19).

The simplest interpretation for the inhibition by G proteins of the phosphorylation of mAChRs is that the phosphorylation sites in mAChRs are masked by the binding of G proteins. However, most of the third internal loop in mAChRs can be removed without affecting the function of the mutated receptors except that the down regulation of mutated receptors is suppressed (20,21). Thus the phosphorylation sites are not likely to be directly involved in the binding of G proteins. Recently Palczewski (22) reported that light activation of a rhodopsin preparation lacking the phosphorylation sites stimulated the phosphorylation by rhodopsin kinase of a synthetic peptide with the sequence of phosphorylation sites in rhodopsin. This result suggests that rhodopsin interacts with rhodopsin kinase at two sites, the phosphorylation site and a kinase-activating site, and that a conformational change of the kinase-activating site is induced by absorption of light. The binding of transducin to rhodopsin may interfere with the interaction of the kinase-activating site with the rhodopsin kinase. We may assume a similar mechanism in that the binding of acetylcholine induces a conformational change in some regions of the mAChR and the conformational change makes possible the binding and activation of either a G protein or the mAChR kinase but not both. The regions near transmembrane segments in the second and third internal loops and the carboxyterminal tail of mAChRs are thought to be involved in the interaction with G proteins and probably also with the mAChR kinase.

Stimulation by G protein $\beta\gamma$ subunits of light- or agonist-dependent phosphorylation of rhodopsin and mAChR.

Stimulation by a low concentration of G proteins of the phosphorylation of mAChRs could be reproduced by $\beta\gamma$ subunits (18). The α subunit alone did not stimulate nor inhibit the phosphorylation. The initial rate of the phosphorylation reaction was increased and the affinity for substrates (ATP and rhodopsin) did not change appreciably. The $\beta\gamma$ subunits originating from different G proteins, Gi, Go, and Gs, gave essentially the same stimulatory effect, and ten different kinds of $\beta\gamma$ subunits isolated from bovine brain had a similar stimulatory effect. Only the $\beta\gamma$ subunit derived from transducin had a much lower activity for stimulation of phosphorylation.

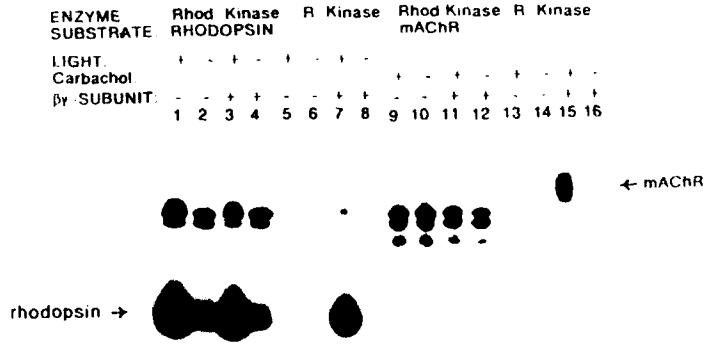

Fig. 3

Effects of G protein $\beta\gamma$ subunits on the phosphorylation of rhodopsin and atrial mAChRs by rhodopsin kinase (Rhod Kinase) and mAChR kinase (R Kinase). Data were taken from Ref. 23.

Rhodopsin was found to be phosphorylated by the mAChR kinase in a light-dependent manner, and the phosphorylation was also stimulated by G protein βγ subunits (Fig. 3)(23). On the other hand, the light-dependent phosphorylation of rhodopsin by rhodopsin kinase was not stimulated by the βγ subunit, consistent with the previous report by Kelleher and Johnson(19). Furthermore, βARs purified from bovine lung were found to be phosphorylated by the mAChR kinase and the phosphorylation was also stimulated by G protein βγ subunits (Kameyama et al. submitted). The phosphorylation of mAChR, rhodopsin and βAR was stimulated by similar concentrations of βγ subunits isolated from porcine brain and the stimulatory effect of transducin βγ subunits was much less for all three substrates. These results are consistent with the assumption that the βγ subunit interacts with and activates the mAChR kinase. Kinetic results for the phosphorylation of rhodopsin by the mAChR kinase are consistent with the assumption that the mAChR kinase interacts with both rhodopsin and βγ subunits in a random order resulting in a formation of the ternary complex of rhodopsin, βγ subunits and the mAChR kinase(23).

We have found that the phosphorylation of mAChRs by the βARK1 expressed in COS-7 cells was activated by G protein βγ subunits (Kameyama et al. submitted). The concentration of βγ subunits required for the stimulation was 1-30 nM, similar to the concentration required for the stimulation of phosphorylation by the mAChR kinase. Thus βARK1 has two properties attributed to the mAChR kinase, that is the phosphorylation of mAChR and the activation by G protein βγ subunits. We do not have any evidence against the assumption that the mAChR kinase is the same as or very similar to the βARK1. Recently, Pitcher et al.(24) reported that the phosphorylation of βARs by the βARK1 expressed in Sf9 cells was stimulated by G protein βγ subunits.

Amino acid sequences of βARK1(15) and rhodopsin kinase(25) deduced from the nucleotide sequences of corresponding cDNAs are similar to each other, but the βARK1 is longer than rhodopsin kinase, having approximately 130 extra amino acid residues in the carboxyterminus. As βARK1 is different from rhodopsin kinase as regards the activation by βγ subunits, it would be reasonable to assume that the extra carboxyterminal segment is responsive for the activation by G protein βγ subunits. So we have constructed a mutant lacking a part of the extra carboxyterminus in βARK1 and found that the mutant could phosphorylate mAChRs in an agonist-dependent manner

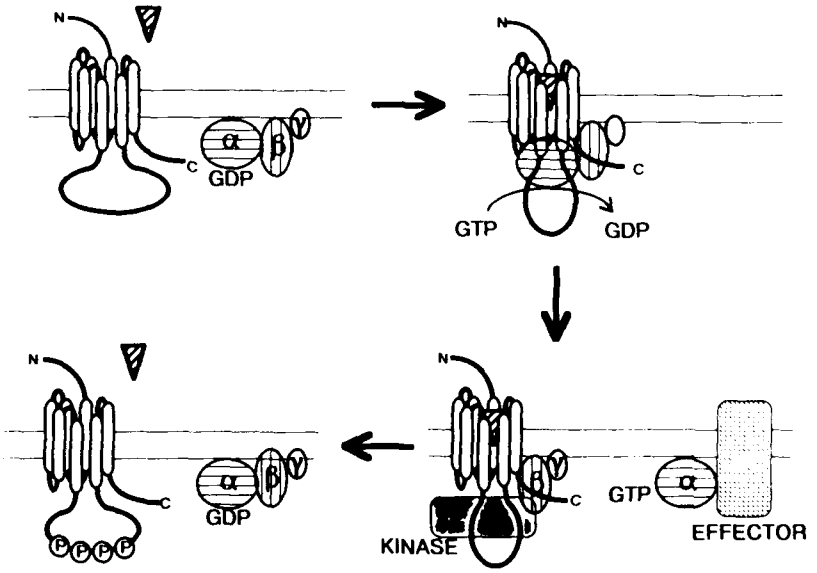

Fig. 4

A schematic model for the interaction of receptors, G protein α and βγ subunits, and receptor kinase.

but the phosphorylation was not stimulated by G protein βγ subunits (Kameyama et al. submitted). This result clearly explains why the phosphorylation by rhodopsin kinase is not stimulated by βγ subunits and suggests that this region is the site of interaction with G protein βγ subunit. Physical interaction between the carboxyterminal peptide of 220 amino acid residues of βARK and G protein βγ subunits has been demonstrated by Pitcher et al.(24).

Hypothesis

The function of mAChRs, as well as other G protein coupled receptors, is to activate G proteins, that is to facilitate the conversion of G proteins from the trimer form ($\alpha_{GDP}\beta\gamma$) to the dissociated form (α_{GTP} + βγ). The activation of G proteins is followed by activation of different kinds of effectors by α_{GTP}. In some cases, βγ subunits are also reported to act on effectors; e.g. the opening of inward-rectifying K^+ channel(26), and facilitation or suppression of activation by α_{GTP} of adenylate cyclase depending on the type of adenylate cyclase(27). The present result suggests that βγ subunits function as activators of G protein coupled receptor kinases and thereby facilitate homologous desensitization.

Agonist-bound mAChRs facilitate the dissociation of GDP from G proteins (G, $\alpha_{GDP}\beta\gamma$) and thereby a ternary complex (aRG or aR$\alpha\beta\gamma$) of agonist (a), mAChR (R) and guanine nucleotide free G proteins is formed. Addition of GTP or GTPγS results in the dissociation of the α_{GTP} or $\alpha_{GTP\gamma S}$ from the ternary complex. It is not known if βγ subunits directly interact with mAChRs or indirectly through α subunits, and if the βγ subunit is also dissociated from aR on addition of GTP or GTPγS. The direct interaction between βγ subunits and rhodopsin has been shown (19,28,29). It is tempting to speculate that the βγ subunit remains bound to aR and the G protein coupled receptor kinase binds to the aRβγ complex and thereafter phosphorylates the receptor. As shown in Fig. 4, we propose a working hypothesis that (1) the kinase and α subunits compete for βγ subunits and the sites of mAChRs which were exposed by binding of agonist, and (2) in a putative complex of aR, βγ and the kinase, the kinase interacts with and is activated by both βγ subunits and the exposed sites of mAChRs.

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References

1. N.M. NATHANSON, Annu. Rev. Neurosci. *10* 195-236 (1987).
2. R.L. HUGANIR and P. GREENGARD, Neuron *5* 555-567 (1990).
3. K. PALCZEWSKI and J.L. BENOVIC, Trends Biochem. Sci. *16* 387-391 (1991).
4. K. HAGA, T. HAGA and A. ICHIYAMA, J. Neurochem. *54* 1639-1644 (1990).
5. L.C. ROSENBAUM, D.A. MALENCIK, S.R. ANDERSON, M.R. TOTA and M.I. SCHIMERLIK, Biochemistry *26* 8183-8188 (1992).
6. H. UCHIYAMA, K. OHARA, K. HAGA, T. HAGA and A. ICHIYAMA, J. Neurochem. *54* 1870-1881 (1990).
7. R.M. RICHARDSON and M.M. HOSEY, Biochemistry *29* 8555-8561 (1990).
8. R.M. RICHARDSON, J. PTASIENSKI and M.M. HOSEY, J. Biol. Chem. *267* 10127-10132 (1992).
9 K. HAGA, H. UCHIYAMA, T. HAGA, A. ICHIYAMA, K. KANGAWA and H.MATSUO, Mol. Pharmacol. *35* 286-294 (1989).
10. A.K.S. HO, Q.-L. LING, R. DUFFIELD, P.H. LAM and J.H. WANG, Biochem. Biophys. Res. Commun. *142* 911-918 (1987).

11. J.L. BENOVIC, F.J. MAYOR, R.L. SOMERS, M.G. CARON and R.J. LEFKOWITZ Nature *321* 869-872 (1986).
12. M.M. KWATRA, J.L. BENOVIC, M.G. CARON, R.J. LEFKOWITZ and M.M. HOSEY Biochemistry *28* 4543-4547 (1989).
13. J.L. BENOVIC, J.W. REGAN, H. MATSUI, F.JR. MAYOR, S. COTECCHIA, L.M.F. LEEB-LUNDBERG, M.G. CARON and R.J. LEFKOWITZ, J. Biol. Chem. *262* 17251-17253 (1987).
14. T. HAGA and K. HAGA, Biomed. Res. *10* 293-299 (1989).
15. J.L. BENOVIC, A. DEBLASI, W.C. STONE, M.G. CARON and R.J. LEFKOWIZ, Science *246* 235-240 (1989).
16. J.L. BENOVIC, J.J. ONORATO, J.L. ARRIZA, W.C. STONE, M. LOHSE, N.A. JENKINS, D.J. GILBERT, N.G. COPELAND, M.G. CARON and R.J. LEFKOWITZ, J. Biol. Chem. *266* 14939-14946 (1991).
17. S.B. LIGGETT, J. OSTROWSKI, L.C. CHESNUT, H. KUROSE, J.R. RAYMOND, M.G. CARON and R.J. LEFKOWITZ, J. Biol. Chem. *267* 4740-4746 (1992).
18. K. HAGA and T. HAGA, FEBS Lett. *268* 43-47 (1990).
19. D.J. KELLEHER and G.L. JOHNSON, Mol. Pharmacol. *34* 452-460 (1988).
20. R.A. SHAPIRO and N.M. NATHANSON, Biochemistry *28* 8946-8950 (1989).
21. S. MAEDA, J. LAMEH, W.G. MALLET, M. PHILLIP, J. RAMACHANDRAN and W. SADEE, FEBS Lett. *269* 386-388 (1990).
22. K. PALCZEWSKI, J. BUCZYLKO, M.W. KAPLAN, A.S. POLANS and J.W. CRABB, J. Biol. Chem. *266* 12949-12955 (1991).
23. K. HAGA and T. HAGA, J. Biol. Chem. *267* 2222-2227 (1992).
24. J.A. PITCHER, J. INGLESE, J.B. HIGGINS, J.L. ARRIZA, P.J. CASEY, C. KIM, J.L. BENOVIC, M.M. KWATRA, M.G. CARON and R.J. LEFKOWITZ, Science *257* 1264-1267 (1992).
25. W. LORENZ, J. INGLESE, K. PALCZEWSKI, J.J. ONORATO, M.G. CARON and R.J. LEFKOWITZ, Proc. Natl. Acad. Sci. U. S. A. *88* 8715-8719 (1991).
26. D.E. LOGETHETIS, Y.KURACHI, J.GALPER, E.J. NEER, and D.E. CLAPHAM, Nature *325* 321-326 (1987).
27. W.-J. TANG and A.G. GILMAN, Science *254* 1500-1503 (1991).
28. W.J. PHILLIPS and R.A. CERIONE, J. Biol. Chem. *267* 17032-17039 (1992).
29. W.J. PHILLIPS, S.C. WONG and R.A. CERIONE, J. Biol. Chem. *267* 17040-17046 (1992).

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REGULATION OF EXPRESSION AND FUNCTION OF MUSCARINIC RECEPTORS

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Summary

The regulation of expression and function of the muscarinic acetylcholine receptor has been studied using several different systems. The role of glycosylation of the m2 receptor was examined by removal of glycosylation sites using site-directed mutagenesis followed by expression in stably transfected cells. The results demonstrated that glycosylation was not required for the synthesis and appearance of the receptors on the cell surface or for the coupling of the receptors to inhibition of adenylyl cyclase activity. Site-directed mutagenesis also was used to demonstrate that the single cysteine in the carboxy terminal domain of the m2 receptor was not required for receptor function, thus rendering unlikely a model suggesting a requirement for palmitoylation of this cysteine in receptor function. The muscarinic receptors expressed in embryonic chick heart were identified by molecular cloning. Two genes were initially identified which are expressed in chick heart and correspond to the chick m2 and m4 receptors. Experiments using the polymerase chain reaction to identify low abundance mRNAs indicate that at least one addition receptor gene is expressed in chick heart. In cell culture, activation of the muscarinic receptors decreases the levels of mRNA encoding the cm2 and cm4 receptors. This probably results from decreased gene transcription due to both mAChR-mediated inhibition of adenylyl cyclase and mAChR-mediated stimulation of phospholipase C.

The elucidation of the factors which regulate the expression and function of muscarinic acetylcholine receptors (mAChR) is of obvious importance in understanding the mechanisms underlying cholinergic transmission. In this chapter, we will describe studies on the expression and function of wild type and mutant muscarinic receptors, the molecular characterization of mAChR expressed in chick heart, and the regulation of mAChR gene expression in response to muscarinic receptor activation.

Elimination of mAChR glycosylation by site-directed mutagenesis

Muscarinic receptors are glycoproteins. The first evidence for this came from the demonstration that solubilized mAChR bind to and can be specifically eluted from lectin columns (1). The purified mAChR from porcine heart is 35-50% carbohydrate by weight (2), and the sequences of all five cloned mammalian mAChR genes contain multiple consensus sites for N-linked glycosylation. Liles and Nathanson (3) demonstrated that treatment of neuroblastoma cells with tunicamycin, an inhibitor of N-linked protein glycosylation, caused a decrease in the number of mAChR binding sites present on the cell surface with little initial change in the total number of mAChR binding sites in the cells. Several experiments suggested that inhibition of glycosylation inhibited transport of newly synthesized mAChR to the cell surface with the newly synthesized receptors accumulating intracellularly prior to their subsequent degradation.

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Because studies with tunicamycin cannot distinguish between a requirement for glycosylation of the mAChR itself and a generalized requirement for cellular glycosylation, we have used site-directed mutagenesis to directly test the role of glycosylation in synthesis and function of the mAChR (4). The sequence of the cloned porcine m2 receptor exhibits three consensus sites for N-linked glycosylation, at asn2, asn3, and asn6. To eliminate any possible effects of charge, we constructed three triple mutants in which the three asparagines were changed to either aspartates, lysines, or glutamines, and expressed the mutant and wild type receptors in stably transfected CHO cells. The lysine triple mutant was poorly expressed and not extensively studied, but the aspartate and glutamine mutants were expressed to the same level as the wildtype receptor. All three triple mutants displayed an affinity for antagonist identical to that of the wildtype receptor, and all of the receptors expressed on the cell surface. Thus, glycosylation is not an obligatory requirement for the normal synthesis and processing of the mAChR. Glycosylation also does not appear to affect the rate of degradation of the mAChR. The rates of receptor disappearance of the asp and gln mutants were similar to that of the wildtype receptor in cyclohexemide-treated cells both in the absence and the presence of carbachol (to induce receptor downregulation). Furthermore, cells expressing the asp and gln mutants exhibit an ability to inhibit adenylyl cyclase activity that is virtually identical to that of the wildtype receptor. Thus, glycosylation is also not required for functional responsiveness of the mAChR. A similar conclusion was reached by Haga (5), who demonstrated that purified m2 receptors which were enzymatically deglycosylated were as active as glycosylated receptors in coupling to G-proteins in reconstituted vesicles.

Role of C-terminal cysteine in mAChR function.

There is evidence that the ß-adrenergic receptor and rhodopsin are palmitoylated on cysteines in the carboxy terminal domain, and that elimination of the palmitate decreases functional activity (6, 7). Because many (but not all) of the G-protein linked receptors contain cysteines in this region, it has been suggested (6) that this palmitate may anchor the carboxy terminal tail to the membrane, thus forming a fourth intracellular loop that would be involved in interaction with G-proteins. To test this hypothesis, we eliminated the sole cysteine in the carboxy tail of the porcine m2 receptor by site-directed mutagenesis and expressed the mutant receptor in CHO cells (8). The mutant receptor was expressed to the same extent and inhibited adenylyl cyclase activity as effectively as the wild type receptor. Studies with the m1 receptor have also demonstrated that elimination of its carboxy domain cysteine does not alter its ability to activate phospholipase C (9), and a number of cloned receptors have no cysteines in their carboxy terminal domains (cited in ref. 8). In addition, while chemical depalmitoylation reduces the ability of rhodopsin to activate transducin, removal of the carboxy terminal cysteines by site-directed mutagenesis does not reduce the functional activity of rhodopsin. Thus, palmitoylation is not an absolute requirement for functional activity of the G-protein-linked receptor superfamily.

Internalization of muscarinic receptors

In a previous study, we demonstrated that activation of protein kinase C in stably transfected Y1 adrenal cells leads to internalization of the m1 but not the m2 receptor (10). We have found that activation of PKC in transiently transfected COS cells also causes internalization of the m1 but not the m2 receptor. Studies with a chimeric m1/m2 receptor demonstrate that the carboxy terminal third of the m1 receptor is required for internalization due to PKC activation (Goldman and Nathanson, unpublished observations).

Functional activity of muscarinic receptors

We have begun to use a novel functional assay to examine the signaling mediated by muscarinic receptors in transiently transfected cells. A muscarinic receptor is transfected into a cell with a plasmid containing a luciferase reporter gene under the control of a cAMP-regulated promoter. Control experiments indicate that increases in intracellular cAMP but not intracellular calcium or PKC activation induce luciferase expression. The determination of the level of luciferase expression following addition of agonist thus allows measurement of changes in the cAMP levels in transfected cells. Surprisingly, both the m1 and cm4 receptors cause large increases in luciferase expression (Migeon and Nathanson, unpublished observations). The mechanism for this increase is under investigation.

Muscarinic receptors in chick heart

As described previously (11), there are pharmacological, biochemical, and immunological differences in the mAChR in chick and mammalian hearts. In order to determine the molecular basis of the differences between mAChR in chick and mammalian hearts, we have isolated the genes encoding several avian mAChR. Initial screening of a chicken genomic library with a mixture of mammalian m1 and m2 receptor probes yielded a gene which corresponded to the avian homolog of the mammalian m4 receptor, termed cm4 (11). Rescreening of the library with a probe from the cm4 gene yielded a gene encoding the chick m2 (cm2) receptor (12). Both solution hybridization and Northern blot analyses demonstrated that both genes were expressed in chick heart (as well as brain). Furthermore, subtype-selective antisera produced following immunization with either specific peptides or fusion proteins demonstrated that both receptor proteins are present in the heart (Parenteau, Tietje, and Nathanson, unpublished observations). Because of the data demonstrating tissue-specific developmental changes in the expression and function of mAChR in chick heart during development, it will be of great interest to use these antibody and nucleic acid probes to determine if there is differential regulation of the mAChR genes during development.

Expression of the cm2 and cm4 receptors in both CHO and Y1 adrenal cells demonstrated that the receptors exhibited the high affinity for pirenzepine characteristic of the mAChR in situ in chick heart. Both receptors only coupled to inhibition of adenylyl cyclase activity in Y1 cells and could not stimulate phospholipase C activity. In CHO cells, both the cm2 and cm4 receptors inhibited adenylyl cyclase and stimulated phospholipase C activity. Thus, as with the mammalian mAChR, the functional specificity of the chick mAChR depends not only on the amino acid sequence encoded by a particular gene but on the type of cell in which that gene is.

In order to determine if any additional mAChR subtypes are expressed in chick heart, we used the polymerase chain reaction (PCR) with degenerate oligonucleotide primers to isolate sequences encoding additional mAChR. A fragment was isolated whose sequence demonstrated the highest degree of amino acid similarity to the mammalian m5 receptor. The gene encoding this fragment has been isolated and partial sequence analysis confirms its identity as the chick m5 receptor. PCR analysis using cm5 specific primers indicates that it is also expressed in both chick heart and brain (Creason, Tietje, and Nathanson, unpublished observations).

Regulation of mAChR gene expression by agonist

Studies initially carried out with the ß-adrenergic receptor demonstrated that long-term agonist activation of the receptor leads to a decrease in the level of mRNA encoding the receptor due to a cAMP-mediated decrease in mRNA stability (13). We have found that activation of mAChR in embryonic chick heart leads to decreases over 6-8 hours in the levels of mRNA encoding the cm2 and cm4 receptors (14). There is no change in the rate of degradation of the mRNAs, suggesting that the decrease in mRNA results from a decrease in the rate of gene transcription. Because activation of chick heart mAChR both inhibits adenylyl cyclase and activates phospholipase C, there are multiple second messenger pathways which have the potential to be involved in the regulation of mAChR mRNA expression. We took advantage of the presence in chick heart of adenosine receptors which inhibit adenylyl cyclase but do not stimulate phospholipase C, and angiotensin II receptors, which stimulate phospholipase C but do not inhibit adenylyl cyclase, to test the roles of these two pathways in mRNA regulation. Each heterologous agonist had modest effects on the level of mRNA, while inhibition with both agonists had a greater effect on mRNA levels. These results suggest that regulation of both the adenylyl cyclase and phospholipase C pathways is required to regulate mRNA expression. Recent studies using pertussis toxin (to inhibit mAChR-mediated inhibition of adenylyl cyclase but not stimulation of phospholipase C) and partial muscarinic agonists (to allow inhibition of adenylyl cyclase but not stimulation of phospholipase C) support the hypothesis that both the adenylyl cyclase and phospholipase C pathways are involved in mAChR-mediated regulation of mAChR gene expression (Habecker, Wang, and Nathanson, unpublished observations).

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References

1. G.S. HERRON and M.I. SCHIMERLIK, J. Neurochem. 41 1414-1420 (1983).
2. G.L. PETERSON, L.C. ROSENBAUM, D.J. BRODERICK and M.I. SCHIMERLIK, Biochemistry 25 3189-3202 (1986).
3. W.C. LILES and N.M. NATHANSON, J. Neurochem. 46 89-95 (1986).
4. C.J. VAN KOPPEN and N.M. NATHANSON, J. Biol. Chem. 265 20887-20892 (1990).
5. K. OHARA , H. UCHIYAMA, K. OHARA, T. HAGA and A. ICHIYAMA, Eur. J. Pharmacol. 189 341-346 (1990).
6. B.F. O'DOWD, M. HANTOWICH, M.G. CARON, R.J. LEFKOWITZ and M. BOUVIER, J. Biol. Chem. 264 7564-7569 (1989).
7. Y.A. OVCHINNIKOV, N.G. ABDULAEV and A.S. BOGACHUK, FEBS Lett. 230 1-5 (1988).
8. C.J. VAN KOPPEN and N.M. NATHANSON, J. Neurochem. 57 1873-1877 (1991).
9. T.M. SAVARESE, C.D. WANG and C.M. FRASER, J. Biol. Chem. 267. 11439-11448 (1992) .
10. N.M. SCHERER and N.M. NATHANSON, Biochemistry 29 8475-8483 (1990).
11. K.M. TIETJE, P.S. GOLDMAN and N.M. NATHANSON, J. Biol. Chem. 265 2828-2834 (1990).
12. K.M. TIETJE and N.M. NATHANSON, J. Biol. Chem. 266 17382-17387 (1991).
13. S. COLLINS, M. BOUVIER, M.A. BOLANOWSKI, M.G. CARON and R.J. LEFKOWITZ, Proc. Natl. Acad. Sci. USA 86 4853-4857 (1989).
14. B.A. HABECKER and N.M. NATHANSON, Proc. Natl. Acad. Sci. USA 89 5035-5038 (1992).

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m1-TOXIN

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Summary

The venom of the Eastern green mamba from Africa, *Dendroaspis angusticeps*, contains a number of toxins which block the binding of ^{3}H-antagonists to genetically-defined m1 and m4 muscarinic acetylcholine receptors. Most of the anti-muscarinic activity of the venom is due to the presence of a newly-isolated toxin, "m1-toxin", which has 64 amino acids and a molecular mass of 7361 Daltons. At present m1-toxin is the only ligand which is known to be capable of fully blocking m1 receptors without affecting m2-m5 receptors. It binds very rapidly, specifically and pseudoirreversibly to the extracellular face of m1 receptors on cells, in membranes or in solution, whether or not the primary receptor site is occupied by an antagonist. Bound toxin can either prevent the binding and action of agonists or antagonists, or prevent the dissociation of antagonists. The toxin is useful for identifying m1 receptors during anatomical and functional studies, for recognizing and stabilizing receptor complexes, and for occluding m1 receptors so that other receptors are more readily studied.

The numbers and approximate locations of m1-m5 receptors in different tissues have been established through the use of antibodies to different intracellular portions of m1-m5 receptors (1-3). However specific agonists and antagonists for these receptors are still needed for other studies, in particular to establish the functions of the predominant m1 and m4 receptors in the forebrain. These two receptors (m1 and m4) have been particularly difficult to distinguish with existing ligands: the most selective antagonist is himbacine, which shows 10-fold higher affinity for m4 than m1 receptors, and the next most selective ligand is pirenzepine, which shows 6-fold higher affinity for m1 than m4 receptors (4,5). In order to obtain more specific ligands for functional and other studies of muscarinic receptors, we have begun to study anti-muscarinic toxins. Most of our data have been presented in thesis (6) or abstract form (7-10).

Toxins have a rich and growing history as ligands for the identification of protein molecules which are otherwise difficult to identify, notably receptors and ion channels. During evolution toxins were clearly selected for their ability to interact with the extracellular faces of such membrane proteins. The use of alpha-bungarotoxin permitted the first quantitative studies of receptors in tissues, in membranes and in solution (11), and nicotinic receptors which bind curarimimetic alpha-neurotoxins are now the most extensively studied of any receptors. Investigators in Karlsson's laboratory in Sweden were the first to demonstrate that there are

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toxins which block muscarinic receptors (12,13). They purified two toxins from the venom of the green mamba, and found that the primary sequence of the more prevalent toxin, "MTX2", was homologous with respect to its disufide bonds with the primary sequences of curarimimetic neurotoxins and fasciculin, a toxin from *D. angusticeps* which inhibits acetylcholinesterase. Since both of their anti-muscarinic toxins blocked only 50-55% of the binding of ^{3}H-quinuclidinyl benzilate (QNB) to rat cortical receptors, Adem et al (12) suggested that their toxins might be specific for one subtype of muscarinic receptor. However subsequent work by Jerusalinsky et al (14) indicated that both toxins blocked m1 receptors in the cortex, and m2 receptors in the heart and brainstem, suggesting that the toxins were not fully subtype-specific. They demonstrated competition between both toxins and other receptor ligands, including the agonist, ^{3}H-oxotremorine-M. They also prepared ^{125}I-MTX2 and estimated its K_d in the cortex as 14 nM. Recent studies by Adem et al (15, an abstract in this symposium) confirm that their first toxin, "MTX1", competes with pirenzepine and other antagonists for muscarinic receptors. Moreover ^{125}I-MTX1 appears, by autoradiography, to bind with high specificity to brain regions rich in m1 receptors. The venom of the green mamba may prove, therefore, to have several toxins which show high specificity for m1 receptors.

Assay of anti-muscarinic toxins It is convenient to assay the anti-muscarinic activity of venom protein fractions by first mixing muscarinic receptors with diluted toxin for long enough to permit maximum toxin-binding, followed by the measurement of residual, unblocked receptors with low concentrations of a radioactive antagonist. In our standard assay for anti-m1 muscarinic activity we incubate membranes from 5 mg of packed CHO cells (which have 0.25-0.5 pmol of pure m1 receptors) with 0-50 pmol of toxin in 0.2 ml of 50 mM phosphate-1 mM EDTA buffer (16) for 30 minutes at 25°C, and then label remaining receptors using 9.8 ml of 0.1 nM ^{3}H-N-methylscopolamine (NMS) in the same buffer, during an additional hour. Membranes with bound NMS are then recovered by filtration and the radioligand is counted. An alternative assay which yields a similar result utilizes membranes from 5 mg of rat hippocampus or cortex (which have about 0.25 pmol of m1 receptors) plus 9.8 ml of 1.0 nM ^{3}H-pirenzepine in 20 mM Tris buffer containing 1 mM $MnCl_2$ (17). For the assay of soluble muscarinic receptors, membranes are first dissolved in 1% digitonin, receptors are exposed in sequence to toxin and 1 nM ^{3}H-QNB, and labeled receptors are recovered by precipitation with protamine sulfate and polyethylene glycol (18).

Three factors complicate assays of the activity of m1-toxin. First, the anti-m1 muscarinic activity of m1-toxin is effectively irreversible. As a result it is not possible to evaluate steady-state binding constants for m1-toxin, and in order to quantify toxin activity, measurements must be made instead of the minimum quantity of toxin necessary to block receptors. In theory, 0.5 pmol of m1-toxin should block 0.5 pmol of m1 receptors, and the concentrations of toxin and receptor should be irrelevant. In practice, the toxin concentration does seem to be important, particularly for comparisons of the blockade of several receptors by one toxin, and in our assay full blockade of pure m1 receptors typically requires a molar ratio of m1-toxin/receptor of 5-10. Comparisons of the specificity of anti-muscarinic toxins for different receptors should therefore be made using approximately equal receptor concentrations. Given this precaution it is useful to define **one unit of anti-muscarinic activity** as the minimum amount of venom protein which blocks a given amount of one muscarinic receptor subtype by at least 95% under standard assay conditions. For lyophilized mamba venom one unit of anti-m1 muscarinic activity is about 10 μg under our standard assay conditions. For pure m1-toxin one unit is about 30 ng, and corresponds to a toxin concentration during toxin-binding of about 20 nM. The second variable which affects assays of m1-toxin activity is that the toxin can bind to receptors which are already labeled with an antagonist. For assay purposes, therefore, m1-toxin must be used before other ligands. In this regard, m1-toxin differs from MTX1 and MTX2, which can apparently be studied by steady-state

competition techniques. These other toxins bind more reversibly and with less obvious allosterism than m1-toxin. Finally, since none of the toxins penetrate membranes, assays of their ability to block receptors in membranes should be carried out with an antagonist like ^{3}H-NMS which does not penetrate membranes.

Purification of m1-toxin Our first purification step is the same as that developed by Adem et al (12) and used by Jerusalinsky et al (14), namely gel filtration of lyophilized and reconstituted venom protein from *D. angusticeps* on Sephadex G-50. These workers utilized the third and major peak of eluted protein, which contains dendrotoxin, fasciculin and many other toxins of about 7000 Daltons, for further purification of anti-muscarinic toxins by cation-exchange chromatography and reversed-phase HPLC. In contrast, we found that the majority of the anti-muscarinic activity of the venom eluted well after this major protein peak. The peak of anti-muscarinic activity was broad but symmetrical, and its position was little affected by changes in the medium which usually decrease protein adsorption. The most active fractions were combined, lyophilized and reconstituted in 10% acetonitrile containing 0.1% trifluoracetic acid. m1-Toxin proved very easy to purify to homogeneity by reversed-phase HPLC on a C-18 column. A toxin with high specificity for m4 receptors has been purified by a different protocol. This "m4-toxin" complicates the recognition and purification of toxins which show specificity for m1 receptors (MTX1, MTX2 and m1-toxin) unless specific assays for anti-m1 muscarinic activity (like those described above) are used.

Physical properties of m1-toxin m1-Toxin runs during polyacrylamide gel electrophoresis in SDS very slightly faster than alpha-bungarotoxin, which has a molecular mass of about 8000 Daltons. The primary sequence of m1-toxin shows 64 amino acids and indicates that the toxin has a molecular mass of 7361 Daltons. The computed isoelectric point of m1-toxin is almost neutral. The toxin has twice as many aromatic amino acids (eight) as MTX2, which may help account for its slower filtration on Sephadex G-50. The most prevalent amino acid in m1-toxin is cysteine; and all eight cysteine residues and four other residues are invariant in sequence in m1-toxin, MTX2, erabutoxin, cobrotoxin, cardiotoxin and fasciculin (13,19). The structure of erabutoxin has been established by X-ray crystallography (19), and shows four tightly bunched disulfide bonds at one end of the molecule, which stabilize three adjacent peptide loops at the other end of the molecule. Given the very similar sizes and key sequence homologies of the toxins named, it is very likely that all have a very similar structure, and that all have a similar evolutionary history. It appears, therefore, that one family of toxin proteins has evolved to the point where it can interact specifically with three major families of acetylcholine-binding proteins: nicotinic and muscarinic receptors, and acetylcholinesterase. m1-Toxin and MTX2 show 63% sequence identity. The sequence of MTX1 has not been published, and cannot yet be compared.

Potential active regions of anti-muscarinic toxins Since the primary structures of more than 50 anti-nicotinic and 2 anti-muscarinic toxins are known, it is tempting to look at the differences between these groups for potential clues as to what features may confer anti-muscarinic activity. Excluding those amino acids which are identical among the short-chain neurotoxins noted above, there are 13 residues which are highly conserved in anti-nicotinic toxins, which are not present in m1-toxin or MTX2. All these are located on two adjacent peptide loops of the toxins, and one face of this region has long been suspected to be the active region of anti-nicotinic toxins (20). By comparison, m1-toxin and MTX2 have 15 highly conserved residues not present in the anti-nicotinic toxins. These are widely dispersed and do not alone indicate a specialized region. Given the additional information, however, that MTX2 can be labeled with iodine without losing activity (14), whereas m1-toxin cannot (see below), it may be speculated that tyrosine-51, which occurs in m1-toxin but not MTX2, is important for the blocking activity of m1-toxin. This residue is on one of the two peptide loops thought to be necessary for anti-nicotinic activity.

Specificity of m1-toxin for m1 receptors One unit of m1-toxin blocks the binding of ^{3}H-NMS to pure m1 receptors on membranes from CHO cells, while amounts up to at least 65 units have no effect on the binding of NMS to similar amounts of m2, m3 or m5 receptors. m1-Toxin begins to show measurable blocking activity on pure m4 receptors at about 5 units, and by 65 units m1-toxin can block about 35% of the m4 receptors on CHO cells. Low concentrations (1-4 units) of m1-toxin can therefore be used to completely occlude m1 receptors, while having no effect on m2-m5 receptors. When increasing concentrations of m1-toxin are tested for their ability to block the binding of radioactive antagonists to mixed populations of muscarinic receptors in membranes from the cerebral cortex, hippocampus and striatum, it is evident that 1 unit of toxin readily blocks some receptors, and that the proportion of total receptors blocked is the same as the proportion previously identified as m1 receptors with m1-specific antibodies (1-3). Experiments of this type allow estimates of the proportion and numbers of total muscarinic receptors which are m1 receptors in different tissues.

At concentrations up to 65 units m1-toxin does not block the binding of ^{3}H-nicotine to nicotinic receptors in membranes from the rat cerebral cortex.

Molecular biology of antimuscarinic toxins It is desirable to clone and express the cDNA for m1-toxin and other trace anti-muscarinic toxins from the venom of *D. angusticeps*. Ducancel et al (21) prepared a cDNA library from the venom glands of *D. angusticeps* and we have repeated their work. They were able to establish the sequence of the cDNA for MTX2 and we are working on the sequence of the cDNA for m1-toxin. It remains to be seen whether the genes for anti-muscarinic toxins can be successfully expressed in heterologous systems to obtain adequate amounts of each toxin for comparative studies.

Antagonist activity of m1-toxin on intact living cells and tissues Sub-K_d concentrations of ^{3}H-pirenzepine can be used to label m1 receptors in the rat hippocampus and cerebral cortex with about 95% specificity (17) because of the 6-fold higher affinity of pirenzepine for m1 than m4 receptors (4,5) and the approximately 3-fold higher concentration of m1 than m4 receptors in these tissues (1-3). When slices of these tissues are placed in in warm oxygenated Krebs solution and are exposed to 1.0 nM ^{3}H-pirenzepine, the radioligand readily diffuses into each tissue and binds reversibly to m1 receptors. When the tissue is exposed first to 50 nM m1-toxin, specific radioligand binding is abolished. These studies demonstrate that m1-toxin can diffuse readily into tissues, as might be expected from its size, and that it acts on the outside of intact cells. Experiments with intact CHO cells expressing only m1 receptors, and exposed to the tertiary ligand ^{3}H-QNB or the quaternary ligand ^{3}H-NMS, demonstrate further that m1-toxin acts on receptors which are on the outside of cells. When carbachol was tested for its ability to enhance the turnover of radioactive phosphoinositides in hippocampal slices, it was found that m1-toxin blocked approximately two-thirds of the agonist-stimulated turnover. These studies indicate that m1-toxin blocks the binding of agonists as well as antagonists to muscarinic receptors.

Rate of dissociation of m1-toxin Most toxins dissociate very slowly from their target proteins, and m1-toxin is not an exception. The duration of binding of m1-toxin was studied in two ways. In one series of experiments membranes with toxin-labeled receptors were washed and then resuspended in the presence of various radioactive antagonists in order to measure the rate of reappearance of free receptors. No recovery of free receptors occurred in 8 hours at 25°C. When the membranes were treated under conditions which cause m1-toxin to dissociate, the receptors were then able to bind antagonists. These studies establish that m1-toxin binds pseudoirreversibly, and that it does not damage m1 receptors. In a second series of experiments pure m1 receptors in solution were labeled with ^{3}H-NMS, half of the receptors were exposed to m1-toxin, and all were then diluted in the presence of 1.0 μM nonradioactive QNB to study the

dissociation of the radioactive antagonist. m1-Toxin stopped the dissociation of ^{3}H-NMS for at least 6 hours at 25°C, indicating the presence of stable receptor complexes containing both NMS and m1-toxin. Thus m1-toxin remains bound to receptors with or without occupied primary receptor sites.

Allosteric binding of m1-toxin m1-Toxin slows the dissociation of several ^{3}H-antagonists from m1 receptors in membranes as well as the dissociation of NMS in solution. The implication of these results is that the toxin binds to the extracellular portions of m1 receptors, and does not project significantly into the receptor pocket which binds primary ligands. Some potential uses of this allosteric effect are noted below.

Rate of binding of m1-toxin It was anticipated that some time would be necessary for the free loops of m1-toxin to intercalate with the extracellular loops of m1 receptors and establish a sufficient number of hydrogen bonds to produce stable toxin-receptor complexes. On the contrary, when we varied the time of toxin-binding, it was found that maximum blockade was achieved in less than 30 seconds, even on ice and with amounts of toxin which subsaturated m1 receptors. These results imply that the fit between the opposing loops of toxin and receptor is excellent and requires little adjustment for optimum interaction.

Site of binding of m1-toxin on m1 receptors Since it is probable that m1-toxin binds to extracellular amino acids on m1 receptors which are different from those in m2-m5 receptors, and since the amino acid sequences of m1-m5 receptors are known, it is possible to speculate as to which regions of m1 receptors (and to a lesser extent, m4 receptors) may account for toxin-binding. Of the 77 amino acids in rat m1 receptors which are believed to be extracellular (22), 31 are not found in m2, m3 or m5 receptors, only 3 are identical in m1 and m4 receptors, and only 7 more are conserved between m1 and m4 receptors. The most distinct region of m1 receptors is its 24-residue amino terminal end, and it seems likely that this region is important for toxin-binding. However the ability of m1-toxin to stabilize the whole receptor molecule in solution strongly suggests additional sites of interaction. The putative three extracellular loops of m1 receptors each show 3-5 residues which are not found in m2, m3 or m5 receptors, and 1 or 2 of these residues are common or conserved in each loop beteen m1 and m4 receptors. Presumably some or all of these loops are involved in toxin-binding. Measurements of the binding of m1-toxin to mutated and chimeric receptors should help disclose the nature of toxin-receptor interactions further. Ultimately it should be possible to determine the exact structure of such complexes by X-ray crystallography.

Labeling of m1-toxin A number of different methods for radio-iodination have been tested, and while these produced radioactive m1-toxin, the toxin was inactivated. For this reason further studies are being attempted with tritiated and fluorescent m1-toxin, antibodies to m1-toxin, and toxin labeled during gene expression.

Use of m1-toxin for anatomical studies We have established by light microscopic autoradiography that 50-100 nM m1-toxin blocks the binding of 1 nM ^{3}H-QNB to muscarinic receptors in parts of the brain like the dentate gyrus and CA1 region of the hippocampus which are known to be particularly rich in m1 receptors, while having no effect on m2 receptors in places like the brainstem. When the toxin is used with concentrations of ^{3}H-pirenzepine that normally label many m1 receptors and a few m4 receptors, the pattern of labeled receptors is the same as that obtained with antibodies against m4 receptors (1). These studies indicate that the binding of m1-toxin remains specific after the freezing, sectioning, thawing and drying of membranes.

m1-Toxin has several properties which are ideal for further anatomical studies: it diffuses readily in intact living tissue, it binds with very high specificity to receptors at synapses, it binds irreversibly so that excess toxin can be fully removed, it can be fixed on receptors allowing subsequent thin-sectioning of tissues for anatomical studies at very high resolution, and the toxin can be recognized with a variety of techniques. These properties should allow detailed examination of the exact locations and numbers of functionally-active m1 receptors on individual nerve cells, without interference from the same receptors at sites of synthesis or passage.

Use of m1-toxin for functional studies Provided that adequate supplies of m1-toxin can be produced, there is reason to believe that m1-toxin will be the antagonist of choice for studying the physiological, pharmacological and biochemical effects of activating m1 receptors. The toxin should also prove useful for completely blocking m1 receptors so that other receptors, especially m4 receptors, can be studied in detail. Since the number of receptor subtypes is then more limited, the use of other, partially selective antagonists for receptor identification is improved. With luck, m1-toxin will prove useful for blocking m1 receptors in the brains of laboratory animals, so that some idea of the behavioral effects of such blockade can be established.

Use of m1-toxin for biochemical, biophysical and binding studies When labeled m1-toxin becomes generally available, it may prove the ligand of choice for counting m1 receptors. Alternatives include MTX1 and MTX2. m1-Toxin is particularly effective for biochemical studies of receptors in solution because it retains its selectivity for m1 receptors, unlike pirenzepine. The allosteric nature of the binding of the toxin should prove useful for stabilizing receptor complexes for biophysical work, and for such unusual purposes as purifying already labeled receptors, and recognizing receptors with genetically-altered primary binding sites.

The ability of m1-toxin to completely block one population of receptors has been useful for several studies. In one series of experiments we have used m1-toxin to block the 40% of muscarinic receptors in the rat striatum which are m1 receptors, leaving 55% of the original total as m4 receptors and about 5% as m2 receptors. This approach permits direct studies of nearly pure m4 receptors associated with normal amounts of their native G proteins. Competition studies between agonists and ^{3}H-NMS for toxin-spared m4 receptors show equal numbers of high and low affinity agonist-binding sites, which suggests dimeric receptors (see 23), and show that oxotremorine-M is the common cholinergic agonist which has the highest affinity and largest K_I/K_H ratio for m4 receptors. This permits direct binding studies and autoradiography of m4 receptors with ^{3}H-oxotremorine-M. Measurements of the dissociation of ^{3}H-pirenzepine show that it dissociates as slowly from m4 as m1 receptors; this permits the combination of m1-toxin and low concentrations of ^{3}H-pirenzepine to be used for the autoradiography of m4 receptors.

In other experiments we have examined the effect of blocking m1 receptors on the binding of 5.0 nM ^{3}H-oxotremorine-M at 25°C to membranes from the rat cortex, because of a lingering question as to whether this ligand is ever useful for direct binding studies of m1 receptors associated with their native G protein(s). The rat cortex shows primarily m1, m4 and m2 receptors (1-3). Blockade of m1 receptors with m1-toxin had no effect on the binding of ^{3}H-oxotremorine-M, in keeping with evidence that the binding of this agonist is usually limited to m2 and m4 receptors, because of its rapid dissociation from m1 receptors. We conclude that this agonist should not be used at low concentrations for binding studies of m1 receptors at 25°C. The binding of oxotremorine-M to m1 receptors is readily studied by competition between the agonist and 1 nM ^{3}H-pirenzepine (17), and its affinity for the guanine nucleotide-sensitive high affinity conformation of m1 receptors would seem to be high enough to allow the toxin to stabilize agonist-receptor complexes. Since we have not seen such stabilization so far, the question arises as to whether m1-toxin may prove useful for distinguishing the high affinity conformation of m1

receptors (for agonists) from the lower affinity conformations of the receptor when it is unoccupied, occupied by an antagonist or occupied at low affinity by an agonist.

Adaptive value of m1-toxin for the green mamba Although m1-toxin is only a trace component of the venom of the green mamba, it is intriguing to ask what benefit the toxin might have for the life of these snakes that would warrent its selection during evolution. The snakes are arboreal, and may use their venom to paralyze frogs, birds and small mammals. One possibility is that a bite to the head injects m1-toxin into the brain, where m1 receptors are important. Another is that m1-toxin, injected peripherally, is capable of blocking that aspect of neurotransmission which is facilitated by m1 receptors in adrenergic and cholinergic ganglia and in the adrenal medulla. Partial blockade of adrenergic fight-fright-flight responses would clearly have adaptive advantage. So far, clinical studies with the ganglionic m1 antagonist, pirenzepine, and experimental work with the m1 agonist, McN-A-343, have not yielded a full answer as to the possible importance of m1 receptors in ganglia. Perhaps the use of m1-toxin in whole animals will yield this answer.

References

1. A.I. LEVEY, C.A. KITT, W.F. SIMONDS, D.L. PRICE and M.R. BRANN, J. Neurosci. 11 3218-3226 (1991).
2. M. LI, S.S., WALL, R.P. YASUDA, W. CIESLA and B.B. WOLFE, Neurosci. Abstr. 17 1532 (1991).
3. S.J. WALL, R.P. YASUDA, M. LI, W. CIESLA and B.B. WOLFE, Dev. Brain Res. 66 181-185 (1992).
4. N.J. BUCKLEY, T.I. BONNER, C.M. BUCKLEY and M.R. BRANN, Mol. Pharm. 35 469-476 (1989).
5. F. DÖRJE, J. WESS, G. LAMBRECHT, R. TACKE, E. MUTSCHLER and M.R. BRANN, J. Pharm. Exp. Ther. 256 727-733 (1991).
6. S.I. MAX, Doctoral Dissertation, University of Miami, Miami, FL (1992).
7. S.I. MAX, J.S. LIANG, and L.T. POTTER, Neurosci. Abstr. 17 389 (1991).
8. S.L. PURKERSON, J.S. LIANG, S.I. MAX, and L.T. POTTER, Neurosci. Abstr. 17 390 (1991).
9. S.I. MAX, J.S. LIANG, and L.T. POTTER, Neurosci. Abstr. 18 1142 (1992).
10. S.L. PURKERSON, H.E. HANCHETT, and L.T. POTTER, Neurosci. Abstr. 18 1144 (1992).
11. R. MILEDI and L.T. POTTER, Nature 233 599-603 (1971).
12. A. ADEM, A. ASBLOM, G. JOHANSSON, P.M. MBUGUA and E. KARLSSON, Biochim. Biophys. Acta 968 340-345 (1988).
13. E. KARLSSON, C. RISINGER, M. JOLKKONEN, C. WERNSTEDT and A. ADEM, Toxicon 29 521-526 (1991).
14. D. JERUSALINSKY, C. CERVENANSKY, C. PENA, S. RASKOVSKY and F. DAJAS, Neurochem. Int. 20 237-247 (1992).
15. A. ADEM, N. BOGDANOVIC, A. ISLAM, B. WINBLAD and E. KARLSSON, (Abstract in this symposium).
16. L.T. POTTER, D.D. FLYNN, H.E. HANCHETT, D.L. KALINOSKI, J. LUBER-NAROD and D.C. MASH, Trends Pharm. Sci. Suppl. 22-31 (1984).
17. L.T. POTTER, C.A. FERRENDELLI and H.E. HANCHETT, Cell. Molec. Neurobiol. 8 181-191 (1988).
18. D.D. FLYNN and L.T. POTTER, Mol. Pharm. 30 193-199 (1986).
19. D. TSERNOGLOU and G.A. PETSKO, FEBS Lett. 68 1-4 (1976).

20. D. TSERNOGLOU, G.A. PETSKO and R.A. HUDSON, Mol. Pharm. 14 710-716 (1978).
21. F. DUCANCEL, E.G. ROWAN, E. CASSAR, A.L. HARVEY, A. MENEZ and J.C. BOULAIN, Toxicon 29 516-520 (1991).
22. E.C. HULME, N.J.M. BIRDSALL and N.J. BUCKLEY, Ann. Rev. Pharmacol. Toxicol. 30 633-673 (1990).
23. L.T. POTTER, L.A. BALLESTEROS, L.H. BICHAJIAN, C.A. FERRENDELLI, A. FISHER, H.E. HANCHETT and R. ZHANG, Mol. Pharmacol. 39 211-221 (1991).

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IMMUNOLOGICAL LOCALIZATION OF m1-m5 MUSCARINIC ACETYLCHOLINE RECEPTORS IN PERIPHERAL TISSUES AND BRAIN

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Summary

Knowledge of the distributions and functions of native m1-m5 muscarinic acetylcholine receptors in tissues is limited. To characterize the family of m1-m5 proteins directly, a panel of subtype-selective antibodies was generated against divergent i3 loop-fusion proteins. Each antibody was shown to bind a single cloned receptor specifically. In peripheral tissues and brain, four receptor proteins (m1-m4) were found to account for the vast majority of the muscarinic binding sites using immunoprecipitation studies with the subtype-specific antibodies. The subtypes were differentially distributed, although most tissues were comprised of a complex mixture of receptors. Moreover, within tissues there were major differences in the precise localization of the subtypes, as determined by immunocytochemistry. The immunological methods described offer a novel approach with exquisite sensitivity and specificity for delineating the distribution of m1-m5 receptors in animal and human tissues.

Molecular cloning of five muscarinic acetylcholine receptor genes (m1-m5) has led to dramatic advances in understanding the structure and pharmacology of the encoded receptor proteins (1-6). Nevertheless, knowledge of the distribution and functions of the native receptor subtypes in different tissues has lagged behind. Several muscarinic receptor genes are expressed in most tissues, and at the extreme, all five mRNAs are present in hippocampus (7-9). Detailed kinetic binding studies have also provided evidence for four distinct binding sites in brain homogenates (10). Thus, the receptor repertoire in different tissues is likely to be a pharmacologically complex mixture of m1-m5 proteins. Because conventional pharmacological approaches cannot readily distinguish m1-m5 (4,11-13), the identities of the receptor proteins in tissues are uncertain. Moreover, conclusions about the molecular identity (i.e., m1-m5) of any particular receptor subclass (i.e., M_1-M_4) involved in ligand binding and/or behavioral and tissue effects of "selective drugs" are only tentative.

Immunological methods offer a novel approach for direct characterization of m1-m5 in tissues. Cloning of the receptors and identification of regions of divergence have made it possible for us to target antibodies to all of the individual receptor subtypes (14,15). Our strategy has been to use recombinant proteins, consisting of unique regions of the five receptors, for immunization of rabbits and purification of the antibodies. The antibodies produced against each fusion protein react with the respective cloned receptor, and appear to be completely subtype-specific. This panel of antibodies to m1-m5 has allowed us to identify and quantify the native receptor subtypes in peripheral tissues (16) and brain (15). The same approach has been used by Wolfe and colleagues to identify several of the native receptor subtypes (17,18). We have also developed immunocytochemical methods for the localization of the receptors with exquisite sensitivity and specificity (15). Here we briefly review the production and characterization of the antibodies, and discuss their use for localization of m1-m5 in peripheral tissues and in brain.

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Production and Characterization of Antibodies to m1-m5

The i3 loops of m1-m5 are excellent targets for subtype-specific antibodies. These regions are highly divergent, large (ranging from 157-203 amino acids), and predicted to be antigenic (19,20). The corresponding segments of the human cDNAs were subcloned into a bacterial expression vector, pGEX-2T. In the recombinant vectors, the receptor i3 loops are fused at their N-terminus to the *Schistosoma japonicum* gene encoding glutathione S-transferase (GST). The i3 loop-GST fusion proteins were expressed in bacteria, affinity-purified on immobilized glutathione, and used to immunize rabbits. Polyclonal antibodies reactive with each i3 loop were affinity-purified using the respective purified fusion proteins.

The specificities of antibodies to the i3 loops have been established by immunoblotting and immunoprecipitation. Immunoblotting with the five fusion proteins has confirmed that the i3 loops are immunologically unrelated. Immunoprecipitation assays have demonstrated the each antibody binds specifically to a single cloned receptor (15), and binding was blocked by the homologous but not heterologous fusion proteins. These experiments demonstrated that antibodies generated against recombinant i3 loops were able to quantitatively bind functional muscarinic receptors with virtually complete subtype-specificity. As described below, immunoprecipitation and immunocytochemical studies also establish the reactivities of the antibodies with the native proteins and have been used to characterize m1-m5 in animal tissues.

Muscarinic Receptor Subtypes in Peripheral Tissues

Regional Distribution of m1-m5 Proteins by Immunoprecipitation

The compositions of muscarinic receptor proteins markedly differ among various peripheral tissues as determined by the immunoprecipitation method described above (16). The distribution and abundance of m1-m5 proteins in rabbit peripheral tissues are in general agreement with pharmacological studies of the binding sites and mRNA expression, as shown in the Table. For example, in atria, the m2 protein accounted for about 90% of the solubilized receptors, with little or none of the other subtypes recovered (16). These findings agree with other immunological studies (18,21) and reports of homogeneous populations of M_2 binding sites and m2 mRNA expression in heart (4,22,23). In salivary glands, immunoprecipitation of m1 and m3 proteins correlates with pharmacological identification of M_1 and M_3 subclasses and expression of m1 and m3 mRNA (4,22,23). These data demonstrate that in certain situations, the correspondence between molecular and pharmacological classifications is good. However, closer inspection of the composition of receptor proteins in this and other tissues reveals significant differences with the distribution of the binding sites and the mRNA.

TABLE I

Muscarinic Proteins, mRNA, and Binding Sites in Peripheral Tissues

Tissue	m1-m5 Proteins[a]	m1-m5 mRNA[b]	M_1-M_4 Binding[c]
Heart	m2 (88%)	m2	M_2
Salivary Glands	m3 (42%)	m3	M_3
	m1 (36%)	m1	M_1
	m2 (12%)		M_2
	m4 (7%)		
Ileum	m2 (69%)	m2	M_2
	m4 (12%)		M_3
	m3 (4%)	m3	
	m1 (3%)		
Lung (peripheral)	m4 (41%)	m4	M_4
	m2 (40%)		

[a]Reference 16 [b]References 4,23 [c]References 22,24-27

Comparison of Receptor Protein, mRNA, and Ligand Binding Sites

The distributions of muscarinic receptor subtype mRNA and protein show some differences. In salivary glands, the m2 and m4 receptor proteins have been detected, but not the respective mRNA. In ileum, the m1 and m4 proteins are present without the mRNAs. Similarly, in lung, the m2 protein is present but not the transcript. Several factors might account for these apparent discrepancies. Even small differences in subregions of tissues analyzed are likely to be important (e.g., different glands, different layers of intestinal tract, and peripheral lung versus larger airways may all have distinct compositions of receptors). Due to varying translational efficiencies and turnover rates, there also may be significant differences in the levels of mRNA and protein, as well as differences in the sensitivities of the techniques used to detect them. Some differences in the location of mRNA vs. protein are also expected because of protein transport. For example, most or all peripheral tissues are richly innervated by sympathetic and/or parasympathetic nerves. Presynaptic receptor proteins on these nerve fibers will be detected in the tissues by immunologic and binding studies. However, because mRNA is spatially restricted to cell bodies, the receptor mRNAs may be extrinsic to the tissues (e.g., in sympathetic ganglia).

Many peripheral tissues have a more complex mixture of receptor proteins than would be predicted from ligand binding studies. In salivary glands, although m1 and m3 are the most abundant subtypes, the m2 and m4 proteins constitute about 20% of the total receptors (16). These findings, together with the pharmacology of the clones (12,13), suggests that the M_2 binding sites in salivary gland (22) might consist of m2 and/or m4 proteins. In addition, m4 protein may contribute to M_1 and possibly M_3 binding sites (12,13). Similar difficulties with attempts to use pharmacological methods to distinguish m1-m5 are evident in other tissues. In ileum, although there are high levels of m2 protein (16,28) and M_2 binding (25,26), the m1, m3, and m4 receptors are also present (16,28). Therefore, the relative contributions of these four proteins to the M_2 and M_3 binding sites in ileum (25,26) are presently unclear. In peripheral lung, the homogeneity of M_4 binding sites (27) belies the presence of m2 and m4 proteins.

Localization of m1-m5 by Immunocytochemistry

Discordance between the results of protein, mRNA, and binding site distributions in peripheral tissues highlights the importance of precise localization of the receptors. Immunocytochemistry has great potential for cellular and subcellular localization of the receptor proteins (15). Examples of the localization of m1, m2, and m4 receptors are shown in Fig. 1.

FIG. 1
Immunocytochemical localization of m1, m2, and m4 in select peripheral tissues of the rat. Shown are m1 receptor in gastric mucosa (left), m2 receptor in heart (middle), and m4 receptor in colon (right). See text for details.

The gastric mucosa of the rat is highly enriched in m1 immunoreactivity, as shown in Figure 1. Interestingly, the m1 receptor is selectively present in the superficial part of the gastric glands (above the dashed line). This indicates that the receptor expression is highly selective in certain epithelial cell types and may be related to maturation of the cells (which divide deep in the crypts). Localization of m1 in stomach suggests this protein may be the site of action for M_1 antagonists, such as pirenzepine, which inhibit gastric acid secretion. As expected, the m2 protein is localized in rat heart. However, the results demonstrate that much of the receptor may be within autonomic nerves (arrows) in addition to muscle cells. Discrete clusters of m2 are observed, suggesting possible subcellular specialization in the cardiac myocyte, although ultrastructural localization would be needed to clarify this localization. The m4 protein is present in nerve fibers that invest the colon, suggesting a presynaptic localization and a possible role in the regulation of neurotransmitter release. These examples illustrate that receptor subtypes are discretely localized within tissues, and can provide important clues to the functions of the receptors. This approach will be fruitful for future investigations of the muscarinic receptor subtypes in peripheral tissues.

Muscarinic Receptor Subtypes in the CNS

Regional Distribution of m1-m5 by Immunoprecipitation

Immunoprecipitation studies in rat brain have demonstrated that m1, m2, and m4 are the most abundant receptor subtypes, and that there are marked regional differences in the exact composition of receptors (15), as shown in Table II.

TABLE II
Muscarinic Proteins, mRNA, and Binding Sites in Rat CNS

Tissue	m1-m5 Proteins[a]	m1-m5 mRNA[b]	M_1-M_4 Binding[c]
Cortex	m1 (34-40%) m2 (19-37%) m4 (15%) m3 (10%)	m1 m2 m4 m3	M_1 (35%) M_2 (11%) M_4 (33%) M_3 (11%)
Hippocampus	m1 (47%) m2 (17%) m4 (15%) m3 (10%)	m1 m2 m4 m3 m5	M_1 (55%) M_2 (12%) M_4 (16%) M_3 (11%)
Striatum	m4 (29%) m1 (29-31%) m2 (12-29%) m3 (6%)	m4 m1 m2 m3	M_4 (46%) M_1 (27%) M_2 (9%) M_3 (8%)
Thalamus	m2 (42-49%) m1 (6-16%) m4 (15%) m3 (6%)	m2 m1 m4 m3 m5	not reported
Brainstem and Cerebellum	m2 (70-84%) m3 (5-6%) m1 (2-5%) m4 (1%)	m2 m3	M_2 (80-90%)

[a]References 15,17,18,28 [b]References 7-9,29-31 [c]References 10,24

There is very good agreement between our findings and those of Wolfe and colleagues using different antibodies to the m1 and m2 subtypes (17,18). The m1 protein is abundant in the cerebral cortex, hippocampus, and striatum, with lower levels in thalamus and hindbrain. The m2 receptor is also present in these regions, and is the predominant subtype in thalamus and hindbrain. We have also detected high levels of the m4 receptor, particularly in striatum (15). Although we did not observe the m3 subtype in our initial studies, the m3 receptor accounts for a small proportion (5-10%) of total binding sites throughout the brain (28, K.Frey and A. Levey, unpublished observations). The m5 receptor has yet to be detected, possibly because it is expressed at very low levels and in select brain regions (9,29). Although these data have all been obtained in rat brain, we have observed a similar distribution of m1, m2, and m4 in human brain (A. Levey, unpublished observations).

Comparison of Receptor Protein, mRNA, and Ligand Binding Sites

The regional distributions of m1-m4 are in good agreement with mRNA and recent estimates of the proportions of M_1-M_4 binding sites by Waelbroeck et al. (10,24)(Table II). However, because each region contains most receptors, the above comparison does not establish a one-to-one correspondence between the molecular subtypes, m1-m4, and the pharmacological classification, M_1-M_4. There are also some significant differences in the ratios of m2/M_2 and m4/M_4 in cortex, suggesting that different populations of receptors might be measured by these methods. Detailed comparisons are probably not warranted, though, because small differences in sampling techniques between studies could lead to dramatic differences in the apparent composition of subtypes (due to the regional heterogeneity of cerebral cortex and other brain regions). For this reason, it would be more profitable to compare tissue localization of the receptors (e.g., by immunocytochemistry and ligand autoradiography) to determine if the molecular and pharmacological classifications are equivalent. Unfortunately, the lack of selective ligands makes equilibrium binding studies for M_1-M_4 troublesome at present.

Localization of m1-m5 by Immunocytochemistry

The differential expression of all five muscarinic receptor mRNAs, and at least m1-m4 proteins in brain, together with the complex organization of the central nervous system, pose major obstacles to a better understanding of the distribution and functions of the subtypes. However, immunocytochemistry provides the resolution necessary to begin to unravel the cellular and synaptic organization of the muscarinic receptor family. Detailed descriptions of the localization of the receptors in brain are beyond the scope of this article. Initial studies have described the localization of m1, m2, and m4 in rat brain (15). Here, select examples of m1-m4 are shown to demonstrate the utility of this approach.

Localization of m1-m4 immunoreactivities in coronal sections of rat forebrain are shown in Figure 2. Despite the co-existence of all four receptors in cerebral cortex, hippocampus, striatum, thalamus, amygdala, and hypothalamus, the subtypes have highly complementary distributions within each region. For instance, the receptor proteins, like the mRNAs (7,8,29), are enriched in different regions and lamina in the cerebral cortex. Also note the localization of m2, m3, and m4 in almost reciprocal structures within both the thalamus and hypothalamus. In hippocampus, despite the presence of each mRNA within the pyramidal neuron layer, the receptor proteins are localized specifically in different CA regions and lamina. Possibly, the amino acid sequence of each receptor protein may control the subcellular distribution of the receptors. These findings indicate that the receptors must have unique roles in neural processing within each region of brain.

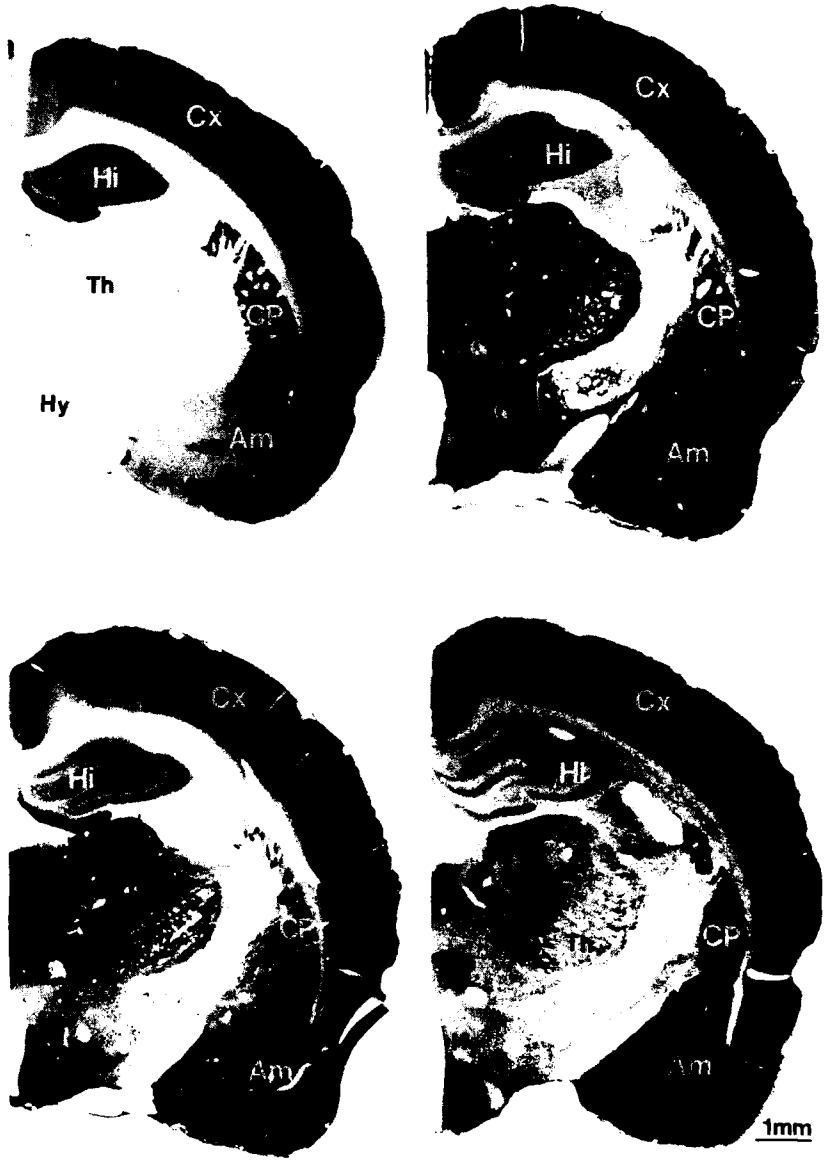

FIG. 2
Immunocytochemical localization of m1-m4 in rat forebrain. Note that m1 (upper left), m2 (upper right), m3 (lower left), and m4 (lower right) receptors are all differentially distributed. Abbreviations: Am, amygdala; CP, caudate-putamen; Cx, cortex; Hi, hippocampus; Hy, hypothalamus; Th, thalamus.

Conclusions

A panel of antibodies has been developed that bind the five cloned muscarinic receptors with virtually complete subtype-specificity. These antibodies have provided an unprecedented

opportunity to characterize the muscarinic receptor family in peripheral tissues and brain. Immunoprecipitation studies can be used to quantify the composition of native subtypes in tissues, and immunocytochemistry can be used to localize the receptors. Utilization of these methods with electron microscopy will also enable determination of the pre- and postsynaptic locations of the subtypes in different tissues. Potentially, these immunological methods can also be used to study the receptors in human tissues directly. Ultimately, this approach may help identify the best targets for more selective muscarinic drugs and guide their therapeutic applications.

Acknowledgments

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References

1. T. KUBO, K. FUKUDA, A. MIKAMI, A. MAEDA, H. TAKAHASHI, M. MISHINA, T. HAGA, K. HAGA, A. ICHIYAMA, K. KANGAWA, M. KOJIMA, H. MATSUO, T. HIROSE and S. NUMA, Nature 323 411-416 (1986).
2. T. KUBO, A. MAEDA, K. SUGIMOTO, I. AKIBA, A. MIKAMI, H. TAKAHASHI, T. HAGA, A. HAGA, A. ICHIYAMA, K. KANGAWA, K. MATSUO, T. HIROSE and S. NUMA, FEBS Lett. 209 367-372 (1986).
3. T.I. BONNER, N.J. BUCKLEY, A.C. YOUNG and M.R. BRANN, Science 237 527-532 (1987).
4. E.G. PERALTA, A. ASHKENAZI, J.W. WINSLOW, D.H. SMITH, J. RAMACHANDRAN and D.J. CAPON, EMBO J. 6 3923-3929 (1987).
5. T.I. BONNER, A.C. YOUNG, M.R. BRANN and N.J. BUCKLEY, Neuron 1 403-410 (1988).
6. E.C. HULME, N.J.M. BIRDSALL and N.J. BUCKLEY, Annu. Rev. Pharmacol. Toxicol. 30 633-73 (1990).
7. N.J. BUCKLEY, T.I. BONNER and M.R. BRANN, J. Neurosci. 8 4646-4652 (1988).
8. D.M. WEINER and M.R. BRANN, Trends. Pharmacol. Sci. (Suppl,Subtypes of Muscarinic Receptors IV) 10 115 (1989).
9. M.T. VILARO, J.M. PALACIOS and G. MENGOD, Neurosci. Lett. 114 154-159 (1990).
10. M. WAELBROECK, M. TASTENOY, J. CAMUS and J. CHRISTOPHE, Mol. Pharmacol. 38 267-273 (1990).
11. I. AKIBA, T. KUBO, A. MAEDA, H. BUJO, J. NAKAI, M. MISHINA and S. NUMA, FEBS Lett. 235 257-261 (1988).
12. N.J. BUCKLEY, T.I. BONNER, C.M. BUCKLEY and M.R. BRANN, Mol. Pharmacol. 35 469-476 (1989).
13. F. DORJE, J. WESS, G. LAMBRECHT, R. TACKE, E. MUTSCHLER and M.R. BRANN, J. Pharmacol. Exp. Ther. 256 727-733 (1991).
14. A.I. LEVEY, T.M. STORMANN and M.R. BRANN, FEBS Lett. 275 65-69 (1990).
15. A.I. LEVEY, C.A. KITT, W.F. SIMONDS, D.L. PRICE and M.R. BRANN, J. Neurosci. 11 3218-3226 (1991).
16. F. DORJE, A.I. LEVEY and M.R. BRANN, Mol. Pharmacol. 40 459-462 (1991).
17. S.J. WALL, R.P. YASUDA, F. HORY, S. FLAGG, B.M. MARTIN, E.I. GINNS and B.B. WOLFE, Mol. Pharmacol. 39 643-649 (1991).
18. M. LI, R.P. YASUDA, S.J. WALL, A. WELLSTEIN and B.B. WOLFE, Mol. Pharmacol. 40 28-35 (1991).
19. R.A. LERNER, Adv. Immunol. 36 1-44 (1984).
20. P. STERN, TIBTECH 9 163-169 (1991).
21. C.S.K. MAYANIL, R.M. RICHARDSON and M.M. HOSEY, Mol. Pharmacol. 40 900-907 (1991).

22. H.N. DOODS, M.J. MATHY, D. DAVIDESKO, K.J. VAN CHARLDORP, A. DE JONGE and P.A. VAN ZWIETEN, J. Pharmacol. Exp. Ther. 242 257-262 (1987).
23. A. MAEDA, T. KUBO, M. MISHINA and S. NUMA, FEBS Lett. 239 339-342 (1988).
24. M. WAELBROECK, M. GILLARD, P. ROBBERECHT and J. CHRISTOPHE, Mol. Pharmacol. 30 305-314 (1986).
25. E. GIRALDO, E. MONFERINI, H. LADINSKY and R. HAMMER, Eur. J. Pharmacol. 141 475-477 (1987).
26. A.D. MICHEL and R.L. WHITING, Eur. J. Pharmacol. 145 305-311 (1988).
27. S. LAZARENO, N.J. BUCKLEY and F. ROBERTS, Mol. Pharmacol. 38 805-815 (1990).
28. S.J. WALL, R.P. YASUDA, M. LI and B.B. WOLFE, Mol. Pharmacol. 40 783-789 (1991).
29. D.M. WEINER, A.I. LEVEY and M.R. BRANN, Proc. Natl. Acad. Sci. U. S. A. 87 7050-7054 (1990).
30. M.T. VILARO, K.-H. WIEDERHOLD, J.M. PALACIOS and G. MENGOD, Neuroscience 40 159-167 (1991).
31. M.T. VILARO, K.H. WIEDERHOLD, J.M. PALACIOS and G. MENGOD, Neuroscience 47 367-393 (1992).

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PHARMACOLOGICAL CHARACTERIZATION OF GUANINE NUCLEOTIDE EXCHANGE REACTIONS IN MEMBRANES FROM CHO CELLS STABLY TRANSFECTED WITH HUMAN MUSCARINIC RECEPTORS M1-M4.

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Summary

We have studied muscarinic agonist stimulated $[^{35}S]GTP\gamma S$ binding and $[\gamma^{32}P]GTP$ hydrolysis (GTPase) in membranes from CHO cells stably transfected with human muscarinic m1-m4 receptors. 'Full' agonists were at least 10-fold more potent at m2 & m4 receptors than at m1 & m3. This pattern was less marked with 'partial' agonists, which had a greater maximal effect at m2 & m4 than at m1 & m3. McN-A343 uniquely was more potent and efficacious at m4 than at m2 receptors. Antagonist affinity constants were estimated by fitting the data from inhibition curves directly to the Schild model. Antagonist affinity estimates were very similar to those measured earlier in binding studies using animal tissues, and confirmed a small degree of m4 selectivity for tropicamide and secoverine. The receptor subtypes activated more than one G-protein subtype; m2 & m4 receptors activated only pertussis (PTX) sensitive G-proteins, while m1 & m3 coupled to both PTX sensitive and insensitive G-proteins. Acetylcholine (ACh) was more potent in stimulating guanine nucleotide exchange in PTX-treated m1 cells than in controls.

The binding of an agonist to muscarinic receptors increases the binding of receptors to G-proteins, which in turn increases the release of GDP from the G-protein and the binding and subsequent hydrolysis of GTP. Guanine nucleotide exchange is therefore among the first measurable G-protein-mediated responses in the signal transduction cascade following muscarinic receptor activation. We have assessed the pharmacology of the exchange process stimulated by activation of human muscarinic receptor subtypes stably transfected into CHO cells. Two measures were used: the hydrolysis of $[\gamma^{32}P]GTP$ by high affinity GTPase, and binding of $[^{35}S]GTP\gamma S$, both using a crude membrane preparation. These measures allow comparison of agonist action at different subtypes using the same response and assay conditions, in contrast to whole cell or tissue assays which require measurement of different responses for the different subtypes. These guanine nucleotide exchange measures provide a convenient, rapid and accurate functional assay system for human muscarinic receptor subtypes. In addition, it is hoped that the relative simplicity of the responses will allow a better understanding of the molecular processes underlying the the pharmacological concepts of apparent agonist affinity and efficacy.

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Methods

CHO cells stably transfected with human m1, m2, m3 or m4 receptors (1) were a gift from Dr. N.J.Buckley. Membranes washed in 20 mM HEPES + 10 mM EDTA were stored in 20 mM HEPES + 0.1 mM EDTA at -70°C and thawed before use. The assay buffer was 20 mM HEPES + 100 mM NaCl + 5 (GTPase) or 10 (GTPγS binding) mM $MgCl_2$. Buffers were pH 7.4.

For the GTPase assay, 5 µg membrane protein was incubated in buffer + 1 mM ATP + [γ^{32}P]GTP (10-100 nM or as indicated) + drugs in a volume of 100 µl at 30°C for 15 minutes. The reaction was stopped by addition of 750 µl of a slurry of 5% charcoal in 20 mM H_2PO_3 + 1 mg/ml BSA. After centrifugation (20,000xg 5 minutes) an aliquot of the supernatant, containing released labelled phosphate, was counted for radioactivity.

For the GTPγS binding assay, 15-50 µg membrane protein was incubated in buffer + GDP (0.1 µM for m1 and m3 receptors, 1 µM for m2 and m4 receptors, or as indicated) + [^{35}S]GTPγS (50-100 pM) + drugs in a volume of 1 ml at 30°C for 30 minutes. Bound label was collected by rapid filtration using a Brandel cell harvester and counted for radioactivity.

When antagonists were used, they were equilibrated with ACh and membranes for 30 minutes before the addition of label. Preincubation with ACh did not reduce its potency or maximal effect.

Data were analysed by non-linear regression analysis (SigmaPlot, Jandel).

Results

GTPγS binding

GDP was required to increase the absolute agonist response (m2 & m4) or to increase the ratio of response to basal activity (m1 & m3) (figure 1). The optimal GDP concentrations were 0.1 µM (m1 & m3) and 1 µM (m2 & m4). GDP also reduced the potency of ACh. The pEC_{50} values for GDP inhibiting ACh potency at m1 & m4 were 6.8 and 6.4 respectively.

Basal GTPγS (0.1 nM) binding appeared to be linear with time up to 3 hours, and ACh-stimulated binding had a half time for association greater than an hour (data not shown). The effect of ACh was catalytic, i.e. the maximal concentration of stimulated GTPγS binding was greater than the maximal concentration of receptors (data not shown).

The subtype-dependent requirement for GDP in the GTPγS binding assay and its effect on ACh potency limit the usefulness of the assay in comparing agonists between subtypes. There should be no problem, however, in using the assay to assess antagonist affinity by the method of Schild (2). Figure 2 shows activation curves for ACh in the presence of various concentrations of pirenzepine and Schild plots of the data.

In further experiments, 8 antagonists were studied by constructing inhibition curves against a fixed ACh concentration. The data were fitted directly to the Schild model (3,4). The Schild slope factors were close to 1 and were constrained to 1 for K_b estimation. Figure 3 shows the pK_b estimates. The results are in complete agreement with pK_i estimates from binding studies (5,6). Points of interest are: 1) Pirenzepine shows a rank order of affinity m2<=m3<=m4<m1, while himbacine shows a rank order m1=m3<m2=m4, so this pair of drugs can be used to distinguish between m1-m4 receptor subtypes. 2) Tropicamide and secoverine had a small degree of overall m4 selectivity.

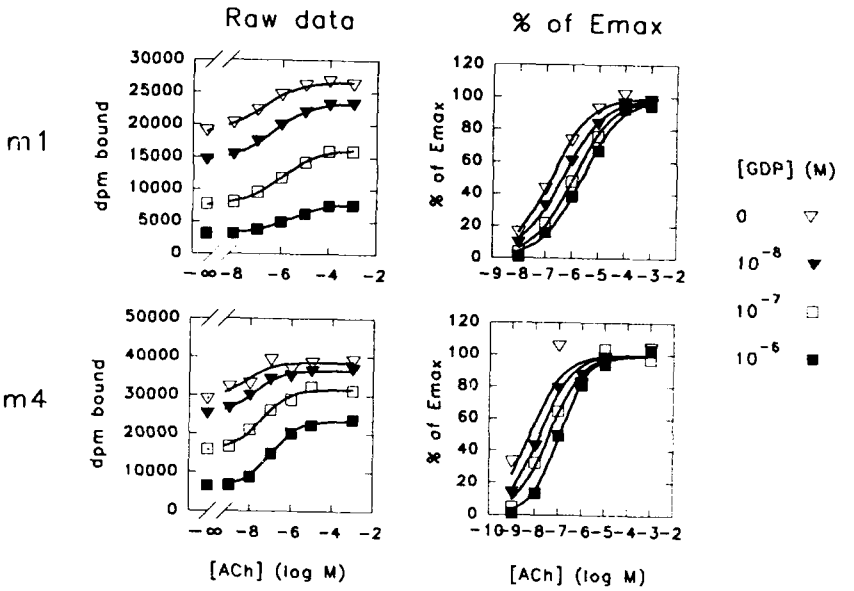

FIG. 1

Effect of GDP on acetylcholine stimulated GTPγS binding in m1 and m4 CHO cell membranes

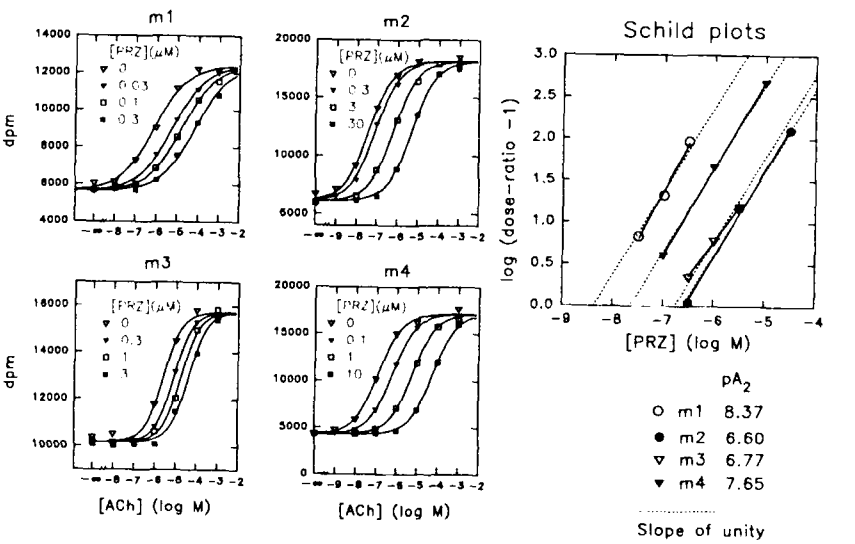

FIG. 2

Acetylcholine stimulation of GTPγS binding at m1-m4 receptors in the presence of various concentrations of pirenzepine, and the corresponding Schild plots.

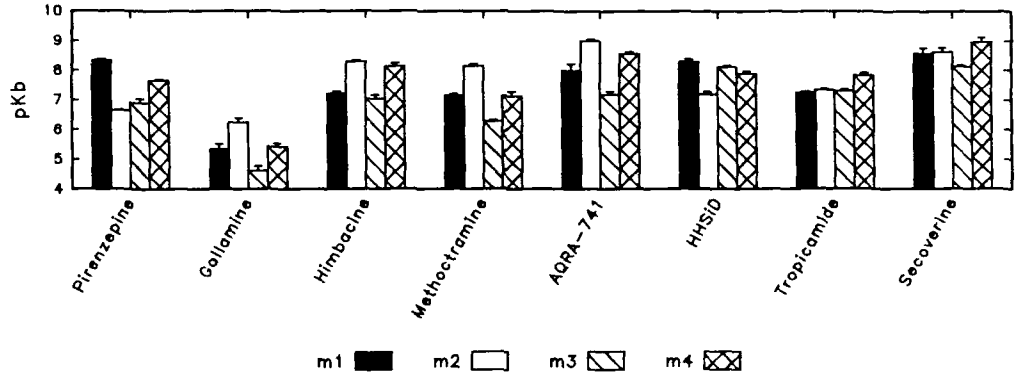

FIG. 3

Log affinity estimates of antagonists at muscarinic receptors. Antagonist titrations were performed on [^{35}S]GTPγS binding stimulated by a fixed acetylcholine concentration and the data were fitted directly to the Schild model. Results are means and s.e.m of 2-5 experiments.

GTPase activity

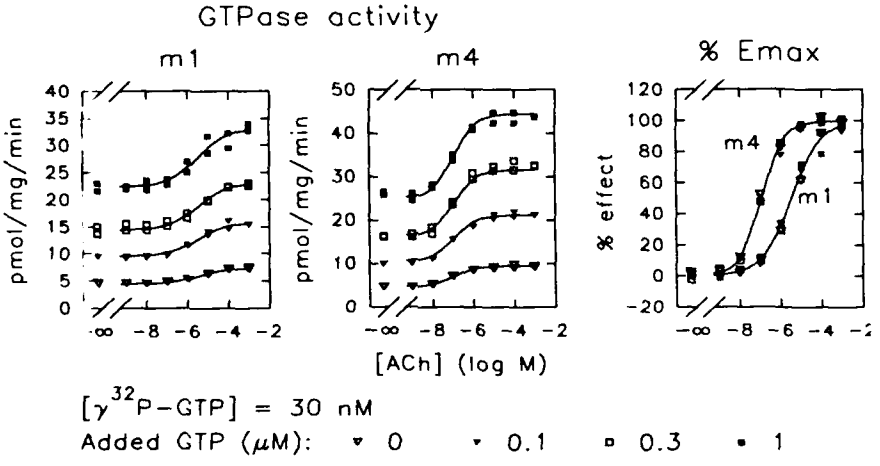

FIG. 4

Effect of substrate (GTP) concentration on stimulation of GTPase activity by acetylcholine in membranes containing m1 or m4 receptors.

The receptor densities of membranes containing m1-m4 receptors were 7, 1, 5, and 3 pmol/mg protein respectively. At a [^{32}P]GTP concentration of 30 nM the stimulation of GTPase activity over basal activity by 1 mM ACh was 116, 54, 88, and 108% respectively. Low affinity GTPase was unaffected by ACh and was defined as GTPase measured in the presence of 30 μM GTP.

The potency of ACh was unaffected by substrate concentrations up to 1 μM (figure 4).

Titrations of eight agonists were performed. The stimulation was expressed as a percent of the effect of 1 mM ACh, which was measured in each assay. The pEC_{50} and Emax values are shown in figure 5. ACh, carbachol and oxotremorine-M were 'full' agonists causing maximal effects of about 100% at all four subtypes. These agonists were at least 10-fold more potent at m2 & m4 receptors than at m1 & m3 receptors. Methyl furmethide showed a similar pattern but had an Emax of about 70% at m1 & m3 receptors. Oxotremorine and arecoline were also 'full' at m2 & m4 receptors, but 'partial' at m1 & m3. Pilocarpine and McN-A 343 were 'partial' at all subtypes, but showed dissimilar patterns of selectivity: 1) they had similar Emax values at m1 receptors, but at m3 receptors pilocarpine had a small but measurable effect while McN-A 343 was ineffective; 2) pilocarpine had greater potency and Emax at m2 than at m4 receptors, while McN-A 343, in contrast, had greater potency and Emax at m4 than at m2 receptors. McN-A 343 behaves in this assay as a selective m4 agonist.

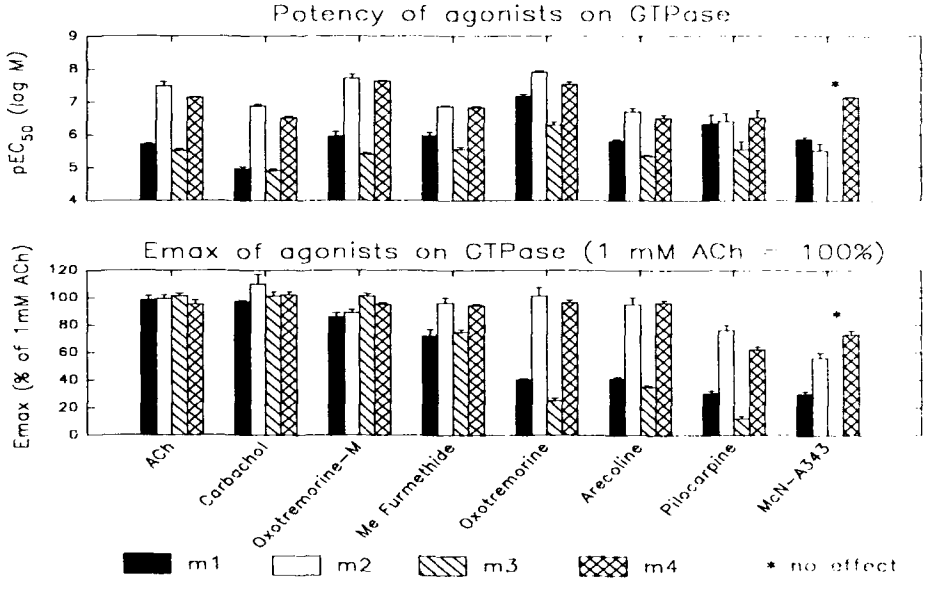

FIG. 5

Agonist potency (-log EC_{50}) and Emax relative to the effect of 1 mM acetylcholine. Results are mean and s.e.m of 3-5 experiments.

Two other general features of these results are of interest. Firstly, full agonists are more potent than might be expected from their apparent affinity in binding studies ('low affinity site') given the proximity of the response to the receptor. This can be seen in a dual labelling experiment in which ACh-stimulated [^{35}S]GTPγS binding was measured in the presence of [^{3}H]N-methyl scopolamine (an antagonist radioligand) (figure 6). These results suggest that there may be a degree of 'receptor reserve' in this response.

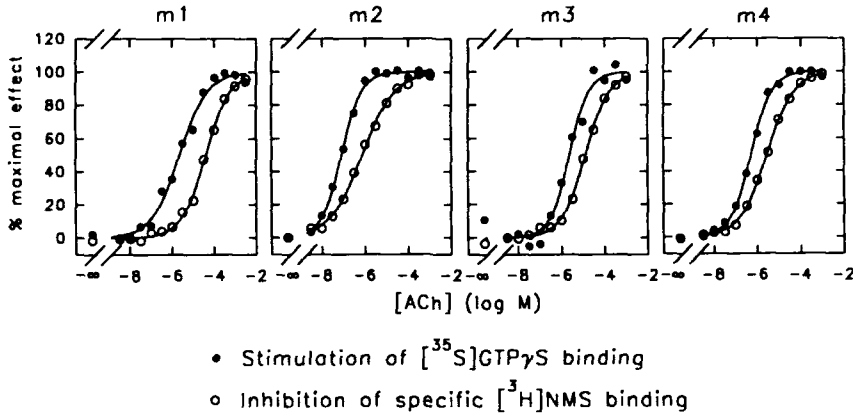

FIG. 6

Dual label experiment with ACh simultaneously inhibiting [^{3}H]NMS binding and stimulating [^{35}S]GTPγS binding

A second feature of interest is that the agonist titrations have 'flat' logistic slope factors: this is most marked with the m1 receptor preparation in which ACh titrations have slope factors of about 0.5, rather than the value of about 1 predicted by most theoretical models of agonism - even the ternary complex model (7,8) (in which predicted agonist curves may be flat due to depletion of free receptor or G-protein) generates minimal slope values of about 0.7, in contrast to the observed value of 0.5 at m1 receptors. One explanation for this effect might be that the receptor binds to a number of different G-proteins with different affinities. Using agonist stimulated photoaffinity labelling with a radioactive photoactivatable analogue of GTP we have found that the m4 subtype does indeed mediate activation of more than one G-protein (Farries et al, submitted for publication). Further evidence that m1 and m3 receptors can activate more than one G-protein is provided in the next section.

Pertussis toxin treatment

In order to study further the possible heterogeneity of G-proteins activated by individual muscarinic receptors, cells were grown in the absence and presence of pertussis toxin (PTX, 100 ng/ml for 15 hours). PTX treatment abolished agonist stimulated photolabelling with the photoactivatable GTP analogue (Farries et al, submitted for publication). In functional studies, PTX treatment reduced basal GTPase and GTPγS binding activities at all subtypes and abolished agonist effects at m2 and m4 receptors. At m1 and m3 receptors, in contrast, 25-35% of the control response remained in the PTX-treated membranes. Most interestingly, ACh had a potency at this residual response 1-2 orders of magnitude greater than at the control response, and the slopes of the curves were steeper (figure 7). One possible explanation of this result is that the increased agonist potency is caused by the altered receptor:G-protein stoichiometry. To test this, m1 and m4 cells were grown in various concentrations of PTX. The m4 membranes showed a graded reduction in response to ACh, but the ACh potency was unaffected (data not shown), in contrast to the results with m1 membranes which were similar to those found earlier. So the increased ACh potency via PTX-insensitive G-proteins cannot be explained simply by altered receptor:G-protein stoichiometry.

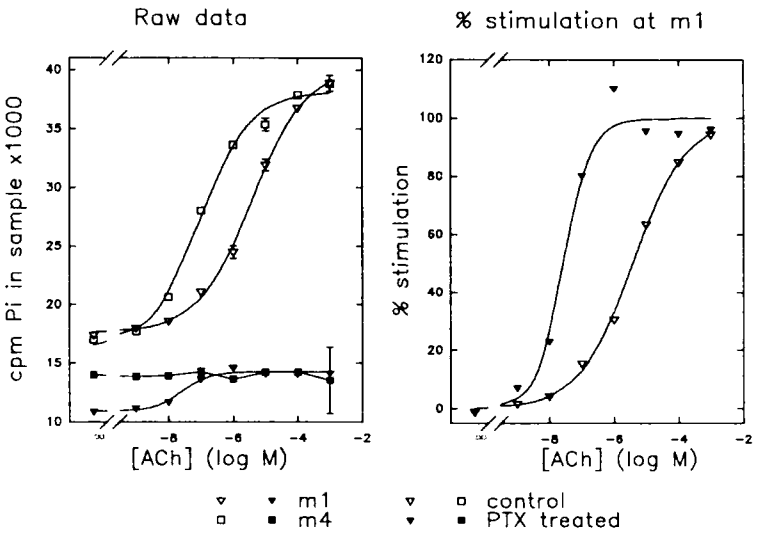

FIG. 7

Acetylcholine stimulation of GTPase activity in membranes from control and PTX treated (100 ng/ml,15 hours) m1 and m4 CHO cells. Results are the mean and s.e.m of triplicate determinations.

Discussion

We have studied agonist stimulated GTPγS binding and GTPase responses using membranes from CHO cells stably transfected with muscarinic m1-m4 receptors. Antagonist affinities for human m1-m4 receptors measured functionally agree very well with their affinities for native rat and rabbit receptors measured in radioligand binding experiments (5). The functional experiments confirm the finding that tropicamide and secoverine have modest m4 selectivity. The relative potencies and efficacies of eight agonists are consistent with previous data(9-12). Perhaps because of the high levels of receptor expression in these cells, even partial agonists such as pilocarpine and McN-A343 produced quantifiable responses. McN-A343 may have a relative selective efficacy and affinity for m4 receptors.

We have found (as have others (18,19)) that muscarinic receptor subtypes interact with more than one subtype of G-protein. m2 & m4 receptors seem to bind only to PTX-sensitive G-proteins. m1 & m3 receptors seem to bind to PTX-sensitive and -insensitive G-proteins, which may have different affinities for the receptor since ACh potency was increased in PTX treated membranes.

These functional responses have many attractive features. Membranes can be prepared and frozen in bulk for convenience, and the assays themselves are easy, quick and very reproducible. The transfected CHO cells used here do not contain endogenous acetylcholinesterase or ACh so the natural neurotransmitter can be used as the reference agonist. Muscarinic guanine nucleotide exchange responses can be measured in membranes from brain, heart and smooth muscle (13-17), but few animal tissues contain single populations of muscarinic receptor subtypes. The GTPase and GTPγS binding responses in membranes from stably transfected CHO cells provide useful functional responses for the characterisation of

ligands at muscarinic receptor subtypes. The challenge is to understand the responses at the molecular level.

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References

1 N.J. BUCKLEY, T.I. BONNER, C.M. BUCKLEY and M.R. BRANN, Mol.Pharmacol. 35 469-476 (1989).
2 O. ARUNLAKSHANA and H.O. SCHILD, Brit. J. Pharmacol. 14 48-58 (1959).
3 D.R. WAUD, Advances in General and Cellular Pharmacology, T. Narahashi and C.P. Bianchi (eds), 145-178, Plenum Press, New York (1976).
4 D.R. WAUD, Methods in Pharmacology, E.E. Daniel and D.M. Paton (eds), 471-506, Plenum Press, New York (1975).
5 S. LAZARENO, N.J. BUCKLEY and F.F. ROBERTS, Mol.Pharmacol. 38 805-815 (1990).
6 F. DORJE, J. WESS, G. LAMBRECHT, R. TACKE, E. MUTSCHLER and M.R. BRANN, J.Pharmacol.Exp.Ther. 256 727-733 (1991).
7 F.J. EHLERT, Mol.Pharmacol. 28 410-421 (1985).
8 T. COSTA, Y. OGINO, P.J. MUNSON, O. ONARAN and D. RODBARD. Mol.Pharmacol. 41 549-560 (1992).
9 F. MITCHELSON, Pharmacol.Ther. 37 357-423 (1988).
10 M. MCKINNEY, D. ANDERSON and L. VELLA-ROUNTREE, Mol.Pharmacol. 35 39-47 (1989).
11 M. MCKINNEY, J.H. MILLER, V.A. GIBSON, L. NICKELSON and S. AKSOY, Mol.Pharmacol. 40 1014-1022 (1991).
12 J.R. HU and E.E. EL-FAKAHANY, Mol.Pharmacol. 38 895-903 (1990).
13 P. ONALI, M.C. OLIANAS, J.P. SCHWARTZ and E. COSTA, Mol.Pharmacol. 24 380-386 (1983).
14 S. GHODSI-HOVSEPIAN, W.S.,JR. MESSER and W. HOSS, Biochem.Pharmacol. 39 1385-1391 (1990).
15 G. HILF and K.H. JAKOBS, Eur.J.Pharmacol. 172 155-163 (1989).
16 G. HILF, P. GIERSCHIK and K.H. JAKOBS, Eur.J.Biochem. 186 725-731 (1989).
17 C. LIEBMANN, S. NAWRATH, M. SCHNITTLER, H. SCHUBERT and K.H. JAKOBS, Naunyn Schmiedebergs.Arch.Pharmacol. 345 7-15 (1992).
18 A. ASHKENAZI, E.G. PERALTA, J.W. WINSLOW, J. RAMACHANDRAN and D.J. CAPON, Cell 56 487-493 (1989).
19 S. OFFERMANNS, M. GOLLASCH, J. HESCHELER, K. SPICHER, A. SCHMIDT, G. SCHULTZ and W. ROSENTHAL, Mol.Endocrinol. 5 995-1002 (1991).

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MUSCARINIC RECEPTOR SUBTYPES: MODULATION OF ION CHANNELS

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Summary

The discovery of five muscarinic receptor subtypes by molecular genetic techniques has resulted in new approaches to understanding their function. This involves the expression of the individual genes encoding each receptor subtype in isolation, such that their effects and mechanisms of action can be studied. The coupling of the receptors with G-proteins and ion channels is the subject of this review and emphasis is placed upon the assignment of genetically defined receptor subtypes with a given physiological function. Activation of inwardly rectifying potassium conductances by m2 and m4 and inhibition by m1, as well as stimulation of calcium-dependent conductances by m1, m3 and m5 are discussed.

Muscarinic receptor activation results in a variety of hyperpolarizing and depolarizing responses, dependent upon the cell type or the tissue under investigation. The diversity of response is due to the multiplicity of ionic conductances modulated by the muscarinic receptors (1-5). These include many types of potassium, calcium, cation or chloride channels. The muscarinic receptors can be divided into a family of five receptor subtypes (m1-m5), identified by molecular genetic approaches (6-9). It is now possible to begin to unravel some of the complexity of muscarinic responses by studying each receptor subtype in isolation and hence to aid determination of the mechanisms involved in the various responses. There appear to be many mechanisms by which muscarinic receptors can bring about ion channel modulation and some of these will be explored in this review.

Most of the information currently available in the literature about the electrophysiological consequences of muscarinic receptor stimulation, has used various muscarinic antagonists in attempts to simplify study, and to assign a given response to a particular receptor subtype. However, while these drugs have been extremely useful to date, they do not allow discrimination between all five of the muscarinic receptor subtypes that have been cloned (10-12). Therefore, it has become expedient to express the receptor subtypes in isolation in cell lines, by transfection with the genes encoding them, and using other molecular methods, such as the use of subtype specific antibodies or antisense oligonucleotides, to identify the receptor in terms of their genetically defined subtype and to assign a given function.

Muscarinic receptors mediate their effects by activating GTP binding proteins (G-proteins), which in turn may directly modulate an ion channel or modulate an enzyme involved in synthesis of a second messenger. Examples of second messenger modulation by muscarinic receptors include inhibition of adenylyl cyclase by m2 and m4, resulting in reduced cAMP levels within the cell. This is mediated via a pertussis toxin (PTX) sensitive G-protein (5, 13-15). m1, m3 and m5 stimulate phospholipase C (PLC), which hydrolyses phosphatidyl inositol to various inositol phosphates such as inositol tris-phosphate (IP3) and diacylglycerol. This action is via a PTX

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insensitive G-protein in most cells examined. IP3 causes release of calcium from intracellular stores and diacylglycerol activates protein kinases and is metabolised to arachidonic acid and derivatives (5, 14-16). m1, m3 and m5 also raise cAMP levels in A9 L cells (5), probably by an indirect mechanism through calcium (17), while m1, m3 and m4 (in the presence of PTX) stimulate large increases in cAMP levels in a pertussis toxin in-sensitive manner (14). The recent observations that $\beta\gamma$ subunits released from activation of G-protein heterotrimers, can selectively enhance Type II and Type IV adenylyl cyclase activity (18, 19), might indicate the mechanism by which these muscarinic receptors are increasing cAMP levels.

Inwardly rectifying potassium conductances, as can be deduced from their name, pass more current into the cell than allowing to pass out of the cell, can be modulated both directly by G-proteins and indirectly by second messengers. These effects have been well characterized in heart (20-22), where only m2 muscarinic receptor mRNA and protein have been detected (23, 24). The activation of an inwardly rectifying potassium conductance (I_{KM}) by muscarinic receptor stimulation in atria, has been shown to be directly activated by the α subunit of the pertussis toxin sensitive G-protein - Gi (25). The $\beta\gamma$ subunits, dissociated on activation of the G-protein have also been shown to modulate this inward potassium conductance (26), however, there does not appear to be any evidence showing that $\beta\gamma$ subunits mediate the muscarinic receptor-induced activation of the channel (25). The m4 muscarinic receptor subtype, shown to be the endogenous receptor in AtT-20 cells (a pituitary derived cell line), has also been shown to stimulate an increase in an inwardly rectifying potassium conductance (27, 28). As in the heart, the muscarinic receptor-mediated increase in inwardly rectifying potassium conductance, appears to differ from the inward potassium conductance present in control conditions (see Figure 1).

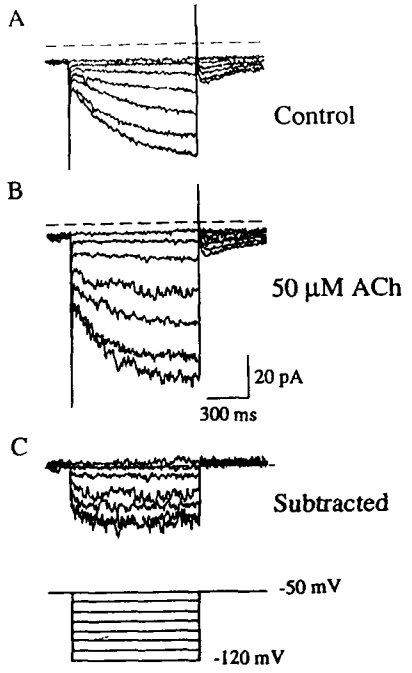

Fig. 1. Stimulation of the m4 muscarinic receptor in the pituitary AtT-20 cell line, activates an inwardly rectifying potassium conductance. The control or background current has slow activation kinetics, shown here in a series of voltage steps from -60mV to -120mV, superimposed for clarity. A) control currents show slow activation and tail currents. B) Inwardly rectifying currents are increased by application of 50 µM ACh. C) Net ACh-induced currents are not time-dependent, displaying fast activation kinetics and no tail currents. (Data taken from reference 28).

In the AtT-20 cell line the background conductance is very slowly activating and displays tail currents on repolarization. This resembles the atrial pacemaker inwardly rectifying conductance I_f (31, 32) and the hyperpolarizing cation conductance I_H in brain (30). The control conductance in AtT-20 cells was not affected by muscarinic receptor stimulation or by application of GTPγS (28), a G-protein activator. On the other hand, the net muscarinic agonist-induced current, had fast activation kinetics and was mimicked by GTPγS application and abolished by pre-treatment

with PTX, indicating that the m4 activated inward rectifier is modulated by a PTX sensitive G-protein. The nature of the G-protein/channel interaction was not addressed. A similar response has been observed in RBL-2H3 cells transfected with the m2 receptor subtype (29). In these cells, both the control and muscarinic agonist-induced inwardly rectifying conductances had fast activation kinetics. Muscarinic stimulation also activates an inward potassium conductance in brain (30), however, neither the kinetic properties nor the mechanism of this effect have been determined.

Another inwardly rectifying conductance (I_f) present in the sino-atrial node pacemaker cells, which has very slow activation kinetics and passes both potassium and sodium, is *reduced* by muscarinic receptor activation (31, 32). As stated previously, only m2 has been detected in atrial tissue and thus this reponse is likely to be mediated by the m2 receptor subtype. The reduction of cellular cAMP levels by the m2 receptor is believed to be responsible for this effect by shifting the activation curve of I_f to negative potentials (32). This effect might reflect a direct action of cAMP on the channel (32) or reflect a reduction in the state of phosphorylation of the ion channels, due to the reduced stimulation of cAMP dependent protein kinase (PKA). The notion that the state of phosphorylation of a channel is involved in its function has been around a long time, and is important for whole-cell recordings, where the addition of ATP in the patch pipette is necessary for maintenance of many potassium and calcium conductances. An example of this can be seen in the muscarinic receptor-mediated decrease in an inwardly rectifying conductance in brain, which could be mimicked by phorbol esters (33). The phorbol esters are presumably activating protein kinase C (PKC), or other protein kinases, which may be important for modulation of this particular inward rectifier. Support for this idea comes from the inhibition of the inward rectifier in m1 receptor transfected RBL-2H3 cells (34). Both raised cAMP and phorbol esters can mimick the muscarinic mediated response (Jones unpublished observations). Further complications arise from the observation that raised intracellular calcium can also inhibit the inward rectifier of the RBL-2H3 cell (35) however, it is likely that the increased calcium is activating protein kinases also. The inhibition of the muscarinic receptor-stimulated atrial inward rectifier, by the SH2-SH3 domains of a GTPase Activating Protein (GAP) (36) usually associated with Ras, gives us an indication that we are just scratching at the surface of the complex nature of receptor mediated channel modulation.

Thus, m2 and m4 appear to directly activate inwardly rectifying potassium channels and can also modulate other inward rectifiers by reducing cAMP levels, possibly by a reduction in the activity of PKA. m1, m3 and m5 can all raise cAMP levels and also stimulate PKC and thus can theoretically modulate the channels also. It remains to be seen if they also couple directly with the ion channel through a G-protein.

Another second messenger mediated effect of muscarinic receptors is the activation of calcium-dependent conductances such as the calcium-dependent potassium, chloride and cation conductances of glandular tissue, for example in salivery or lacrimal glands (37, 38). These conductances are activated indirectly by the stimulation of PLC and the resultant release of calcium by IP3 from intracellular stores. The m1, m3 and m5 muscarinic receptors are obvious candidates for this response due to their stimulation of phosphatidyl-inositol metabolism. Activation of calcium-dependent potassium and chloride conductances have been demonstrated with these receptors in transfected A9 L (10, 39, 40), CHO (14), NG108-15 cells (41) and Xenopus oocytes (6). The electrophysiological effects could be mimicked by intracellular application of IP3 or raised intracellular calcium (42, 43), but were unaffected by cAMP or phorbol esters (42) (see figure 2). These actions are mediated by a PTX in-sensitive G-protein. In the A9 L and CHO cells the raised intracellular calcium concentration was shown to be due to release of intracellular calcium (14, 42). In lacrimal gland cells however, it appears that calcium is initially released from intracellular stores, but sustained elevations in intracellular calcium concentration appear to be due to influx of calcium from the extracellular solution and are thought to involve IP4-induced calcium entry (44). m2 and m4 did not produce activation of calcium-

dependent conductances in any of the above studies.

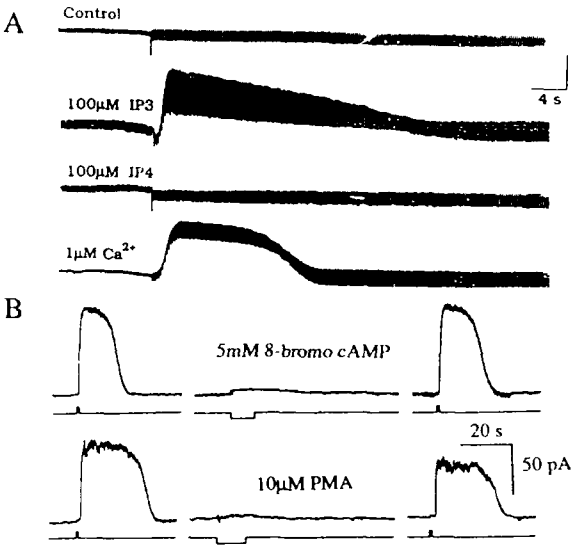

Figure 2. m1, m3 and m5 activate calcium-dependent potassium conductances in A9 L cells. This action is mediated by IP3 formation which releases calcium from intracellular stores. A) Application of IP3 or high intracellular calcium via the patch pipette, mimicks the ACh-induced effect in A9 L cells. B) Neither cAMP nor phorbol esters (applied at downward deflections of lower traces) mimick the ACh-induced effect (10μM ACh applied at upward deflections of lower traces).

Inhibition of the M-current by muscarinic receptors has also been studied in terms of the genetic subtypes. The M-current is a voltage and time-dependent potassium conductance that can be modulated by a variety of G-protein coupled receptors (1, 45, 46, 50). In NG 108-15 cells, m1 and m3 but not m2 or m4 inhibit this current (41). This would indicate either a direct G-protein/channel coupling or the complement of second messengers associated with PI breakdown. The response is PTX in-sensitive and has been shown to be mimicked by IP3 in hippocampal neurons (47), but not in sympathetic ganglia or in NG 108-15 cells (48, 49). In the latter, phorbol esters mimick the muscarinic response (49, 50, 51). These differences may reflect the different levels of phosphorylation and ability of IP3 to release calcium in various tissues and not separate modulatory mechanisms.

In the early experiments with injections of mRNA into Xenopus oocytes, the m2 muscarinic receptor was shown to activate a non-specific cation conductance, the mechanism by which this occurred was not known (7). This conductance appears to be similar to the cation conductance activated by muscarinic receptors in smooth muscle and in chromaffin cells (52, 53). The cation conductance activated in the chromaffin cells was mediated via a PTX sensitive G-protein and is thought to be mediated by m4.

Calcium conductances are modulated by muscarinic receptors, probably by both direct G-protein coupling and by second messenger mediation (See 54-57 for calcium channel reviews). Coupling of muscarinic receptor-induced inhibition of calcium currents has been shown recently, to be mediated specifically by the G-protein α subunit of Go1 and involves the β3 subunit, in two very elegant studies by Kleuss et al., using antisense oligonucleotides in GH3 cells (58, 59). There is evidence to indicate that this effect is mediated directly by the G-protein (60, 61, 62). The response is PTX sensitive and believed to be activated by the m4 muscarinic receptor in

GH3 cells. A PTX sensitive inhibition of calcium currents has been observed in m2 and m4-transformed NG-108-15 cells (63). Reduced cAMP levels, however, are thought to be responsible for the inhibition of calcium currents in cardiac tissue by muscarinic receptor stimulation (64), and from our knowledge of second messenger modulation by muscarinic receptors, and the fact that only m2 is present in heart, this would indicate that m2 and m4 could mediate such an effect. Thus, m2 and m4 probably directly inhibit calcium channels via Go, and also modulate them by changes in their phosphorylation states via changes in cAMP levels. Muscarinic receptor induced *increases* in calcium conductances have been observed in T-type calcium conductances in hippocampus (65), and were shown to be modulated via a direct action of a PTX sensitive G-protein. N or L-type calcium currents are also increased on muscarinic receptor stimulation in smooth muscle and can be mimicked by application of diacylglycerol (66). This would indicate that m1, m3 or m5 might be involved, however, the possibility of direct G-protein modulation cannot be ruled out.

TABLE 1 MUSCARINIC RECEPTOR EFFECTS

m1 m3 m5	m2 m4
stimulate PLC (5, 13-16)	inhibit adenylyl cyclase (5, 13-16)
increase intracellular calcium (42, 43)	lower cAMP levels (5, 13-16)
raise cAMP levels (5, 13-17)	raise cAMP levels (m4 in presence of PTX) (14)
release arachidonic acid (16)	
inhibit inward rectifier (m1) (34)	stimulate inward rectifier (28, 29)
activate Ca-dependent K^+ and Cl^- (14, 39-42)	inhibit calcium currents (63)
inhibit m-current (m1, m3) (41)	activate cation conductance (m2) (7)
stimulate release (69)	
PTX-insensitive	PTX-sensitive

These effects only reflect studies with cloned receptor subtypes expressed in various cell lines.

In examining each of the possible mechanisms for modulation of the inward rectifier, calcium conductances and the m-current, the dual modulation by direct G-protein coupling and by second messenger mediation are recurring (Figure 3). This suggests the notion that perhaps direct G-protein coupling with ion channels influences channel opening or alters the probability of opening, while the indirect mechanism via phosphorylation by protein kinases might shift the voltage-dependence of their activation. This latter possibility obviously only affects voltage-dependent conductances. This simple explanation may aid us in designing experiments, that not only test which receptor subtype is involved in a given response, but could aid in the separation of the two modulatory mechanisms quite readily. This method has already proven useful, in attempts to resolve the actions of muscarinic and adrenergic receptors on calcium channels (67).

Predicting the effects of the various muscarinic receptor subtypes in terms of their physiological effects on a given cell, is important for the targeting of particular subtypes in development of therapeutic agents. This necessitates a correlation of the modulatory effect of a receptor subtype with a hyperpolarization or depolarization of the membrane and an indication of where in the cell this is occurring. One way to aid this rather difficult process is to study the effects of the various receptor subtypes on release of neurotransmitters.

Muscarinic receptors regulate acetylcholine release as well as other neurotransmitters such as glutamate, GABA or dopamine (68). In the central nervous system, muscarinic receptors inhibit release of acetylcholine in a manner indicative of pharmacologically defined M2 receptors and has been thought of as the pre-synaptic receptor. However, other receptors have also been implicated (68). Inhibition of other neurotransmitters such as GABA and glutamate appears to be

via M2 or M3 receptors. An enhancement of release has also been demonstrated in central and peripheral neurons and these have been indicated to be due to an M1 pharmacology. Protein kinase C and PI metabolism have been implicated in this response. These observations lead us to the suggestion that m1, m3 and m5 could stimulate release, while m2 and m4 might be inhibitory. This is supported by studies showing that m1 receptors transfected in RBL-2H3 cells stimulate release of secretory granules (69). The mechansims involved in m1-stimulated release are not clear, but yet again involve the breakdown of phosphatidyl inositol and protein kinases. In terms of the muscarinic receptor-induced inhibition of transmitter release, activation of inwardly rectifying potassium channels by m2 or m4 in neuronal terminals, would exert an inhibitory effect on neurotransmitter release by hyperpolarizing the membrane.

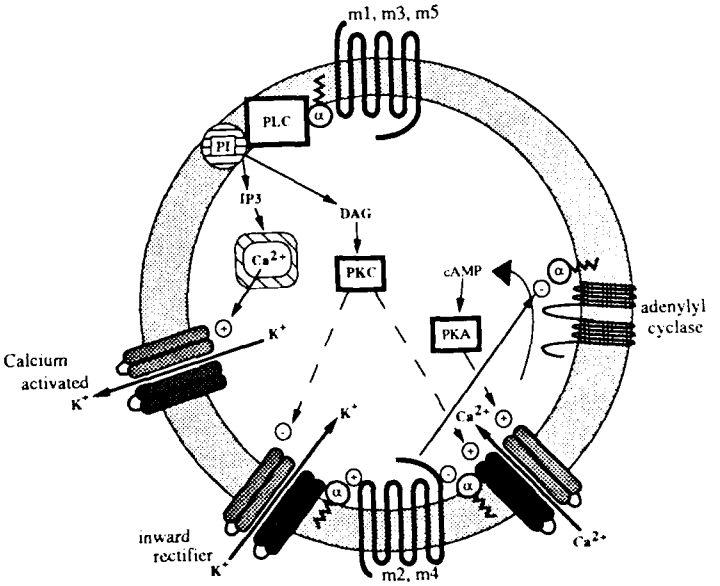

Figure 3. Cartoon summarizing the actions of the five muscarinic receptor subtypes. Solid lines represent known pathways, dashed lines indicate suggested pathways. Note the dual regulation of the inward rectifier and calcium channel.

In order to complete the predictions about which muscarinic receptor subtype should be targeted for a given neuronal pathway, we need to also establish exactly what the subtype does *in situ*. This necessitates the use of molecular probes, such as subtype selective antibodies or antisense oligonucleotides, to identify the receptor subtype mediating a given response in a given neuronal population. This method relies on the antibody or antisense oligonucleotide preventing the activation of the receptor stimulated response, the former by blocking access of the G-protein to the receptor, the latter by knocking out the synthesis of the protein by inactivating the mRNA encoding it. These approaches have been found to be sucessful for several G-protein interactions (58, 59, 70, 71) and can also be applied to muscarinic receptor subtypes. In some cell types, for example in the dopamine containing neurons of the substantia nigra, these approaches would not be necessary, as only m5 mRNA has been detected in these cells (72) and thus an examination of the actions of muscarinic agonists should reveal their effects. Initial studies reveal a complex array of electrophysiological effects (73, 74).

References

1. D.A. BROWN, Annu. Rev. Physiol. 52 215-242 (1990).
2. M.J. CHRISTIE and R.A. NORTH, Trends Pharmacol. Sci. suppl. Subtypes of Muscarinic Receptors III, 30-34 (1988).
3. R.A. NICOLL, Science 241 545-551 (1988).

4. R.A. NORTH, Muscarinic Receptor Subtypes, J.H. Brown (eds), Humona Press (1989).
5. S.V.P. JONES, A.I. LEVEY, D.M. WEINER, J. ELLIS, E.A. NOVOTNY, S. YU, F. DORJE, J. WESS and M.R. BRANN, Molecular Biology of Receptors Which Couple to G-proteins, M.R. Brann (ed), 170-197, Birkhauser, Boston (1992).
6. T. KUBO, K. FUKUDA, A. MIKAMI, A. MAEDA, H. TAKAHASHI, M. MISHINA, T. HAGA, K. HAGA, A. ICHIYAMA, K. KANGAWA, M. KOJIMA, H. MATSUO, T. HIROSE and S. NUMA, Nature 323 411-16 (1986).
7. T. KUBO, A. MAEDA, K. SUGIMOTO, I. AKIBA, A. MIKAMI, K. FUKUDA, H. TAKAHASHI, T. HAGA, K. HAGA, A. ICHIYAMA, K. KANGAWA, H. MATSUO, T. HIROSE, and S. NUMA, FEBS Lett. 209 367-72 (1986).
8. T.I. BONNER, N.J. BUCKLEY, A.C. YOUNG and M.R. BRANN, Science 237 527-532 (1987).
9. T.I. BONNER, A.C. YOUNG, M.R. BRANN and N.J. BUCKLEY, Neuron 1 403-410 (1988).
10. M.R. BRANN, N.J. BUCKLEY, S.V.P. JONES and T.I. BONNER, Mol. Pharmacol. 32 450-455 (1987).
11. N.J. BUCKLEY, T.I. BONNER, C.M. BUCKLEY and M.R. BRANN, Mol. Pharmacol. 35 469-476 (1989).
12. F. DORJE, J. WESS, G. LAMBRECHT, R. TACKE, E. MUTSCHLER and M.R. BRANN J. Pharm. Exp. Ther. 256 727-733 (1991).
13. E.G. PERALTA, A. ASHZENAZI, J.W. WINSLOW, J. RAMACHANDRAN and D.J. CAPON Nature 334 434-437 (1988).
14. S.V.P. JONES, C.J. HEILMAN and M.R. BRANN Mol. Pharmacol. 40 242-247 (1991).
15. M.R. BRANN, J. WESS and S.V.P. JONES J. Gen. Physiol. 45 106-115 (1990).
16. B.R. CONKLIN, M.R. BRANN, N.J. BUCKLEY, A.L. MA, T.I. BONNER and J. AXELROD Proc. Natl. Acad. Sci. USA 85 8698-8702 (1988).
17. C.C. FELDER, R.Y. KANTERMAN, A.L. MA and J. AXELROD, J. Biol. Chem. 264 20356-20362 (1989) .
18. W.J. TANG and A.G. GILMAN, Science 254 1500-1503 (1991).
19. A.D. FEDERMAN, B.R. CONKLIN, K.A. SCHRADER, R.R. REED and H.R. BOURNE Nature 356 159-161 (1992).
20. H.C. HARTZELL, Prog. Biophys. Molec. Biol. 52 165-247 (1988).
21. A.M. BROWN and L. BIRNBAUMER, Annu. Rev. Physiol. 52 197-213.
22. G. SZABO and A.S. OTERO, Annu. Rev. Physiol. 52 293-305 (1990).
23. F. DORJE, A. LEVEY, E. MUTSCHLER and M.R. BRANN, Mol. Pharmacol. 40 459-462 (1991).
24. N.J. BUCKLEY, T.I. BONNER and M.R. BRANN, J. Neurosci. 8 4646-4652 (1988).
25. G.E. KIRSH, A. YATANI, J. CODINA, L. BIRNBAUMER and A.M. BROWN, Am. J. Physiol. 254 1200-1205 (1988).
26. D. KIM, D.L. LEWIS, L. GRAZIADEI, E.J. NEER, D. BAR-SAGI and D.E. CLAPHAM, Nature 337 557-560 (1989).
27. A.G. DOUSMANIS and P. S. PENNEFATHER, Biophysical J. 55 546 (1989).
28. S.V.P. JONES, Neurosci. Letts. in press (1992).
29. S.V.P. JONES, Neurosci. Soc. 17 67 (1991).
30. D.A. McCORMICK and D.A. PRINCE, J. Physiol. (Lond.) 392 147-165 (1987).
31. D. DIFRANCESCO and C. TROMBA, J. Physiol. (Lond). 405 477-491 (1988).
32. D. DIFRANCESCO and P. TORTORA, Nature 351 145-147 (1991).
33. N. UCHIMURA and R.A. NORTH, J. Physiol. (Lond). 422 369-380 (1990).
34. S.V.P. JONES, 8th International Cholinergic Symposium, Montreal 19 (1992).
35. M. MUKAI, I. KYOGOKU and M. KUNO, Am. J. Physiol. 262 C84-C90 (1992).
36. G.A. MARTIN, A. YATANI, R. CLARK, L. CONROY, P. POLAKIS, A.M. BROWN and F. McCORMICK, Science 255 192-194 (1992).
37. A. MARTY, Trends Neuroscience 10 373-377 (1987).
38. O.H. PETERSEN and D.V. GALLACHER, Ann. Rev. Physiol. 50 65-80 (1988).
39. S.V.P. JONES, J.L. BARKER, T.I. BONNER, N.J. BUCKLEY and M.R. BRANN, Proc. Natl. Acad. Sci. USA 85 4056-4060 (1988).
40. S.V.P. JONES, J.L. BARKER, N.J. BUCKLEY, T.I. BONNER, R. COLLINS and

M.R. BRANN, Mol. Pharmacol. 34 421-426 (1988).
41. K. FUKUDA, H. HIGASHIDA, T. KUBO, A. MAEDA, I. AKIBA, H. BUJO, M. MISHINA and S. NUMA, Nature 335 355-358 (1988).
42. S.V.P. JONES, J.L. BARKER, M.B. GOODMAN and M.R. BRANN, J. Physiol. (Lond). 421 499-519 (1990).
43. E. NEHER, A. MARTY, K. FUKUDA, T. KUBO and S. NUMA, FEBS Letters 240 88-94 (1988).
44. A.P. MORRIS, D.V. GALLACHER, R.F. IRVINE and O.H. PETERSEN, Nature 330 653-655 (1987).
45. D.A. BROWN, Ion Channels T. Narahashi (ed) volume 1, Plenum Press, New York and London (1988).
46. D.A. BROWN, Trends Neuroscience 11 294-299 (1988).
47. P. DUTAR and R.A. NICOLL, Neurosci. Lett. 85 89-94 (1988).
48. D.A. BROWN and H. HIGASHIDA, J. Physiol. (Lond.) 397 185-207 (1988).
49. D.A. BROWN, N.V. MARRION and T.G. SMART, J. Physiol. (Lond.) 413 469-488 (1989).
50. B. HILLE, Q. J. Exp. Physiol. 74 785-804 (1989).
51. P.J. PFAFFINGER, M.D. LEIBOWITZ, E.M. SUBERS, N.M. NATHANSON, W. ALMERS and B. HILLE, Neuron 1 477-484 (1988).
52. C.D. BENHAM, T.B. BOLTON and R.J. LANG, Nature 316 345-347 (1985).
53. M. INOUE and H. KURIYAMA, J. Physiol. (Lond). 436 511-529 (1991).
54. A.C. DOLPHIN, Annu. Rev. Physiol. 52 243-255 (1990).
55. G. SCHULZ, W. ROSENTHAL and J. HESCHELER, Annu. Rev. Physiol. 52 275-292 (1990).
56. W. TRAUTWEIN and J. HESCHELER, Annu. Rev. Physiol. 52 257-274 (1990).
57. A.M. BROWN, A. YATANI, Y. IMOTO, J. CODINA, R. MATTERA and L. BIRNBAUMER, Ann. N.Y. Acad. Sci. (1990).
58. C. KLEUSS, J. HESCHELER, C. EWEL, W. ROSENTHAL, G. SCHULZ and B. WITTIG, Nature 353 43-48 (1991).
59. C. KLEUSS, H. SCHERUBL, J. HESCHELER, G. SCHULZ and B. WITTIG, Nature 358 424-426 (1992).
60. D.J. BEECH, L. BERNHEIM, A. MATHIE and B. HILLE, Proc. Natl. Acad. Sci. USA 88 652-656 (1991).
61. L. BERNHEIM, D.J. BEECH and B. HILLE, Neuron 6 859-867 (1991).
62. M. TOSELLI, J. LANG, T. COSTA and H.D. LUX, Pflugers Arch. 415 255-261(1989).
63. H. HIGASHIDA, M. HASHII, K. FUKUDA, M.P. CAULFIELD, S. NUMA and D.A. BROWN, Proc. Roy. Soc. Lond. Ser. B 242 68-74 (1990).
64. H.C. HARTZELL and R. FISCHMEISTER, Mol. Pharmacol. 32 639-645 (1987).
65. M. TOSELLI and H.D. LUX, Experentia suppl. 57 97-103 (1989).
66. M.B. VIVAUDOU, L.H. CLAPP, J.V. WALSH and J.J. SINGER, Faseb J. 2 2497-2504 (1988).
67. D.J. BEECH, L. BERNHEIM and B. HILLE, Neuron 8 97-106 (1992).
68. M. RAITERI, M. MARCHI and P. PAUDICE, Ann. N.Y. Acad. Sci. 604 113-129 (1990).
69. S.V.P. JONES, O.H. CHOI and M.A. BEAVEN, FEBS Letts. 289 47-50 (1991).
70. M. ALREJA, E. J. NESTLER and G. K. AGHAJANIAN, Neurosci. Soc. abstr. 16 356 (1990).
71. P.M. LLEDO, V. HOMBURGER, J. BOCKAERT and J.-D. VINCENT, Neuron, 8 455-463 (1992).
72. D.M. WEINER, A. LEVEY, and M.R. BRANN, Proc. Natl. Acad. Sci. USA. 87 7050-7054 (1990).
73. M.G. LACEY, P. CALABRESI and R.A. NORTH, J. Pharm. Exp. ther. 253 395-400 (1990).
74. S.V.P. JONES, N.L. SILVA and M.R. BRANN, Neurosci. Soc 16 356 (1990).

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CHARACTERIZATION OF MUSCARINIC AGONISTS IN RECOMBINANT CELL LINES

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Summary

Using recombinant CHO cells that express Hm1 - Hm5 receptors, reference muscarinic agonists have been characterized with respect to their activity in receptor binding and second messenger assays. In whole cell [^{3}H]-N-methyl scopolamine binding, no agonist was found to be truly subtype selective, although some showed marked differences between several of the subtypes (e.g. m1 vs. m2). As a functional index of receptor activation, phosphatidylinositol (PI) turnover was measured for m1, m3, and m5 receptors while inhibition of forskolin-stimulated cAMP accumulation was measured for m2 and m4 receptors. Both full and partial agonists were delineated in PI turnover, but all agonists showed similar responses on cAMP. Alkylation studies with propylbenzylcholine mustard showed that both efficacy and potency were markedly affected in the functional assays by the number of free receptors. Thus, receptor reserve appears to play a major role in the determination of subtype selectivity for agonists using functional measures. Even with these limitations, however, the use of transformed cell lines is playing a pivotal role in the discovery of selective agonists.

Pharmacological classification of muscarinic receptors is based upon the profile of activity of selective antagonists (1). Receptors possessing high affinity and pharmacological sensitivity for pirenzepine are termed M_1, while those having high affinity for AF-DX 116 and low affinity for pirenzepine are termed M_2. M_3 receptors show lower affinity for both pirenzepine and AF-DX 116 and appear more sensitive to hexahydrosiladifenidol (HHSiD). However, these early studies mainly relied on the use of tissue preparations which subsequently were shown, in many cases, to contain multiple receptor subtypes in varying amounts.

More recent receptor characterization based upon molecular cloning, sequencing, and expression studies have demonstrated the existence of five distinct receptor proteins (m1-m5) each encoded for as a specific gene product (2,3,4). Further, each of these five subtypes has now been stably expressed in CHO cells. In general, m1, m3, and m5 receptors appear to be coupled to phospholipase C which, when activated, results in

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the production of inositol trisphosphate and diacylglycerol, while m2 and m4 receptors are negatively coupled to adenylyl cyclase.

Muscarinic agonists have been suggested to be of therapeutic utility in the symptomatic treatment of Alzheimer's Disease (5). Based upon the "cholinergic hypothesis" (6), a new generation of nonselective muscarinic agonists have been synthesized and tested preclinically (7). These compounds may be efficacious in humans, but will still have considerable side effects due to their nonselective nature. Subtype selective agonists, such as m1 agonists, should retain efficacy while having fewer unwanted effects (8). The use of recombinant cells has become an invaluable tool in the search for these novel selective agonists.

Receptor Binding Studies

Receptor binding studies examining the subtype selectivity of muscarinic antagonists in recombinant cell lines have appeared in the literature (9,10,11). However, very little information has been reported for agonists (12). In trying to characterize agonist selectivity using receptor binding, several issues need to be addressed. For example, whether to use agonist (e.g. cis-methyldioxolane (CMD), oxotremorine-M) or antagonist (e.g. N-methyl scopolamine (NMS), quinuclidinyl benzilate, (QNB)) ligands in membranes or whole cell preparations. Unlike brain or heart tissue, agonist binding in CHO cells does not appear to be possible for all five subtypes. We are able to observe measurable binding in m2 cells with [^{3}H]-CMD, but not in m1 cells. The reason for this is not clear, but it may be that the state of the receptor is different for the two subtypes due to differences in G protein coupling. Alternatively, if the percentage of high affinity sites labeled by agonists is extremely small in m1 cells, it may be that specific binding might not be measurable under the assay conditions used.

Whole Cell [^{3}H]-NMS Receptor Binding

Using whole cell, the five subtypes were found to have Kd values for [^{3}H]-NMS of 0.11-0.63nM and Bmax values ranging from 210-2450 fmol/mg protein. (Binding was performed in a Krebs HEPES buffer, pH 7.4, with an incubation period of 60 minutes at 37°C. GF/B filters were rapidly washed 3X with chilled 0.9% saline). Agonist displacement of nonselective labeled antagonists, such as [^{3}H]-NMS, yields Ki values mainly in the uM range. In inhibition studies (Table 1), it was found that there were two groups of agonists: those showing no differences in affinity between subtypes (AF-102B, arecoline, BM-5, CI-979, and RS-86) and those showing some selectivity between specific subtypes (carbachol, L-670,207, McN-A-343, oxotremorine, and pilocarpine). For these studies, selectivity was defined as greater than 3-fold differences in affinity. The maximal difference between any pair of subtypes was only about 6-fold. For example, k_{app} values for carbachol were: 34.9uM, 5.8uM, 43.1uM, 10.9uM, and 11.4uM for m1, m2, m3, m4 and m5 receptors respectively. Whether the use of labeled selective antagonists

(e.g. [^{3}H]-pirenzepine or [^{3}H]-AF-DX 116) would better define agonist selectivity is not known. This type of data does not yet appear to exist in the literature for recombinant cell lines. However, the present data shows that no agonist was truly subtype selective.

TABLE 1.

Subtype Selectivity of Muscarinic Agonists As Measured By Whole Cell [^{3}H]-N-Methyl Scopolamine Binding In Transformed CHO Cells*.

A. Agonists showing no difference in affinity between subtypes.

Compound	Selectivity	References
AF-102B	m1 = m2 = m3 = m4 = m5	13
Arecoline	(for all compounds)	14,15
BM-5		16
CI-979		7,8
RS-86		17

B. Agonists showing some selectivity between specific subtypes.

Compound	Selectivity	References
Carbachol	m2 = m4 = m5 > m1 = m3	1,14
L-670,207	m2 = m4 > m1 = m3 > m5	18
McN-A-343	m1 = m4 = m5 > m3 > m2	1,14
Oxotremorine	m1 = m2 = m5 > m3 = m4	14
Pilocarpine	m1 = m3 = m5 > m2 = m4	14

*Selectivity defined as greater than 3-fold difference in affinity.

Functional Measurement of Second Messenger Activity

Phosphatidylinositol (PI) Turnover

Muscarinic agonists stimulate the formation of inositol phosphates following activation of m1, m3, and m5 receptors. To measure PI hydrolysis, cells were incubated for 48 hours with [^{3}H]-myo-inositol at which time agonists were added for a 15 minute period. After the addition of TCA, total [^{3}H]-inositol phosphates were determined by ion exchange chromatography. As shown for CHO Hm1 cells (Fig. 1), full agonists such as carbachol produced maximal responses while arecoline, oxotremorine, and pilocarpine produced lesser responses and can be considered as partial agonists. The high number of receptors in Hm1 cells accounts for the marked activity of all four agonists. EC_{50} values were all in the uM range with oxotremorine being the most potent agonist (0.6uM). These responses were blocked by the muscarinic antagonist scopolamine. Similar effects were seen in m3 and m5 cells with lower maximal responses observed in m5 cells due to a lower number of total receptors.

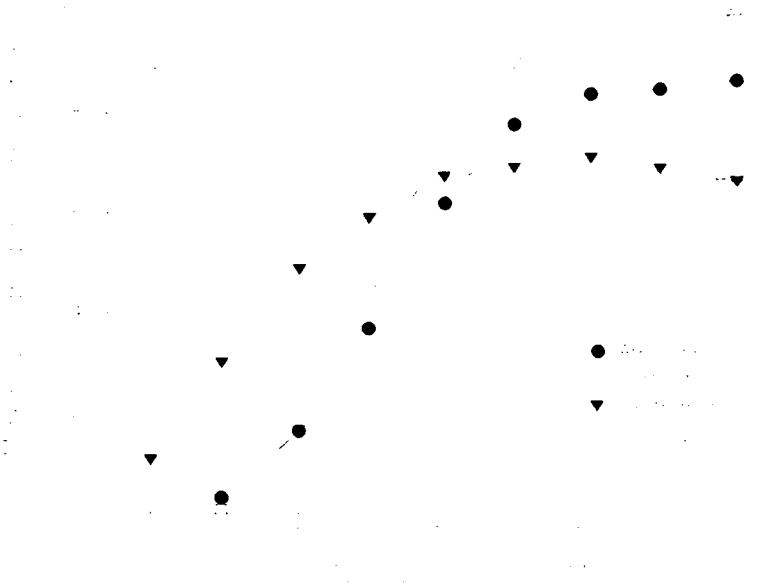

FIG.1

Agonist-stimulated production of total [^{3}H]-inositol phosphates in CHO Hm1 cells.

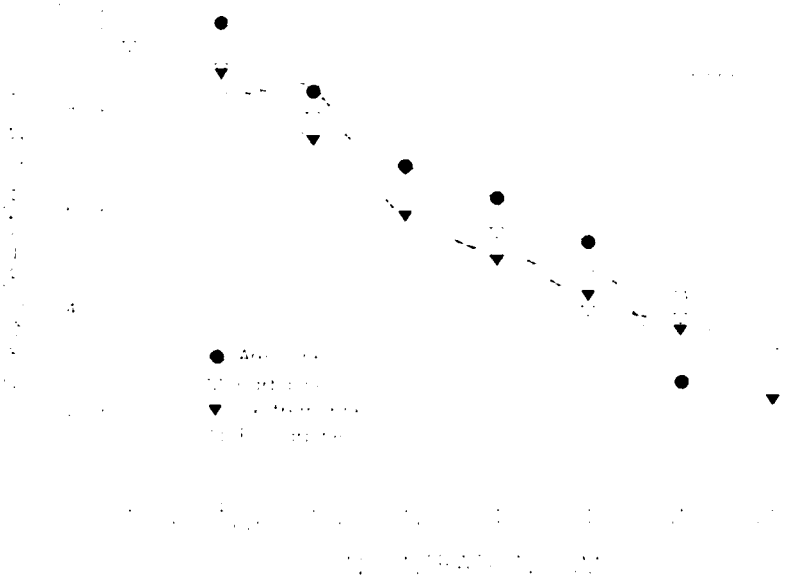

FIG.2

Inhibition of forskolin-stimulated cAMP formation in Hm2 CHO cells.

cAMP Accumulation

Receptors of the m2 and m4 subtypes appear to be negatively coupled to adenylyl cyclase. To measure effects on cAMP accumulation, cells were stimulated with 5uM forskolin in the presence of 0.1uM IBMX and various concentrations of agonist. cAMP was determined by a scintillation proximity assay. As shown in Fig. 2, the four agonists examined in CHO Hm2 cells reduced forskolin-stimulated cAMP formation, with a maximal inhibition of approximately 60%. In contrast to PI turnover, there was no apparent differences in the maximal effect produced by any agonist. EC_{50} values ranged from 2 - 6uM. Scopolamine was able to block these responses. Similar results on both efficacy and potency were also obtained in Hm4 cells (data not shown).

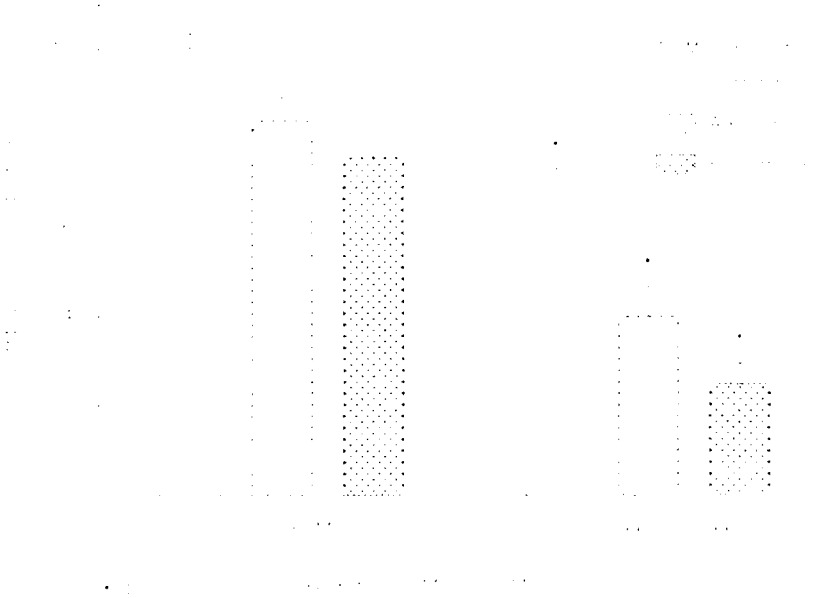

FIG.3

The effect of alkylation with PrBCM on agonist-induced formation of total [^{3}H]-inositol phosphates in Hm1 CHO cells

Role of Receptor Reserve on Functional Selectivity

The theory of receptor reserve has been reviewed in general (19) and more specifically shown to affect the activity of various oxotremorine analogues (20). Full agonists need to occupy only a fraction of the total available receptors, while partial agonists need to occupy a much greater proportion to produce a response and may not produce as great a maximal response as a full agonist. The results in PI turnover shown in Fig.1 give an example of full versus partial agonists with carbachol producing a maximal effect and pilocarpine a partial response. In this case, Hm1 cells have an extremely high

density of receptors (about 2900 fmol/mg protein). The maximal responses of arecoline, oxotremorine, and pilocarpine are much closer to the full agonist carbachol in the Hm1 cells than is observed in rat cortical slices for example. To examine the effect of receptor number on agonist-induced responses, alkylation experiments using propylbenzylcholine mustard (PrBCM) were performed. In Hm1 cells, concentrations of 0.001-10 uM PrBCM decreased the number of receptors up to 90% as measured by [^{3}H]-NMS binding in a concentration dependent manner. As shown in Fig. 3, 1.0uM PrBCM (which reduced the number of free receptors by about 75%) markedly decreased the ability of carbachol, arecoline, and pilocarpine to stimulate total [^{3}H]-inositol phosphate formation. Potency, as well as efficacy was affected. Using carbachol as the example, the EC_{50} under unalkylated conditions was 2 uM, while in the presence of 1.0uM PrBCM it was 12uM and with 10.0 uM PrBCM it rose to 102 uM. Thus, not only is efficacy affected, but potency of agonists is markedly affected by the total number of receptors available for occupation.

Discussion

A new generation of nonselective muscarinic agonists (7,8) which overcome the problems of limited oral activity, poor CNS penetration, and short duration of action associated with classical agonists may provide an adequate test of the cholinergic hypothesis in the symptomatic treatment of Alzheimer's disease (6). However, limiting side effects may not allow high enough drug levels to be achieved in the CNS for true clinical efficacy. Development of m1 selective agonists may allow high drug levels without the unwanted peripheral effects. Thus, the determination of subtype selectivity in recombinant cell lines has become crucial for the identification of these selective agonists.

Characterization of subtype selectivity can be performed using receptor binding and functional second messenger assays. For muscarinic antagonists (9,10,11) results using either type of assay have clearly defined selectivity. Pirenzepine appears to be m1 selective, although having only a 3-4-fold higher affinity than at m4 receptors. AF-DX 116 and methoctramine are clearly m2 selective, while HHSiD has some selectivity for m3 (1). These results are reliable and reproducible from laboratory to laboratory. However, demonstration of agonist selectivity appears to be more controversial and more difficult to determine in a reliable manner.

Agonist results from binding studies using labeled, nonselective antagonists (e.g. NMS, QNB) appear to underestimate selectivity, with most agonists having Ki values in the uM range and showing little or no selectivity. Some, for example bethanechol, may even have values in the high uM range (data not shown), yet still produce robust physiological effects in vivo. Agonist displacement of labeled muscarinic agonists is limited by current ligands. Most available ligands appear to be m2 selective (21,22) and reproducible agonist binding has not been achieved for all subtypes using recombinant CHO cells.

It may be possible that the development of new agonist ligands would advance the search for subtype selective agonists and should be actively pursued.

Measurement of activity in functional second messenger assays can also be used to determine subtype selectivity. In our studies, we have chosen to measure PI turnover (m1, m3, and m5) and inhibition of forskolin-stimulated cAMP accumulation (m2 and m4). Ideally, it would be best to have one functional assay common to all five subtypes to overcome methodological issues associated with each assay. Under the current experimental conditions used, the assay measuring PI turnover is more sensitive to agonists than the cAMP assay. Thus, rank orders of potency must be used for determining selectivity and not absolute values. This issue has already been addressed to some degree (Lazareno, et al. - this issue).

However, it appears that the total number of receptors is critical to measuring functional responses. In cells with a large receptor reserve, both full agonists and partial agonists will produce marked effects (Fig. 1, Hm1 cells), while in cells with few available receptors only high efficacy agonists will show a response. Since both efficacy and potency are affected by receptor number, unless one has cells with an equal number of receptors, compounds can appear to be selective when they functionally are not (and nonselective when they are selective). We addressed this issue by alkylating the receptors with PrBCM. An alternative method would be to establish new cell lines having equal numbers of receptors. In any case, in the search for selective agonists, this issue must be clearly addressed.

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References

1. E.C. HULME, N.J.M. BIRDSALL, and N.J. BUCKLEY, Annu. Rev. Pharmacol. Toxicol. 30 277-295 (1990).
2. T. KUBO, K. FUKUDA, A. MIKAMI, A. MAEDA, M. TAKAHASHI, M. MISHINA, T. HAGA, K. HAGA, A. ICHIYAMA, K. KANGAWA, H. MATSUO, T. HIROSE, and S. NUMA, Nature 323 411-416 (1986).
3. E.G. PERALTA, A. ASHKENAZI, J.W. WINSLOW, D.H. SMITH, J. RAMACHANDRAN, and D.J. CAPON, EMBO J. 6 3923-3929 (1987).
4. T.I. BONNER, N.J. BUCKLEY, A.C. YOUNG, and M.R. BRANN, Science 237 527-532 (1987).
5. W.H. MOOS, R.E. DAVIS, R.D. SCHWARZ, and E.R. GAMZU, Med. Res. Rev. 8: 353-391 (1988).
6. R.T. BARTUS, R.L. DEAN, B. BEER, A.S. LIPPA, Science 217 408-417 (1982).
7. R.D. SCHWARZ, L.L. COUGHENOUR, R.E. DAVIS, D.T. DUDLEY, W.H.MOOS, M.R. PAVIA,and H. TECLE, Cholinergic Basis for Alzheimer Therapy, R. Becker and E. Giacobini (eds.), 347-353 (1991).

8. R. DAVIS, C. RABY, R. SCHWARZ, D. DUDLEY, D. LAUFFER, P. REECE, J. JAEN and H. TECLE, Progress in Brain Research, In press, (1992).
9. N.J. BUCKLEY, T.I. BONNER, C.M. BUCKLEY, and M.R. BRANN, Mol. Pharmacol. 35 469-476 (1989).
10. F. DORJE, J. WESS, G. LAMBRECHT, R. TACKE, E. MUTSCHLER and M.R. BRANN, J. Pharmacol. Exp. Ther. 256 727-733 (1991).
11. C. BOLDEN, B. CUSACK, and E. RICHELSON, J. Pharmacol. Exp. Ther. 517 576-580 (1992).
12. E.A. NOVOTNY and M.R. BRANN, Trends Pharmacol. Sci. (December Suppl.) 4 116 (1989).
13. A. FISHER, D.GURWITZ, D. BARAK, P. HARING, I. KARTEN, R. RANDEIS, Z. PITTEL, D. MARCIANO, H. MESHULAM, Z. VOGEL, and E. HELDMAN, BioMed. Chem. Lett. 2 839-844 (1992).
14. M. SOKOLOVSKY, Adv. Drug Res. 18 432-508 (1989).
15. R. BAKER and J. SAUNDERS, Annu. Rep. Med. Chem. 24 31-40 (1989).
16. B. RINGDAHL, The Muscarinic Receptors, J.H. Brown (ed.), 151-218 (1989).
17. J.M. PALACIOS, C. BALLIGER, A. CLOSSE, A. ENZ, G, GMELIN, and J.MALANOWSKI, Eur. J. Pharmacol. 125 45-62 (1986).
18. J. SAUNDERS and S.B. FREEDMAN, Trends Pharmacol. Sci. (December Suppl.) 4 70-75 (1989).
19. R.F. RUFFOLO, J. Auton. Pharmacol. 2 277-295 (1982).
20. B. RINGDAHL, M. ROCH, and D.J. JENDEN, J. Pharmacol. Exp. Ther. 242 464-471 (1987).
21. A. CLOSSE, H. BITTER, D. LANGENEGGER and A. WANNER, Naunyn-Schmiedeberg's Arch. Pharmacol. 335 372-377 (1987).
22. G. GILLARD, M. WAELBROECK, and J. CHRISTOPHE, Mol. Pharmacol. 32 100-108 (1987).

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WAL 2014 - A MUSCARINIC AGONIST WITH PREFERENTIAL NEURON-STIMULATING PROPERTIES

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Summary

The ability of WAL 2014 to elicit muscarinic responses was investigated in various in vitro and in vivo models. In CHO cells transfected with human m1- or m3-receptor genes, WAL 2014 was clearly more effective in stimulating the M_1-mediated PI response. In isolated tissue preparations, WAL 2014 exhibited full agonist properties in the rabbit vas deferens (putative M_1 receptor) and behaved like a partial agonist at M_2 receptors in the atrium and M_3 receptors in the ileum of guinea-pigs. In the pithed rat, in which the increase in blood pressure is mediated through a stimulation of M_1 receptors in sympathetic ganglia, WAL 2014 produced a full dose response curve, whereas the reference compounds RS 86 and arecoline exhibited a bell-shaped behaviour. This is in accord with the view that WAL 2014 selectively activates M_1 receptors in sympathetic ganglia, whereas conventional agonists in the same dose range stimulate both ganglionic M_1 and vascular M_3 receptors. The preferential neuron-stimulating properties were confirmed by EEG recording in the rabbit, in which muscarinic activation occured at doses similar to those for ganglionic stimulation in the pithed rat. On the other hand, higher doses of WAL 2014 were needed to elicit muscarinic effects in peripheral effector organs, i.e. bradycardia, urinary bladder contraction and increase in airway resistance. It is concluded that WAL 2014 due to its preferential neuronal activity is a promising candidate for a cholinergic substitution therapy in Alzheimer's disease.

The argument in favour of cholinergic replacement therapy is based on the finding that in Alzheimer's disease there is a correlation between the loss of cholinergic neurons in the basal forebrain and the degree of memory impairment (1). However, given the lack of suitable agents, the cholinergic approach has not been adequately tested in clinical trials. Available cholinergic agents are lacking selectivity resulting in severe side effects (2). The goal is, therefore, to discover and develop a cholinomimetic agent with selectivity for the CNS. We focused

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on the development of an M_1-selective agonist, because postsynaptic muscarinic receptors are of the M_1 subtype and are not significantly reduced in Alzheimer's disease (1). Moreover, M_1 agonists are not likely to cause serious peripheral side effects since M_1 receptors are not dominant in muscarinic effector organs such as heart, smooth muscle tissues and exocrine glands.

In this paper we describe the pharmacological profile of the quinuclidine derivative WAL 2014, (R)-3-(2-propynyloxy)-1-azabicyclo[2.2.2]octane,(E)-2-butenedioate (German patent application, DE 38 39 385 9), a new muscarinic agonist. This compound has favourable pharmacokinetic characteristics (high oral bioavailability, lack of metabolism, good blood-brain barrier penetration) and shows a clear preference for M_1 receptor-mediated neuronal responses as demonstrated in various in vitro and in vivo models.

Material and Methods

PI turnover in cell lines. Recombinant CHO cells were maintained in Dulbecco's medium (DMEM) with 4.5 g/l glucose, supplemented with 10 % heat-inactivated fetal calf serum, MEM nonessential amino acids, penicillin (100 units/ml), streptomycin (100 µg/ml), and 2 mM glutamine. Medium was changed every 3-4 days, and confluent cells were subcultured 1:10 using trypsin (0.05 %)-EDTA (0.02 %).
Subconfluent CHO cells expressing human M_1 receptors (CHO-Hm1 cells) and human M_3 receptors (CHO-Hm3 cells), respectively, were labeled with myo-[^{3}H]inositol for 24 h. The cells were stimulated with different concentrations of agonist for 20 min at 37°C in a modified Krebs-Ringer buffer containing 10 mM LiCl. Incubations were terminated by aspiration of the medium and lysis of the cells with methanol. The lysates were applied to DOWEX anion exchange mini-columns. Total inositol phosphates (IP_x) were eluted with 2.5 ml 1 M ammonium formate / 0.1 M formic acid and measured by liquid scintillation counting. To determine the cell-associated radioactivity, the remaining cell monolayer was lysed with 1 ml 0.1 M NaOH / 1 % Triton X-100. The basal level of total IP_x formed in the absence of agonist was subtracted from each measured value. The PI turnover was defined as the radioactivity in the IP_x fraction, expressed as a percentage of the total cell-associated radioactivity.

Isolated tissues. Functional in vitro activity of WAL 2014 and carbachol at muscarinic receptor subtypes was determined by the use of M_1-receptor-mediated inhibition of neurogenic twitch contractions in rabbit vas deferens, M_2-receptor-mediated negative inotropism in electrically driven guinea-pig left atria and M_3-receptor-mediated contraction of guinea-pig ileum longitudinal smooth muscle preparation. These methods have been described in detail earlier (3, 4, 5).

Effects in vivo. Studies in the pithed rat and the anaesthetized guinea pig were carried out as described in detail elsewhere (6).
Thiry fistula dog: 4 conscious beagle dogs of both sexes (9-12 kg b.w.), chronically equipped with an ileal Thiry fistula (7), were used. Contractility of the ileal fistula was elicited and

recorded as described by Schiavone et al. (8). Drugs were dissolved in 1 ml sterile saline, and administered in increasing doses through a previously cannulated cephalic vein of the forelimb, with a 30 min minimum interval between subsequent doses. The effects on intestinal motility were measured by planimetry of the area under the pressure curve for 2 min periods; a single period before each administration was taken as basal period. Each dog received a single dose of a test compound once, with a 4-day interval between consecutive experimental sessions.

EEG experiments. The cortical EEG was recorded from chronically implanted non-restrained rabbits of both sexes. Online EEG analysis by power spectrum analysis was performed on a PDP 11/23 (9). The relative power of the theta-frequency band (3.8 - 7.5 Hz) in percent of the total power was calculated. Epochs of 10 minutes duration were averaged and the absolute differences from the basal predrug value plotted for an observation period of 120 minutes. Analysis of variance and Dunnett-test were performed.

Results

PI turnover in cell lines expressing human muscarinic receptor subtypes. Table 1 reports the EC_{50}-values and relative efficacies of carbachol and WAL 2014 for stimulating PI turnover in CHO cells expressing high and low densities of Hm1 and Hm3 receptors. It is evident that WAL 2014 is an effective agonist in CHO-Hm1 cells, reaching 85 % and 64 % of the maximum carbachol response at high and low receptor level, respectively. In contrast, in CHO-Hm3 cells WAL 2014 was a very weak partial muscarinic agonist yielding only 44 % of the maximal carbachol response at a high receptor density and complete ineffectiveness at a low receptor density.

TABLE 1

Receptor Subtype	Density pmol/mg membrane protein	Carbachol EC_{50} (μM)	WAL 2014	Relative efficacy (%)
Hm1	6.0	0.5	4.0	85
	2.1	0.7	4.0	64
Hm3	5.8	0.3	50.0	44
	0.5	6.0	not active	

Agonist-Induced PI Turnover in CHO Cells. Figures represent the means of duplicate determinations in three separate experiments. The maximum response to carbachol in each experiment was used as 100 % efficacy.

Muscarinic effects in isolated tissue preparations. Table 2 summarizes the results of the activity of carbachol and WAL 2014 in three isolated tissue preparations: the rabbit vas deferens as a neuronal M_1 model, the guinea-pig atrium as a cardiac M_2 model, and the guinea-pig ileum as a smooth muscle M_3 system. The results demonstrate the preference of WAL 2014 for the neuronal M_1-mediated response in the vas deferens preparation exhibiting both higher potency and full intrinsic activity, whereas in the

atrium and in the ileum WAL 2014 was a partial agonist with intrinsic activities of 0.5 and 0.6, respectively.

TABLE 2

	WAL 2014		Carbachol	
	pD_2	i.a.	pD_2	i.a.
Rabbit vas deferens	5.28±0.15	1.0	6.73±0.04	1.0
Guinea-pig atrium	4.65±0.07	0.52±0.03	7.14±0.06	1.0
Guinea-pig ileum	4.61±0.10	0.60±0.10	6.74±0.03	1.0

Muscarinic Activities of WAL 2014 and Carbachol in Isolated Tissue Preparations. pD_2 = -log ED_{50}; i.a. = intrinsic activity; mean values ± SD of at least 6 independent experiments

Muscarinic effects in vivo. WAL 2014 and the reference agonists RS 86 and arecoline were investigated in the **pithed rat**. In this model, muscarinic agonists with preferential action at M_1 receptors, such as McN-A-343 (10), cause a dose-related rise in arterial blood pressure and heart rate through stimulation of sympathetic ganglia. As shown in Figure 1, WAL 2014 induced a pronounced, dose-dependent hypertensive effect. In contrast, RS 86 and arecoline exhibited bell-shaped dose response curves and failed to produce full pressor responses. A qualitative difference between WAL 2014 and the reference agonists was also observed with respect to their effects on heart rate in the pithed rat model. Whereas RS 86 and arecoline exhibited an initial bradycardia followed by a tachycardia, WAL 2014 was devoid of any bradycardic action and produced only a dose related increase in the heart rate (data not shown).

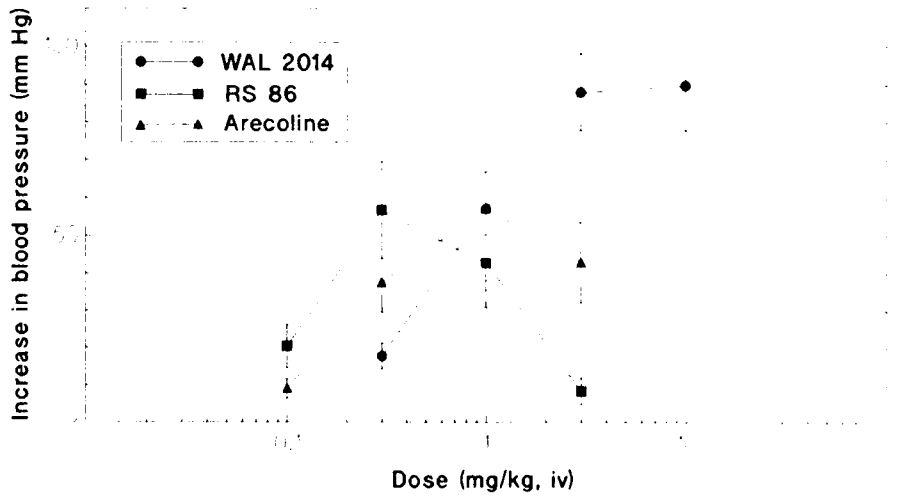

FIG. 1
Effects of WAL 2014, RS 86 and Arecoline on Blood Pressure in the Pithed Rat after Intravenous Administration. Mean values ± SEM of at least 4 animals.

To determine the direct effects of WAL 2014, RS 86 and arecoline on muscarinic effector organs experiments were carried out in the **anaesthetized guinea-pig**. Figures 2a, b and c show the dose response curves of the three agonists for bronchoconstriction, bradycardia and urinary bladder contraction. For comparison, the pressor response of the three cholinomimetics in the pithed rat is included in the diagrams. It is evident that the three compounds display different activity profiles: RS 86 evoked all the investigated muscarinic responses at comparable doses (Fig. 2b), while arecoline showed a preference for postganglionic effector organs (Fig. 2c).

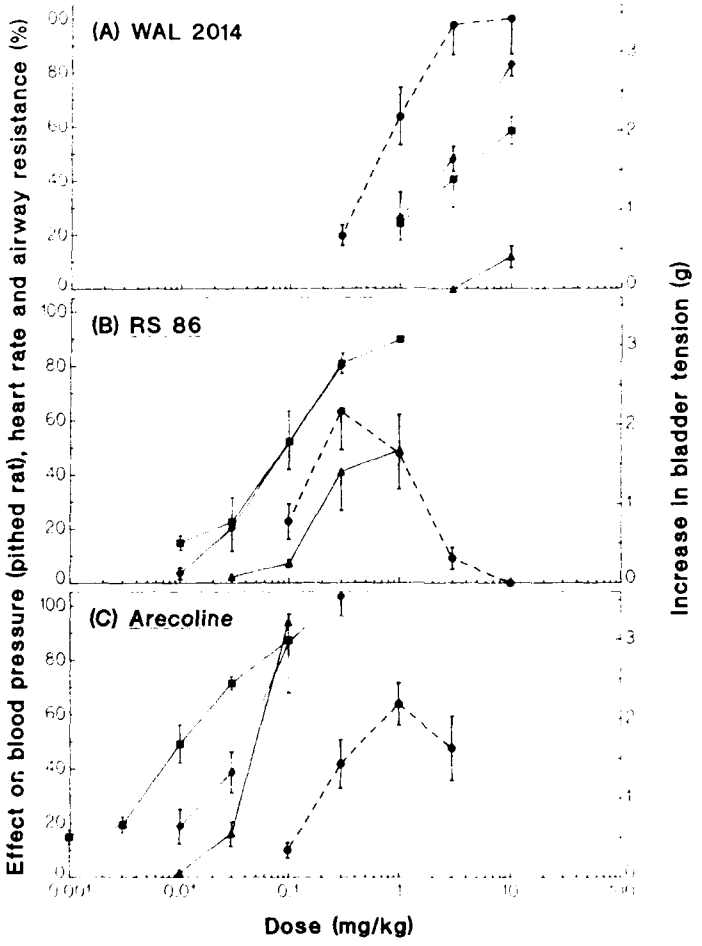

FIG. 2
Effects of WAL 2014 (A), RS 86 (B) and Arecoline (C) on Muscarinic Effector Systems in the Anaesthetized Guinea Pig: Increase in Bladder Tension (◆-◆), Decrease in Heart Rate (■-■), and Increase in Airway Resistance (▲-▲). Compounds were administered by combined intravenous and intraarterial injection (6). Mean values ± SEM of 4 animals. The pressor response in the pithed rat (●---●, see fig. 1) was included for comparison.

On the other hand, WAL 2014 exhibited a preference for the neuronal pressor response in the pithed rat, since neuronal activation occured at lower doses than those needed to stimulate the bladder, the heart and in particular the bronchi (Fig. 2a).

The effects of the three agonists were also evaluated in the **Thiry fistula dog**. Compared to RS 86 and arecoline, WAL 2014 showed a qualitatively different behaviour: While the two reference compounds enhanced the motility of the fistula by increasing basal tone, WAL 2014 inhibited ileal motility by reducing the ongoing phasic contractile activity (Fig. 3).

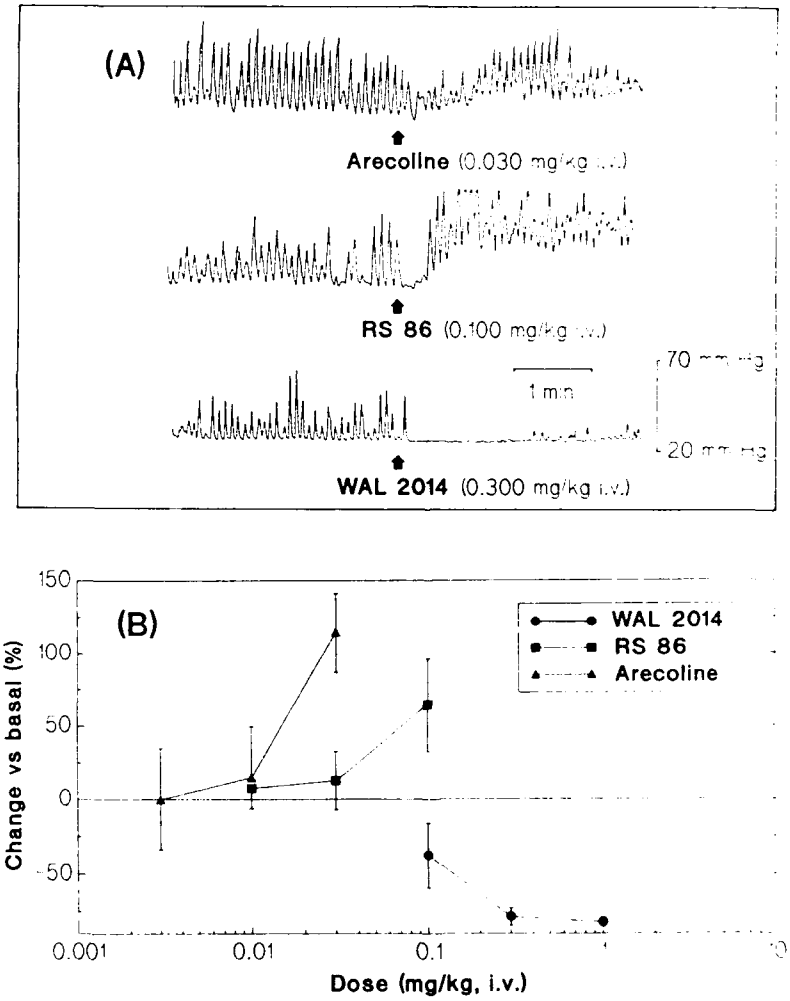

FIG. 3
Effects of WAL 2014, RS 86 and Arecoline on Intestinal Motility in the Thiry Fistula Dog. Panel A: Recordings of phasic contractile activity in single animals before and after i.v.-injections of test compounds. Panel B: % difference in motility calculated from the area under the pressure curve before and after i.v.-injection of test compounds (mean ± SEM of 4 experiments).

To investigate the ability of WAL 2014 to elicit a central neuronal response, the EEG was recorded in rabbits. WAL 2014 provoked a significant increase in theta power activity (Fig. 4). This effect, which is blocked by low doses of scopolamine, is typical of cholinomimetics and reflects an augmented hippocampal arousal. The EEG changes induced by WAL 2014 were observed at doses equivalent to those eliciting a peripheral neuronal response in the pithed rat (compare Fig. 1 and 4).

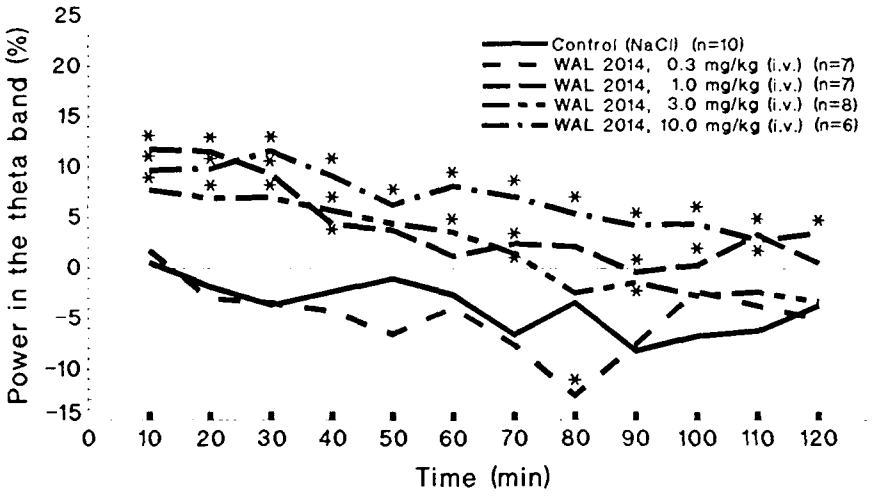

FIG. 4
Effects of WAL 2014 on EEG-pattern of Rabbits. The difference of power in theta band expressed as % of predrug value.
* = significantly different from control ($p>0.05$)

Discussion and Conclusions

In transfected CHO cells, WAL 2014 stimulated Hm1 receptors more effectively than Hm3 receptors. In isolated tissues, it displays full agonistic properties in the neuronal M_1 model of the rabbit vas deferens, and behaves as a partial agonist at atrial M_2 and ileal M_3 receptors. The preferential stimulation of M_1 receptors can be explained primarily by a functional selectivity, since binding studies in rat tissue preparations revealed similar K_i-values in hippocampus (M_1; 6,6 μM), heart (M_2; 6,6 μM) and lacrimal gland (M_3; 29,6 μM). Perhaps the most striking findings demonstrating the M_1-selectivity of this agonist come from in vivo studies in the pithed rat and the Thiry fistula dog. In the pithed rat, M_1 receptor stimulation in sympathetic ganglia causes a rise in blood pressure and heart rate through the release of norepinephrine, whereas direct activation of muscarinic receptors in the endorgans induces hypotension (via endothelial M_3 receptors) and bradycardia (via cardiac M_2 receptors). Similarly, in the Thiry fistula dog ileal motility suppression mediated by M_1 receptors located on intramural inhibitory neurons can be overridden by a direct motility-enhancing effect through M_3 receptor activation in smooth muscle. In both models the effect of WAL 2014 contrasts with that of the reference agonists and is indicative of a preferential activity at neuronal M_1

receptors. In the pithed rat, WAL 2014 produces true dose response curves both for its pressor and tachycardic activity, whereas conventional agonists produce "mixed responses". In the Thiry fistula dog, WAL 2014 suppresses ileal motility, whereas RS 86 and arecoline exhibit a motility-enhancing effect. In addition, the neuron-stimulating properties of WAL 2014 are further illustrated by the finding that a low dose of the M_1 antagonist pirenzepine (0.3 mg/kg) effectively inhibits the pressor response in the pithed rat.

The pharmacological profile of WAL 2014 is comparable in many instances to that of the M_1 agonist McN-A-343 both with respect to in vitro studies in isolated organs (11) and in vivo studies in the pithed rat (12, 13) and Thiry fistula dog (8). The two agents, however, clearly differ in their ability to induce CNS effects. Due to its quaternary ammonium structure and hydrophilic nature the pharmacological actions of McN-A-343 are largely limited to peripheral organs. In contrast, WAL 2014 readily crosses the blood brain barrier as evidenced by the typical cholinergic EEG activation which occurs in the same dose range as the peripheral ganglionic activation in the pithed rat.

We conclude that, based on its preferential neuronal M_1 receptor-stimulating activity, WAL 2014 appears to be a promising candidate for cholinergic replacement therapy in Alzheimer's disease.

References

1. E.K. PERRY, Psychopharmacology: The Third Generation of Progress, H.Y. Meltzer (ed), 887-895, Raven Pr., N.Y. (1987)
2. V. KUMAR and M. CALACHE, Clin. Pharmacol., Ther. and Tox. 29 23-27 (1991)
3. M. ELTZE, G. GMELIN, J. WESS, C. STROHMANN, R. TACKE, E. MUTSCHLER and G. LAMBRECHT, Eur. J. Pharmacol. 158 233-242 (1988)
4. G. LAMBRECHT, R. FEIFEL, M. WAGNER-RÖDER, C. STROHMANN, ZILCH, R. TACKE, M. WAELBROECK, J. CHRISTOPHE, H. BODDEKE and E. MUTSCHLER, Eur. J. Pharmacol. 168 71-80 (1989)
5. F. DÖRJE, N.M. RETTENMAYR, E. MUTSCHLER and G. LAMBRECHT, Eur. J. Pharmacol. 203 417-420 (1991)
6. H.N. DOODS, M. ENTZEROTH and N. MAYER, Eur. J. Pharmacol. 192 147-152 (1991)
7. L. THIRY, Sitzungsberichte Akad. Wien, Mathem.-Naturw. Kl. 1 43-44 (1964)
8. A. SCHIAVONE, A. SAGRADA, R. MICHELETTI and A. GIACHETTI, Br. J. Pharmacol. 94 566-572 (1988)
9. F.J. KUHN, G. SCHINGNITZ, E. LEHR, E. MONTAGNA, H.D. HINZEN and A. GIACHETTI, Arch. Int. Pharmacodyn. Ther. 292 13-24 (1988)
10. A.P. ROSZKOWSKY, J. Pharmacol. Exp. Therap. 132 156-170 (1961)
11. B. JOKISCH-MERHLING, R. FEIFEL, M. ELTZE, J.F. RODGRIGUES DE MIRANDA, G. LAMBRECHT and E. MUTSCHLER, Trends Pharmacol. Sci. 10 (Suppl.) 109 (1989)
12. R. HAMMER and A. GIACHETTI, Life Sci. 31 2991-2998 (1982)
13. J. WESS, G. LAMBRECHT, U. MOSER and E. MUTSCHLER, Life Sci. 35 553-560 (1984)

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NEW FUNCTIONALLY SELECTIVE MUSCARINIC AGONISTS

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Summary

The muscarinic pharmacology of two novel agonists related to McN-A-343, 4-F-PyMcN and 4-F-PyMcN$^+$, has been studied by the use of pharmacological and radioligand binding techniques. Both compounds were potent agonists at M_1 receptors in rabbit vas deferens (pEC_{50} = 6.24 and 6.96) and rat duodenum (pEC_{50} = 5.47 and 6.38), but very weak partial agonists or competitive antagonists at guinea-pig cardiac M_2 and ileal M_3 receptors. There was no receptor reserve for 4-F-PyMcN in rabbit vas deferens, for which the potency (pEC_{50} = 6.24) and apparent affinity (pK_A = 5.99 and 6.21) were similar. 4-F-PyMcN$^+$ showed only limited binding selectivity between four muscarinic receptor binding assays with apparent affinity constants (pK_i) of 5.8, 5.2, 5.6 and 5.7 for M_1, M_2, M_3 and M_4 muscarinic receptor subtypes. The two novel functionally M_1-selective agonists may provide useful tools with which to study muscarinic receptor mechanisms. The non-quaternary compound, 4-F-PyMcN, might become a starting point for the development of drugs that selectively affect M_1 receptors involved in central cholinergic function.

Much interest has been focused in the last decade on the development of selective muscarinic agents (1). Candidates of selective agonists include 4-(3-chlorophenylcarbamoyloxy)2-butynyltrimethylammonium chloride (McN-A-343, FIG. 1) and analogues. McN-A-343 actually served as one of the earliest indicators of the existence of muscarinic receptor subtypes (2). Different from other muscarinic agonists, McN-A-343 is potent in stimulating muscarinic M_1 receptors both in the CNS and peripheral nervous system and has less activity at M_2 and M_3 receptors (2-6). The selectivity displayed by McN-A-343 in functional tests does not extend to binding studies where only minor differences in the affinity of the compound for receptor subtypes has been demonstrated (7-9). Many analogues of McN-A-343 have been prepared, but only a few of these possess selectivity, ef-

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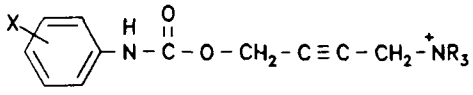
ficacy and potency similar to that of the parent compound (3,10-12). In particular, no closely related, biologically active analogues of McN-A-343 capable of penetrating into the CNS are presently known. Such analogues have been suggested as potential therapeutic agents in conditions associated with cognitive impairments, e.g. Alzheimer's disease (13). To this end, we have synthesized and pharmacologically characterized a series of novel McN-A-343 analogues. We hoped to alter the functional selectivity of McN-



McN-A-343	4-Cl-McN	4-F-PyMcN$^+$	4-F-PyMcN
X = 3-Cl	X = 4-Cl	X = 4-F	X = 4-F
$^+NR_3$ = $^+N(CH_3)_3$	$^+NR_3$ = $^+N(CH_3)_3$	$^+NR_3$ = 	$^+NR_3$ = 

FIG. 1
Chemical structures of McN-A-343, 4-Cl-McN and two amino terminally modified derivatives, 4-F-PyMcN and 4-F-PyMcN$^+$.

A-343 in favour of the M_1 subtype and to discover tertiary M_1-selective agonists capable of crossing the blood-brain barrier. The new compounds were assayed for muscarinic activity on the rabbit vas deferens (putative M_1 receptors) as well as on the guinea-pig atria (M_2 receptors) and ileum (M_3 receptors) (Moser et al., this volume).

Two of the McN-A-343 derivatives, 4-F-PyMcN and 4-F-PyMcN$^+$ (FIG. 1), were the subject of a more detailed pharmacological characterization using both functional and competition radioligand techniques. In particular, we attempted to use the rat duodenum to obtain muscarinic potencies of McN-A-343 and its analogues. This isolated preparation has been described by Micheletti et al. (14) as a new functional M_1 receptor model, but subsequently the same investigators have raised the possibility that the muscarinic receptor mediating relaxation in rat duodenum might be an M_4 receptor (15,16).

Methods

Functional activity at muscarinic receptor subtypes was determined by the use of the M_1 receptor-mediated inhibition of neurogenic twitch contractions (single pulses at 0.05 Hz) and inhibition of evoked noradrenaline release (trains of pulses at 1 Hz) in rabbit vas deferens, M_2 receptor-mediated negative inotropism in paced guinea-pig atria (2 Hz) and M_3 receptor-mediated contraction of guinea-pig ileum longitudinal smooth muscle preparation. These methods have been described in detail earlier (3,17,18 and Grimm et al., this volume). Muscarinic activities at putative M_1 receptors, mediating relaxation in rat duodenum, were determined as described by Micheletti et al. (14). Muscarinic binding selectivity was assessed using [^{3}H]-N-methyl scopolamine ([^{3}H]-

NMS) binding to homogenates of NB-OK 1 cells (M_1) and rat heart (M_2), pancreas (M_3) and striatum (M_4) (19).

Data analysis. Results are given as arithmetic means (n = 4 - 16).

The potencies and apparent efficacies of the agonists were expressed by their pEC_{50} and intrinsic activity (i.a.) values. The apparent affinity (pK_A) of partial agonists was assessed by the technique of Waud (20) and by the low calcium concentration method described by Giao-T. Rico (21) and Eglen and Whiting (22).

Antagonism by 4-F-PyMcN$^+$ (3-100 μM) and 4-F-PyMcN (3-30 μM) in guinea-pig ileum and atria [arecaidine propargyl ester (APE) as agonist] as well as pirenzepine antagonism (30 - 300 nM) in rat duodenum (4-F-PyMcN$^+$ as agonist) was evaluated by Schild analysis (23). Since only one concentration of p-fluoro-hexahydro-sila-difenidol (p-F-HHSiD; 300 nM), AQ-RA 741 (300 nM), guanylpirenzepine (300 nM) and himbacine (1 μM) was tested in rat duodenum, their pA_2 values (TABLE IV) were derived from the equation pA_2 = -log [antagonist] + log (DR-1) (24). [^{3}H]-NMS competition binding data were analyzed by a computer-assisted curve-fitting procedure, and unlabeled drug apparent pK_i values were calculated (19).

The following drugs were used: pirenzepine dihydrochloride, guanylpirenzepine dihydrochloride and 11-({4-[4-(diethylamino)-butyl]-1-piperidinyl}acetyl-5,11-dihydro-6H-pyrido(2,3-b)(1,4)-benzodiazepine-6-one (AQ-RA 741) (Thomae, Biberach/Germany), himbacine hydrochloride (Dr. W. C. Taylor, Sydney/Australia). All other drugs were synthesized in our laboratories.

Results and Discussion

All agonist responses were determined to be muscarinic in nature in that their activities were blocked by: pirenzepine (0.1 μM, pA_2 = 7.8 - 8.2) in rabbit vas deferens and rat duodenum, AQ-RA 741 (300 nM, pA_2 = 8.3 - 8.5) in guinea-pig atria and p-F-HHSiD (0.5 μM, pA_2 = 7.6 - 8.1) in guinea-pig ileum. Tetrodotoxin (TTX) (0.1 and 1 μM) did not block agonist activities in guinea-pig atria and ileum. The potency (pEC_{50}), apparent efficacy (i.a.) and affinity (pA_2) for APE, McN-A-343 and analogues at muscarinic receptors in rabbit vas deferens (M_1) and guinea-pig atria (M_2) and ileum (M_3) are shown in TABLE I.

APE was the most potent compound possessing a small preference for M_2 receptors (17,18). In contrast, the effects of McN-A-343 and analogues differed clearly in the three functional assays. 4-Cl-McN and 4-F-PyMcN$^+$ had comparable potency to McN-A-343 at M_1 receptors in rabbit vas deferens, whereas the tertiary compound 4-F-PyMcN was about 5-fold less potent. In both the atria and ileum McN-A-343 and 4-Cl-McN behaved as partial or full agonists, being more potent and more efficacious in the M_3 assay. 4-F-PyMcN and 4-F-PyMcN$^+$ were competitive antagonists or very weak partial agonists at M_2 and M_3 receptors. These results suggest that 4-F-PyMcN$^+$ represents a functionally M_1-selective agonist with greater selectivity than the parent compound McN-A-343. The compound 4-F-PyMcN is the first tertiary close analogue of McN-A-343 which acts as a relatively potent M_1-selective agonist. It has been reported that the twitch inhibitory potency of muscarinic agonists in rabbit vas deferens increases up to 20-fold on lowering of the extracellular calcium concentration from normal (2.5 or 1.8 mM) to 1.0 mM (17). This is illustrated for the full agonist 4-Cl-McN and the partial agonist 4-F-PyMcN in FIG. 2. In addition, the left-side shift of the dose-response curve

TABLE I

In Vitro Functional Activity of Muscarinic Agents at M_1 Receptors in Rabbit Vas Deferens (RVD), M_2 Receptors in Guinea-Pig Atria (GPA) and M_3 Receptors in Guinea-Pig Ileum (GPI)

	RVD/M_1	GPA/M_2			GPI/M_3		
Compound	pEC_{50}	pEC_{50}	i.a.[a]	pA_2	pEC_{50}	i.a.[a]	pA_2
APE[a]	7.74	8.19	1.00	-	7.58	1.00	-
McN-A-343	6.77	4.87[b]	0.49	-	5.51[c]	0.83	-
4-Cl-McN	7.13	5.26	0.77	-	5.71	1.00	-
4-F-PyMcN$^+$	6.96	-	-	5.51[d]	5.38	0.26	5.40[e]
4-F-PyMcN	6.24[f]	6.05	0.26	5.58[g]	5.46	0.30	5.63[h]

a Intrinsic activity: the maximum response to arecaidine propargyl ester (APE) = 1.00.

b, c pK_A values determined by the technique of Waud (20) and using APE as full agonist, were: M_2 = 4.79, M_3 = 5.17.

d, e, g, h Slopes of Schild plots (1.07, 1.15, 0.90, 1.00, respectively) were not significantly different from unity ($P>0.05$).

f Partial agonism: i.a. = 0.86.

of 4-F-PyMcN is accompanied by an increase in the apparent efficacy. Consequently the affinity constant (pK_A) of 4-F-PyMcN could be derived from a double-reciprocal regression (21,22). The results from these experiments are shown in TABLE II. The affinity estimate of 4-F-PyMcN was 5.99, a value not significantly different ($P>0.05$) from the pK_A value (6.21) obtained by the technique of Waud (20) and from the pEC_{50} value of 6.24. The affinity of 4-F-PyMcN for M_1 receptors in rabbit vas deferens was only 3-fold higher than that for M_2 and M_3 receptors (TABLE I). It thus appears that the ability of 4-F-PyMcN to selectively stimulate M_1 receptors in rabbit vas deferens is not based only on a greater affinity for this subtype.

4-Cl-McN (pEC_{50} = 6.49) and 4-F-PyMcN$^+$ (pEC_{50} = 6.32) reduced the field stimulation-evoked noradrenaline overflow from the rabbit vas deferens with similar high potency (TABLE II). Pirenzepine (0.1 μM) inhibited the agonist-induced responses with high affinity pA_2 values (e.g., 7.74, 4-F-PyMcN$^+$ as agonist). These results suggest that the prejunctional muscarinic receptors in rabbit vas deferens inhibiting the ATP-induced twitch response at 0.05 Hz (3,17,18) and the receptors inhibiting the evoked release of noradrenaline are of the same subtype (putative M_1 receptors) (see also, Grimm et al., this volume).

4-F-PyMcN$^+$ was also compared to McN-A-343 with regard to its ability to displace [^{3}H]-NMS from muscarinic receptors. The respective agonist binding parameters are summarized in TABLE III. The McN-A-343/ and 4-F-PyMcN$^+$/[^{3}H]-NMS competition curves at M_1 and M_3 receptors were best fitted to a single affinity site. However, the displacement isotherm at sites in heart (M_2) and striatum (M_4) were better described

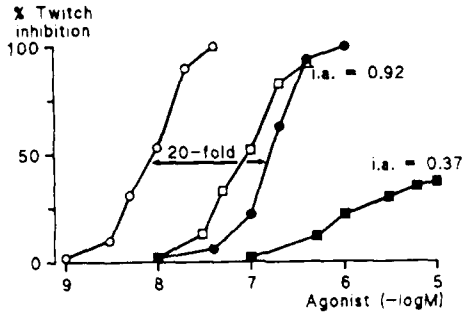


FIG. 2. Effects of lowering the extracellular calcium concentration from normal (1.8 mM, closed symbols) to 1.0 mM (open symbols) on responses of rabbit vas deferens to 4-Cl-McN (circles) and 4-F-PyMcN (squares). The graph shows data obtained from one typical experiment.

TABLE II

Agonist Affinity Constants (pK_A) and Potencies (pEC_{50}) for Inhibition of Neurogenic Responses (NR) in Rabbit Vas Deferens (RVD) and Potencies (pEC_{50}) for Inhibition of Noradrenaline (NA) Release in RVD and for Relaxation in Rat Duodenum (RD/RL). n.d. = not determined

	RVD/pK_A[a]		pEC_{50}		
Compound	Waud	Low Ca^{2+}	RVD/NR	RVD/NA	RD/RL[b]
McN-A-343	5.17[c]	--	6.77	6.49[d]	6.47
4-F-PyMcN$^+$	--	--	6.96	6.32[e]	6.38
4-F-PyMcN	6.21	5.99	6.24	n.d.	5.47

a pK_A values of 4-F-PyMcN were determined as described by Waud (20) and using the low calcium concentration method (21,22). 4-Cl-McN was used as full agonist.

b 4-F-PyMcN$^+$ and 4-F-PyMcN produced a relative maximum response that was indistinguishable from McN-A-343.

c pK_A was estimated using irreversible antagonism (25).

d pEC_{50} for 4-Cl-McN. Maximal inhibition = 91%.

e Maximal inhibition = 83 %.

using a two site model. The pK_i values for McN-A-343 at M_1 and M_3 and for 4-F-PyMcN$^+$ at M_3 receptors (TABLE III) were similar to those pK_A and pA_2 values (TABLE I and II) estimated in functional studies. The pK_i values of the two agonists at the low affinity, but not high affinity site were similar to the pK_A and pA_2 values, respectively, obtained at cardiac M_2 receptors in functional experiments (TABLE I). In addition, the pK_L for McN-A-343 at M_4 receptors in rat striatum (5.6; TABLE III), but not pK_H (6.7) was very similar to the apparent pK_A value (5.4) estimated in the same tissue by measuring M_4 receptor-mediated inhibition of adenylate cyclase (26). 4-F-PyMcN$^+$ had an up to 3-fold higher affinity for muscarinic receptors than McN-A-343. In terms of affinity (denoted as pK_i or pK_L), McN-A-343 was preferential for the M_4 receptor (8-fold M_4 over M_2), whereas 4-F-PyMcN$^+$ had a 4-fold higher affinity for M_1 than M_2 receptors.

TABLE III

Inhibition of [^{3}H]-N-Methyl Scopolamine Binding to Muscarinic Receptor Subtypes

	M_1[a]	M_2[a]		M_3[a]	M_4[a]	
Compound	pK_i	pK_H[b]	pK_L[c]	pK_i	pK_H[b]	pK_L[c]
McN-A-343	5.3	6.0 (41)	4.7	5.3	6.7 (27)	5.6
4-F-PyMcN$^+$	5.8	6.2 (53)	5.2	5.6	6.9 (22)	5.7

a Receptor source: M_1, NB-OK 1 cells; M_2, rat heart; M_3, rat pancreas; M_4, rat striatum.

b Binding to high affinity sites; the relative proportions (%) of these sites are given in parenthesis.

c Binding to low affinity sites.

McN-A-343, 4-F-PyMcN and 4-F-PyMcN$^+$ were further evaluated for muscarinic activity using the rat duodenum. The three agonists produced concentration-dependent relaxation, which was not blocked by hexamethonium (100 μM) but inhibited by pirenzepine (0.1 μM, pA_2 = 7.77 - 7.90) and TTX (1 μM). The maximum relaxation induced by 4-F-PyMcN and 4-F-PyMcN$^+$ and that induced by McN-A-343 did not differ significantly. There was no significant difference between the potencies of McN-A-343 (pEC_{50} = 6.47) and 4-F-PyMcN$^+$ (pEC_{50} = 6.38), but they were about 10 times more potent than 4-F-PyMcN. These results were similar to that found in rabbit vas deferens (TABLE II). The McN-A-343-induced relaxation was often followed by a sustained contraction, whereas the only response to 4-F-PyMcN and 4-F-PyMcN$^+$ was a relaxation. The two opposing effects to McN-A-343 limit its use in the determination of affinities of muscarinic antagonists in rat duodenum.

To characterize further the receptor responsible for relaxation in rat duodenum, we studied the antagonism by a series of muscarinic antagonists endowed with selectivity for subtypes of muscarinic receptors. 4-F-PyMcN$^+$ was used as agonist. The results were compared with that obtained in binding studies at M_1 receptors in NB-OK 1 cells. Examination of the data in TABLE IV clearly shows that the inhibition of 4-F-PyMcN$^+$-induced relaxation by the antagonists followed the same profile as the binding affinities obtained at M_1 receptors in NB-OK 1 cells: pirenzepine > p-F-HHSiD = AQ-RA 741 > guanylpirenzepine > himbacine. In addition, the antimuscarinic potencies of the antagonists relative to pirenzepine (affinity ratios in TABLE IV) obtained in binding and relaxation experiments, respectively, are very similar. These results strongly suggest that the muscarinic receptors mediating NANC relaxation in rat duodenum are of the M_1 subtype. Our results are in disagreement with Micheletti et al. (15,16) who suggested that it might be an M_4 receptor. The reason for this discrepancy is unclear, but it may relate to the use as agonist by Micheletti et al.(14-16) of McN-A-343, which causes a biphasic response in rat duodenum (see above). Further experiments are needed to clarify this issue.

TABLE IV

Affinities of Antagonists for Muscarinic Receptors Mediating Rat Duodenum Relaxation (pA_2) and for Inhibiting [^{3}H]-N-Methyl Scopolamine Binding to M_1 Receptors in NB-OK 1 Cells (pK_i)

	NB-OK 1 cells		Rat duodenum[a]	
Compound	pK_i	Ratio[b]	pA_2	Ratio[b]
Pirenzepine	8.30[d]	-	7.90[c]	-
p-F-HHSiD	7.80[d]	3.2	7.27	4.3
AQ-RA 741	7.80	3.2	7.28	4.2
Guanylpirenzepine	7.60	5.0	7.14	5.7
Himbacine	7.14[d]	14.5	6.80	12.5

a 4-F-PyMcN$^+$ was used as agonist.
b Antilog of {(pA_2 or pK_i of pirenzepine) - (pA_2 or pK_i of antagonist)}.
c Slope of Schild plot (1.06) was not significantly different from unity ($P>0.05$).
d Data taken from Waelbroeck et al. (19,27).

In conclusion, the present results describe two novel muscarinic agonists, 4-F-PyMcN and 4-F-PyMcN$^+$, structurally related to McN-A-343. Their high functional M_1 selectivity makes these compounds suitable for the study of muscarinic receptor mechanisms. The non-quaternary compound, 4-F-PyMcN, might become a starting point for the development of drugs that selectively affect muscarinic M_1 receptors involved in central cholinergic function.

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References

1. R.R. LEVINE and N.J.M. BIRDSALL (eds), Subtypes of Muscarinic Receptors IV, Trends Pharmacol. Sci. 10 (Suppl.) (1989).
2. A.P. ROSZKOWSKI, J. Pharmacol. Exp. Ther. 132 156-170 (1961).
3. M. ELTZE, G. GMELIN, J. WESS, C. STROHMANN, R. TACKE, E. MUTSCHLER and G. LAMBRECHT, Eur. J. Pharmacol. 158 233-242 (1988).
4. S. SUGITA, N. UCHIMURA, Z.-G. JIANG and R.A. NORTH, Proc. Natl. Acad. Sci. USA 88 2608-2611 (1991).
5. J. HU and E.E. EL-FAKAHANY, Mol. Pharmacol. 38 895-903 (1990).
6. A.P.D.W. FORD, W.B. LEVINE, G.S. BAXTER, G.C. HARRIS, R.M. EGLEN and R.L. WHITING, Mol. Neuropharmacol. 1 117-127 (1991).

7. L. MEI, J. LAI, H.I. YAMAMURA and W.R. ROESKE, J. Pharmacol. Exp. Ther. 256 689-694 (1991).
8. H. WEI, W.R.ROESKE, J. LAI, F. WANIBUCHI, K. HIDAKA, S. USUDA and H.I. YAMAMURA, Life Sci. 50 355-363 (1992).
9. S.B. FREEDMAN, M.S. BEER and E.A. HARLEY, Eur. J. Pharmacol. 156, 133-142 (1988).
10. B. RINGDAHL, The Muscarinic Receptors, J.H. Brown (ed), 151-218, The Humana Press, Clifton (1989).
11. C. MELLIN, H.M. VARGAS and B. RINGDAHL, J. Med. Chem. 32 1590-1593 (1989).
12. B.M. NILSSON, H.M. VARGAS and U. HACKSELL, J. Med. Chem. 35 2787-2798 (1992).
13. J.M. PALACIOS, H.W.G.M. BODEKKE and E. POMBO-VILLAR, Acta Psychiatr. Scand., Suppl. 366 27-33 (1991).
14. R. MICHELETTI, A. SCHIAVONE and A. GIACHETTI, J. Pharmacol. Exp. Ther. 244 680-684 (1988).
15. R. MICHELETTI, L. GIUDICI, M. TURCONI, A. DONETTI, Br. J. Pharmacol. 100 395-397 (1990).
16. R. MICHELETTI, A. SCHIAVONE, O. ANGELICI, P. DURANTI, L. GIUDICI, E. CERLDA and A. DONETTI, Life Sci. 47 PL55-PL58 (1990).
17. F. DÖRJE, N.M. RETTENMAYR, E. MUTSCHLER and G. LAMBRECHT, Eur. J. Pharmacol. 203 417-420 (1991).
18. G. LAMBRECHT, R. FEIFEL, M. WAGNER-RÖDER, C. STROHMANN, H. ZILCH, R. TACKE, M. WAELBROECK, J. CHRISTOPHE, H. BODEKKE and E. MUTSCHLER, Eur. J. Pharmacol. 168 71-80 (1989).
19. M. WAELBROECK, M. TASTENOY, J. CAMUS and J. CHRISTOPHE, Mol. Pharmacol. 38 267-273 (1990).
20. D.R. WAUD, J. Pharmacol. Exp. Ther. 170 117-122 (1969).
21. J.M. GIAO-T. RICO, Eur. J. Pharmacol. 13 218-229 (1971).
22. R.M. EGLEN and R.L. WHITING, J. Pharmacol. Exp. Ther. 240 404-409 (1987).
23. R.J. TALLARIDA and R.B. MURRAY, Manual of Pharmacologic Calculations with Computer Programs, Springer-Verlag, New York (1987).
24. R.F. FURCHGOTT, Catecholamines, H. Blaschko and E. Muscholl (eds), Handbook of Experimental Pharmacology, Vol. 33, 283-335, Springer-Verlag, Berlin (1972).
25. R. MICHELETTI and R. SCHIAVONE, J. Pharmacol. Exp. Ther. 253 310-314 (1990).
26. M. MCKINNEY, J.H. MILLER, V.A. GIBSON, L. NICKELSON and S. AKSOY, Mol. Pharmacol. 40 1014-1022 (1991).
27. M. WAELBROECK, J. CAMUS, M. TASTENOY, E. MUTSCHLER, C. STROHMANN, R. TACKE, G. LAMBRECHT and J. CHRISTOPHE, Eur. J. Pharmacol. - Mol. Pharmacol. Sect. 206 95-103 (1991).

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THE DESIGN OF NOVEL MUSCARINIC PARTIAL AGONISTS THAT HAVE FUNCTIONAL SELECTIVITY IN PHARMACOLOGICAL PREPARATIONS *IN VITRO* AND REDUCED SIDE-EFFECT PROFILE *IN VIVO*.

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Summary

Antagonist/agonist binding ratios (NMS/Oxo-M ratio) were used as an index of the efficacy of novel compounds acting at muscarinic receptors. These binding ratios have been used with a range of functional pharmacological assays to investigate the effects of varying the efficacy of muscarinic agonists. This strategy has been used as a means of obtaining functional receptor selectivity by exploiting differences in effective receptor reserves. The oxadiazole and pyrazine muscarinic agonists L-670,548 (NMS/Oxo-M ratio 1100) and L-680,648 (NMS/Oxo-M ratio 690) are amongst some of the most potent and efficacious agonists known. Decreasing the efficacy of compounds from these series, resulted in compounds with functional selectivity. The chloropyrazine L-689,660 (NMS/Oxo-M ratio 28) was an agonist on the rat superior cervical ganglion (M_1), a partial agonist on the guinea-pig ileum (M_3), but was an antagonist in the guinea-pig atria (M_2). Synthesis of compounds with even lower predicted efficacy, such as the cyclopropyloxadiazole L-687,306 (NMS/Oxo-M ratio 15), maintained agonist activity in the ganglion, but showed antagonist activity in the M_3 ileal, as well as the M_2 atrial preparations. When tested *in vivo* these compounds did not produce many of the side effects associated with more efficacious agonists, particularly those associated with the cardiovascular system. However, they were active in reversing scopolamine-induced deficits in a variety of behavioural paradigms. This approach shows how functional selectivity for muscarinic receptor subtypes can be achieved *in vitro*, that *in vivo* reduces the dose-limiting side effects normally associated with muscarinic agonists.

Three important factors have provided impetus for research into muscarinic receptors. The first was the discovery of the involvement of muscarinic receptors in cardiovascular, respiratory, and secretory functions, and importantly in neurotransmission within the central nervous system. The second was the the discovery of the neurochemical deficit in the acetylcholine containing neurones in Alzheimer's disease that resulted in the development of the 'Cholinergic Hypothesis' (1,2). This prompted the search for centrally active muscarinic M_1 agonists in an analogous fashion to the neurotransmitter replacement therapy seen with L-dopa in Parkinson's disease. Finally, and most recently, was the cloning and pharmacological characterisation of five distinct muscarinic receptors and the demonstration of their distribution in discrete areas of the CNS and peripheral tissues (3,4).

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The identification and characterisation of five different muscarinic receptor subtypes offered the opportunity to design agents which would stimulate specific pathways in the CNS selectively and thereby avoid many of the peripheral and centrally mediated side-effects associated with centrally active muscarinic agonists. The discovery of truly receptor-selective muscarinic agonists, in contrast to antagonists, has been relatively unsuccessful to date. However, in addition to pursuing this goal, we have also examined the potential for manipulation of efficacy as a means of obtaining selectivity and reducing side effects. This approach explores the profile of partial agonists and their ability to exhibit functional selectivity between different tissues on the basis of differences in effective receptor reserve (5).

Results

The key to our investigations of functional selectivity has been the establishment of two biological methodologies. In biochemical studies this was the development of the agonist/antagonist binding ratio (NMS/Oxo-M ratio), that have examined the ability of test compounds to distinguish between high and low affinity states of the muscarinic receptors present in rat cerebral cortex (6). The NMS/Oxo-M ratio has been proposed as an index of cortical efficacy since it was shown to reflect the ability to stimulate cortical phosphatidyl-inositol turnover. The second was in the choice of three functional pharmacological models that differed in their receptor reserve as assays for muscarinic agonist activity These included depolarisation of the rat superior cervical ganglion (M_1) (7), negative inotropy in the guinea-pig atrium (M_2) and contraction of the guinea-pig ileum (M_3).

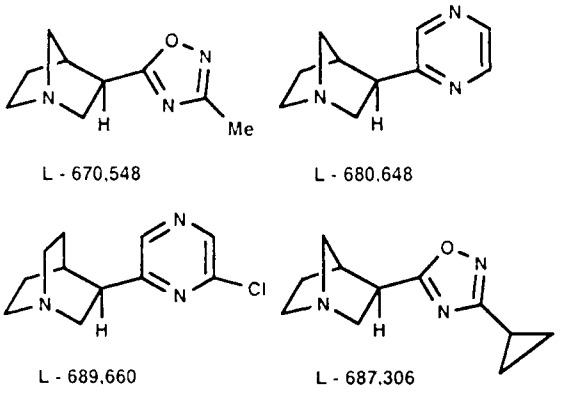

FIG 1

Novel oxadiazole and pyrazine muscarinic agonists.

We previously described a novel series of muscarinic agonists in which the ester moeity of arecoline was replaced by the oxadiazole group. (8,9). This group of compounds included the most potent and efficacious muscarinic agonists known, including the methyl oxadiazole L-670,548 (Fig 1) and the amino oxadiazole L-670,207 (10,11). In a more recent study we also described a related series of azanorbornyl muscarinic agonists in which the oxadiazole group was replaced by a pyrazine (12). This series also resulted in the identification of a number of very potent, highly efficacious compounds.

The pyrazine derivative L-680,648 (Fig 1) displayed high affinity in receptor binding assays ([^{3}H] NMS: Kapp 0.064μM) and an NMS/Oxo-M ratio of 690 (Table I). This value was predictive of a high efficacy agonist as demonstrated by its consistent ability to maximally stimulate cortical phosphatidyl-inositol (PI) turnover (EC_{50} 0.30μM, maximum response 160% of that observed

with 1mM carbachol) and show full agonist activity in all three *in vitro* pharmacological preparations (Table II). Both L-670,548 and L-680,648 showed good evidence of penetration into the CNS as evidenced by an *ex vivo* binding assay (13) (ED_{50} 0.0069 and 0.028mg/kg respectively). The high efficacy of both compounds was however, associated with potent *in vivo* activity at all muscarinic receptor subtypes. This resulted in a diverse spectrum of cholinomimetic activity, including salivation, hypothermia, piloerecetion, tremor and cardiovascular side-effects. In conscious rats, L-670,548 typically included transient marked hypotension and bradycardia, immediately followed by reflex tachycardia and a centrally driven increase in blood pressure (0.001-0.1mg/kg iv). The peripheral hypotensive and bradycardic effects were blocked by N-methylscopolamine and the centrally mediated increases in blood pressure by atropine.

TABLE I

N-Methylscopolamine/oxotremorine-M Binding Ratio in Rat Cerebral Cortex

	[^{3}H] NMS Kapp (nM)	[^{3}H] Oxotremorine-M Kapp (nM)	NMS/Oxo-M Ratio
Muscarine	19,000	4.8	4000
L-670,548	100	0.09	1100
L-680,648	64	0.093	690
L-689,660	37	1.3	28
L-687,306	25	1.7	15
Atropine	1.0	0.48	2.1

Results are expressed as an apparent affinity constant (Kapp) which was corrected for ligand occupancy. The methodology was as previously described (6). The values are geometric means of 3-6 determinations

The *in vivo* profile of the efficacious compounds described above, clearly limited their potential clinical utility. In order to investigate the efficacy threshold for these effects, we examined a series of compounds in which efficacy was progressively reduced. During the course of these studies we identified compounds that had a distinct profile that differed from those full agonists described above. A typical example of this series was the quinuclidine pyrazine derivative L-689,660 (Fig 1). This chlorine substituted compound retained high affinity in the binding assays ([^{3}H] NMS Kapp 0.037 μM, Table I), but had a low NMS/Oxo-M value of 28, indicative of partial agonist activity. Indeed, L-689,660 did not induce a stimulation of cortical PI turnover in rat cerebral cortex at doses up to 1mM, and inhibited, dose-dependently, the stimulation induced by carbachol. These actions were consistent with the behaviour of a partial agonist in a system with a limited receptor reserve. This efficacy level resulted in L-689,660 exhibiting functional selectivity in the pharmacological assays. At M_1 receptors in the rat ganglion, L-689,660 was a potent and efficacious agonist (EC_{50} 50nM) and retained full agonist activity compared with muscarine (Table II). Similarly, at M_3 receptors in the guinea-pig ileum L-689,660 was a potent agonist, although it was not fully efficacious relative to carbachol (relative maximum 0.64). In contrast, at M_2 receptors in the atrium L-689,660 lacked efficacy (relative maximum <0.2 of carbachol) and was shown to act as a competitive antagonist of the response to carbachol with a pA_2 of 7.8.

TABLE II

Pharmacological Evaluation of Novel Oxadiazole and Pyrazine Muscarinic Agents.

	M_1 Rat Superior Cervical Ganglion		M_2 Guinea-Pig Atria			M_3 Guinea-pig Ileum		
	EC_{50} (nM)	R.M.	EC_{50} (nM)	R.M.	pA_2	EC_{50} (nM)	R.M.	pA_2
Muscarine	90	1.2	200	1.0	-	120	1.0	-
L-670,548	2.0	0.9	3.0	0.95	-	1.2	0.95	-
L-680,648	2.7	0.9	1.5	0.9	-	2.1	1.0	-
L-689,660	50	1.2	-	-	7.2	32	0.64	-
L-687,306	63	0.55	-	-	8.00	-	-	8.15

Detailed methods were as previously described (10). EC_{50}: the concentration required to produce half of the relative maximum response. Results are expressed as a geometric mean. R.M.: relative maximum response of the test compound relative to the maximum response to 1μM muscarine. Results are from at least three independent determinations.

TABLE III

Muscarinic Receptor Binding Selectivity of Lead Oxadiazole and Pyrazine Muscarinic Agonists.

	Cerebral Cortex M_1 [^{3}H] Pirenzepine Kapp (μM)	Heart M_2 [^{3}H] NMS Kapp (μM)	Lachrymal Gland M_3 [^{3}H] NMS Kapp (μM)
Pirenzepine	0.015	0.63	0.21
AFDX-116	0.49	0.11	1.8
McN-A-343	5.5	41	14
L-670,548	0.25	0.12.	0.38
L-689,660	0.021	0.11	0.092
L-687,306	0.011	0.067	0.042

Results are expressed as an apparant affinity constant (Kapp) which has been corrected for ligand occupancy. All binding studies were performed in rat tissue and are the geometric means of at least three determinations. Inhibition studies were performed in the presence of 100μM GppNHp.

L-689,660 demonstrated both peripheral and central cholinergic stimulation in rodents, producing salivation (ED_{100} 0.4 mg/kg ip) and hypothermia (ED_{50} 0.34mg/kg ip), although the effects were less marked than L-670,548. In conscious rats, L-689,660 produced only modest effects on cardiovascular function. The predominant change was a very transient fall (-25% maximum) in blood pressure (0.01-3mg/kg iv). A modest tachycardia was also observed. L-689,660 displayed

good activity in the *ex vivo* binding assay in mouse (ED_{50} 0.86mg/kg) and, in the rat, induced a dose-related increase in the intensity of hippocampal slow wave activity at 0.1mg/kg and above. This latter response was blocked by scopolamine at 0.3mg/kg. This finding confirmed that L-689,660 penetrated the blood brain barrier readily and had sufficient efficacy to stimulate central muscarinic responses thought to involve M_1 muscarinic receptors (14). L-689,660 retained activity in a number of rodent behavioural models. For example, in a rat conditioned-suppression of drinking test of long term memory, a dose of 0.3 and 1.0mg/kg of L-689,660 reversed a scopolamine induced deficit in performance (0.6mg/kg).

We subsequently studied the effects of reducing the efficacy of compounds still further when we identified a novel oxadiazole derivative L-687,306 (Fig1) which was even lower down on the efficacy scale (Fig 2). This compound was a potent inhibitor of binding (Kapp 0.025μM) and had a NMS/Oxo-M ratio of 15, consistent with a low intrinsic activity partial agonist. This compound had greater functional selectivity than both L-670,548 and L-689,660. L-687,306 was a competitive antagonist at both cardiac M_2 receptors (pA_2 8.00) and ileal M_3 receptors (pA_2 8.15), but retained partial agonist activity on the rat superior cervical ganglion (efficacy 0.55 relative to muscarine). At doses up to 30mg/kg there was no salivation, diarrhoea, tremor, hypothermia or other side effects normally associated with muscarinic stimulation *in vivo*. Furthermore, in cardiovascular studies L-687,306 produced little evidence of agonist changes in mean arterial blood pressure in conscious rats at doses up to 3 mg/kg iv. The compound penetrated well into the CNS and had an ED_{50} in the *ex vivo* binding assay of 1.5mg/kg. Despite the low efficacy of this compound, L-687,306 retained behavioural activity and was able to reverse a scopolamine (0.3mg/kg) induced deficit in a mouse passive avoidance test at dose of 0.1-10 mg/kg.

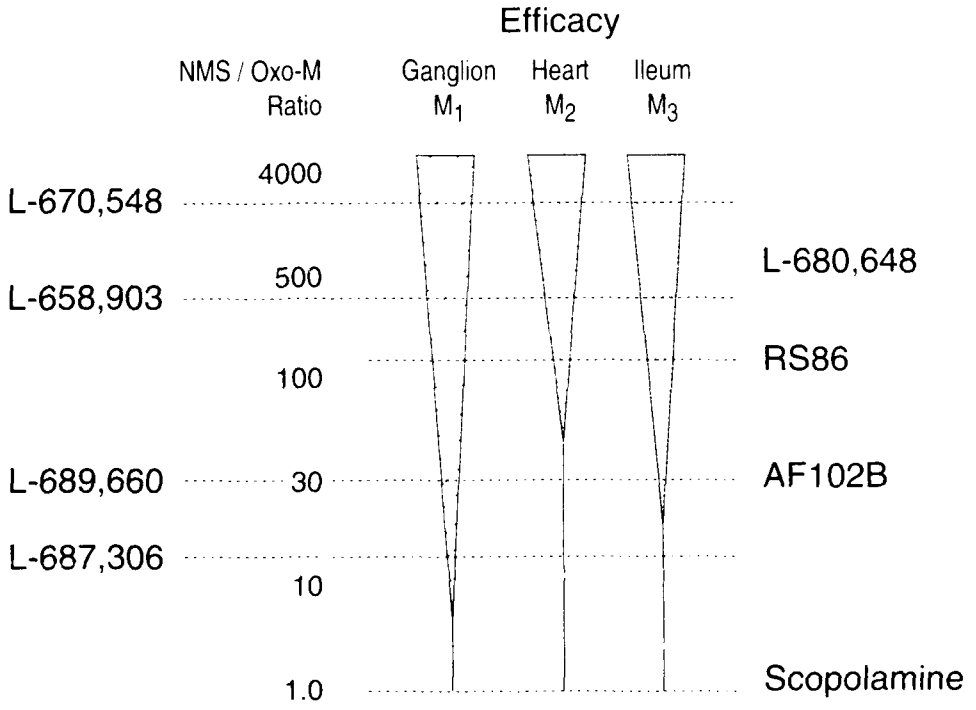

FIG. 2

Relationship between the NMS/Oxo-M ratio and functional activity in the three pharmacological preparations. The width of the bar for each tissue represents the relative agonist activity of compounds with a particular NMS/Oxo-M ratio, for each particular functional assay.

The functional selectivity observed with L-689,660 and L-687,306 was related to differences in effective receptor reserve between the functional assays we used and not to any differences in receptor affinity. Neither compound exhibited any clear muscarinic receptor selectivity in binding assays for M_1, M_2 or M_3 receptors (Table III) or indeed to cloned muscarinic m1, m2 or m3 receptors expressed stably in CHO cells (unpublished observations).

Discussion

These studies show that low efficacy muscarinic agonists such as L-689,660 and L-687,306 display functional muscarinic subtype selectivity that can be demonstrated clearly using tissues with differing receptor reserves. This phenomenon can be illustrated graphically as a sliding efficacy scale relating the NMS/Oxo-M ratio of these compounds, and the relative differences in the receptor reserve between the three functional pharmacological assays (Fig 2). The results clearly show that it is possible to design compounds with varying levels of efficacy in order to obtain a specific functional profile.

The functional selectivity observed in vitro with these compounds appears to be associated with a reduced side-effect profile and a marked reduction in cardiovascular side-effects at CNS active doses, compared to that seen with the higher efficacy compounds. In the case of L-687,306 this resulted in the lack of all of the peripherally mediated side effects normally associated with agonist activity. Importantly however, both L-689,660 and L-687,306 retained activity in behavioural tests of memory function.

The clinical value of functional receptor selectivity achieved by controlling efficacy is difficult to predict. The approach is limited by the relative agonist potency in target versus side-effect tissues being dependent upon their effective receptor reserves and these cannot be manipulated. When pathological changes occur, the effective receptor reserve in target tissues relative to others that mediate side-effects may alter. In addition, the prolonged administration of an agonist may itself result in alterations in receptor/effector coupling, although this possibility is likely to reduce with the efficacy of the agonist used. Consequently, the pharmacological profile of a partial agonist may change with the progression of a disorder and prolonged drug therapy. Nevertheless, clinical evaluation of partial agonist compounds with a range of low efficacies could give a unique opportunity to test the cholinergic hypothesis by restricting the dose-limiting side effects associated with high efficacy agonists, and thereby indicate whether this strategy will provide agents of potential therapeutic benefit.

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References

1. R.T. BARTUS, R.L. DEAN, B. BEER AND A.S. LIPPA, Science 217 408-417 (1982).

2. M. TRAUB and S.B. FREEDMAN, Dementia 3 189-192 (1992).

3. N.J. BUCKLEY, T.I. BONNER AND M.R. BRANN, J. Neurosci. 8 4646-4652 (1988).

4. N.J. BUCKLEY, T.I. BONNER, C.M. BUCKLEY AND M.R. BRANN, Mol. Pharmacol. 35 469-476 (1989).

5. T.P. KENAKIN, Adv. Drug. Res. 15 71-109 (1986).

6. S.B. FREEDMAN, E.A. HARLEY AND L.L. IVERSEN, Br. J. Pharmacol. 93 437-445 (1988).

7. N.R. NEWBERRY, AND T. PRIESTLEY, Br. J. Pharmacol. 93 817-826 (1987).

8. J. SAUNDERS AND S.B. FREEDMAN, Trends. Pharmacol. suppl. 10 70-75 (1989).

9. L. STREET, R. BAKER, T. BOOK, C. KNEEN, A. MACLEOD, A.K. MERCHANT, G. SHOWELL G, J. SAUNDERS, R.H. HERBERT, S.B. FREEDMAN, AND E.A. HARLEY, J. MED. CHEM. 33 2690-2697 (1990).

10. S.B. FREEDMAN, E.A. HARLEY, S. PATEL, N.R. NEWBERRY, M.J. GILBERT, A.T. MCKNIGHT, J.J. MAGUIRE, N.T. MUNDUNKOTUMA, R. BAKER, L. STREET, A. MACLEOD, J. SAUNDERS AND L.L. IVERSEN, Br. J. Pharmacol. 101 575-580 (1990).

11. S.B. FREEDMAN, E.A. HARLEY, R. MARWOOD AND S. PATEL, Eur. J. Pharmacol. 187 193-199 (1990).

12. L. STREET, R. BAKER, T. BOOK, A.J. REEVE, J. SAUNDERS, T. WILSON, R. MARWOOD, S. PATEL AND S.B. FREEDMAN, J. MED. CHEM. 35 295-305 (1992).

13. S.B. FREEDMAN, E.A. HARLEY AND S. PATEL, Eur. J. Pharmacol. 174 253-260 (1990).

14. J.C.BARNES AND F.F. ROBERTS, Eur. J. Pharmacol. 195 233-240 (1991).

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THERAPEUTIC POTENTIAL OF CNS-ACTIVE M_2 ANTAGONISTS: NOVEL STRUCTURES AND PHARMACOLOGY

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Summary

Clinical trials with muscarinic agonists or acetylcholine esterase inhibitors for the treatment of Alzheimer's dementia have shown disappointing or equivocal results (1). An alternative treatment of this disease is the development of drugs which enhance the release of acetylcholine. It is believed, that of the five muscarinic receptor subtypes so far identified in the brain, M_2 receptors are located presynaptically in the cortex and hippocampus and upon stimulation inhibit the release of acetylcholine. Based on this hypothesis, we initiated a drug discovery program with the aim of identifying selective and centrally active M_2 antagonists which are capable of enhancing cholinergic transmission. These efforts resulted in the successful design and synthesis of novel muscarinic antagonists able to cross the blood brain barrier. Moreover, these compounds show few peripheral effects and possess a superior M_2 versus M_1 selectivity. The prototype of this novel class of M_2 selective compounds, BIBN 99, could be a valuable tool to test the hypothesis that lipophilic M_2 antagonists show beneficial effects in the treatment of cognitive disorders.

In the course of our work on tricyclic muscarinic antagonists we discovered compounds exhibiting selectivity for the cardiac muscarinic receptor, the most prominent member being AF-DX 116 (2-5). This molecule exhibits the following selectivity profile: m2>m1, m4>m3>m5. The search for follow-up compounds has been pursued with the aim of improving M_2 affinity and of increasing M_2/M_3 selectivity. The systematic variation of the side chain of AF-DX 116 has resulted in several compounds with the desired improved potency and selectivity, e.g. AQ-RA 741 (4, 6, 7).

Evidence has accumulated in recent years that selective muscarinic blockers may play an important role in the field of Alzheimer's disease. For instance, it has been shown that M_2 antagonists, such as AF-DX 116, enhance the release of acetylcholine in certain brain areas like cortex and hippocampus both in vivo and in vitro (8, 9, 10). This is due to a blockade of presynaptic receptors in the cortex and hippocampus which presumably belong to the M_2 subtype (11, 12). Following the so-called cholinergic hypothesis of Alzheimer's disease, the use of selective M_2 antagonists could be a new strategy to improve memory and learning. According to this concept, a potential therapeutic agent should possess good penetration through the blood brain barrier and a high selectivity for M_2 versus M_1 receptors, since the

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drug should not counteract its presynaptic action by blocking postsynaptic M_1 receptors. A schematic representation of this working hypothesis is depicted in Fig. 1. Some of our work with respect to the design and pharmacological characterization of compounds with the profile described is summarized in this paper.

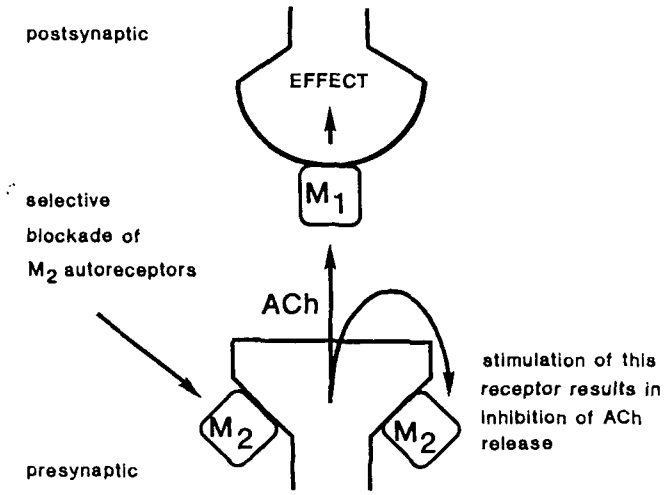


Fig 1 Schematic representation of muscarinic receptors involved in cholinergic transmission in the forebrain

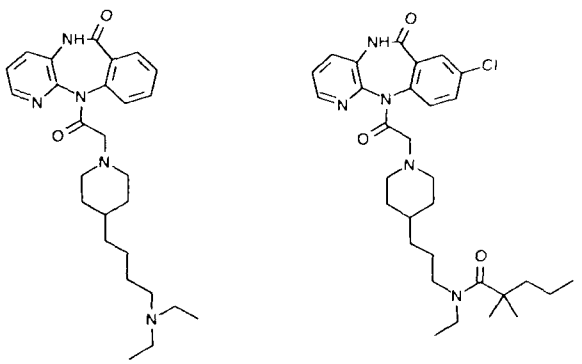
Design of lipophilic M_2 antagonists

According to our working hypothesis, M_2 antagonists suited for the treatment of Alzheimer's disease should possess the following properties:
- M_2 affinity: $K_i < 100$ nM
- Partitition coefficient log $P_{app.} > 1.0$ at pH 7.4 (Prerequisite for CNS penetration)
- M_1/M_2 selectivity > 10 (No M_1 postsynaptic blocking activity)

Our synthetic program for the synthesis of compounds endowed with properties as described above has been started with AQ-RA 741 as a lead (Fig. 2). This compound is a rather hydrophilic molecule with log $P_{app.}$ - 0.1 at pH 7.4. The pK_a values of the tertiary amino groups of the side chain have been found to be 7.15 and 10.3. Hence, under physiological conditions the molecule is doubly protonated to a large extent. As a consequence, the compound shows good water solubility and does not penetrate the blood brain barrier to an appreciable extent.

In order to generate highly lipophilic analogs of AQ-RA 741 we envisaged the following structural modifications:
- Exchange of the terminal tertiary amino group for a carboxamide group
- Introduction of small lipophilic substituents into certain positions of the pyridobenzo--diazepinone tricycle
- Exchange of the pyridine ring for a benzene ring in the tricycle

Following this line we have synthesized compounds depicted in the general formula of Fig. 3. Binding affinities K_i(nM) as well as M_1/M_2 selectivity ratios and partitition coefficients of the newly synthesized compounds are summarized in Tables 1 and 2.



AQ-RA 741 BIBN 99

Fig. 2: Structural formulas of compounds AQ-RA 741 and BIBN 99

As can be seen, replacement of the terminal diethylamino group of the side chain of AQ-RA 741 by a C_2H_5N-CO-R entity resulted in the desired lipophilicity without adversely affecting selectivity and M_2 affinity. This finding is exciting in so far as up to now the presence of a proximal protonated nitrogen atom in the side chain has been considered as essential for the interaction with the muscarinic receptor. Highest M_2 affinity was attained with compounds containing a geminal dimethyl group adjacent to the carboxamide carbonyl. To our surprise, not only lipophilicity but also M_1/M_2 receptor selectivity could be significantly increased by these structural modifications. In the series of compounds with an unsubstituted tricyclic ring, BIBN 77 displays highest receptor affinity and a M_1/M_2 selectivity ratio of 20.

From structure-activity relationships established in the course of our work on hydrophilic M_2 antagonists we knew that the introduction of small substituents into position 8 of the pyrido-benzodiazepinone system does not adversely influence M_2 receptor affinity. Accordingly, this variation has been used for the generation of a set of more lipophilic compounds represented by substances BIBN 97, 98, 99 and 120. As can be seen from table 1, the introduction of a chlorine atom or a methyl group into position 8 of the tricycle leads to M_2 antagonists with favourable selectivity ratios.

Exchange of the pyrido- for a benzene ring (X=CH, Fig. 3) furnished compound BIBN 140 which shows high receptor affinity and a pronounced M_1/M_2 selectivity ratio. In view of their high potency and M_2 selectivity, compounds BIBN 99 and BIBN 140 have been selected for further investigation.

Pharmacology of BIBN 99 and BIBN 140

Receptor binding studies using either rat tissues or CHO-cells expressing the different receptor subtypes revealed that both BIBN 99 and BIBN 140 are highly selective M_2 antagonists.The affinity data presented in Table 2 suggest that BIBN 99 is one of the most selective M_2 antagonists described so far. This compound possesses approximately a 5-,25-,30- and 50-fold higher affinity for m2 (M_2) than m4, m3(M_3), m1(M_1) and m5 receptors, respectively. Especially striking is the 30- and 40-fold m2 versus m1 selectivity of BIBN 99 and BIBN 140, since such a selectivity profile is not shared by any M_2 antagonists previously synthesized. In receptor binding experiments BIBN 99 and BIBN 140 show a 3- to 11 fold higher affinity for the M_2(m2) receptor than AF-DX 116 but possess an equal affinity for the presynaptic receptor in the hippocampus mediating acetylcholine release (Table 2). Although BIBN 99 and BIBN 140 exhibit a higher affinity for cardiac M_2 receptors compared to AF-

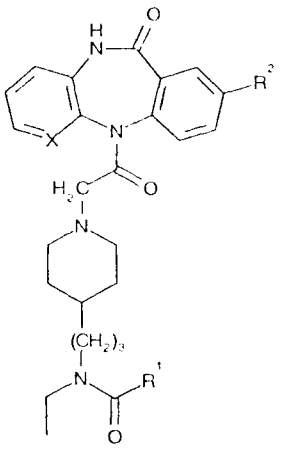


Fig. 3: Structural formula of new lipophilic M_2 antagonists

DX 116, their potency in antagonizing the vagally mediated bradycardia in rats is less pronounced (see Fig.4 and Table 2) This finding can be explained by a rapid decline of the plasma levels of both compounds due to their high lipophilicity. Consequently, it might be

Table 1

Binding affinities (K_i) and M_1/M_2 selectivity ratios for lipophilic M_2 selective muscarinic antagonists as described in the general formula of Fig. 3

Code BIBN	R^1	R^2	X	K_i[nM] M_2[a]	selectivity M_1/M_2[b]	Log Pc
55	$-CH_3$	H	N	200	4	1.36
131	$-CH_2-CH_3$	H	N	173	14	1.86
132	$-(CH_2)_2-CH_3$	H	N	66	14	2.28
56	$-C(CH_3)_3$	H	N	62	14	2.43
77	$-C(CH_3)_2-CH_2-CH_3$	H	N	25	20	2.74
91	$-C(CH_3)_2-(CH_2)_2-CH_3$	H	N	17	15	2.61
97	$-C(CH_3)_3$	8 Cl	N	200	10	2.64
98	$-C(CH_3)_2-CH_2-CH_3$	8 Cl	N	75	13	2.62
99	$-C(CH_3)_2-(CH_2)_2-CH_3$	8 Cl	N	30	33	>2[d]
120	$-C(CH_3)_2-CH_2-CH_3$	8 CH_3	N	25	24	2.06
140	$-C(CH_3)_2-(CH_2)_2-CH_3$	H	CH	12	17	1.20

[a] affinity for rat cardiac M_2 binding sites
[b] M_2 selectivity: $K_i(M_1) / K_i(M_2)$
[c] Partition coefficient at pH 7.4 (octanol/water)
[d] due to insolubility exact value not measurable

Table 2

Affinity of AF-DX 116, AQ-RA 741, BIBN 99 and BIBN 140 for cloned and pharmacologically defined muscarinic receptors

	Pharmacologically defined receptors					
	cortex	pK_i[a] heart	gland	pA_2[b] heart	pK_b[c] hippocampus	log ED_{50}[d] heart
	M_1	M_2	M_3	M_2	M_2	M_2
AF-DX 116	6.3	7.1	5.7	6.9	6.8	6.4
AQ-RA 741	7.7	8.3	6.9	8.5	7.5	7.2
BIBN 99	6.2	7.6	6.1	7.2	6.7	5.1
BIBN 140	6.7	7.9	6.2	7.5	6.9	6.0
	Cloned and expressed receptors					
				pK_i[e]		
		m1	m2	m3	m4	m5
AF-DX 116		6.1	7.0	5.8	6.3	5.3
AQ-RA 741		7.3	8.3	7.0	8.1	5.9
BIBN 99		6.0	7.5	6.1	6.8	5.8
BIBN 140		6.5	8.1	6.4	7.3	6.5

a affinity (-log mol/l) for rat cortical M_1, cardiac M_2 and glandular M_3 binding sites
b affinity (-log mol/l) for guinea-pig atrial muscarinic receptors mediating inotropy
c affinity (-log mol/l) for presynaptic receptors mediating ACh-release in rat hippocampus
d dose (-log mol/kg) necessary to antagonize the bradycardia induced by stimulation of the vagus nerve in the rat by 50 %
e affinity (-log mol/l) for muscarinic binding sites expressed in CHO-cells.

expected that the peripheral effects such as an increase in heart rate would be negligible for these compounds. On the other hand BIBN 99 and BIBN 140 are capable of antagonizing the centrally mediated pressor effects of arecoline in rats, whereas AF-DX 116 and AQ-RA 741 are inactive (data not shown). This observation clearly demonstrates the ability of BIBN 99 and BIBN 140 to penetrate the brain. In order to investigate the possible therapeutic potential of the novel lipophilic M_2 antagonists we selected BIBN 99 for evaluation in the Morris maze task using both age-impaired and unimpaired animals. Figure 5 demonstrates the very high efficacy of BIBN 99 in age-impaired animals. A dose of 0.25 mg/kg (s.c.) improved the latency time of age-impaired animals in this paradigm to a level seen with normal animals. Not too surprisingly, BIBN 99 was without effects in age-unimpaired or young animals (13).

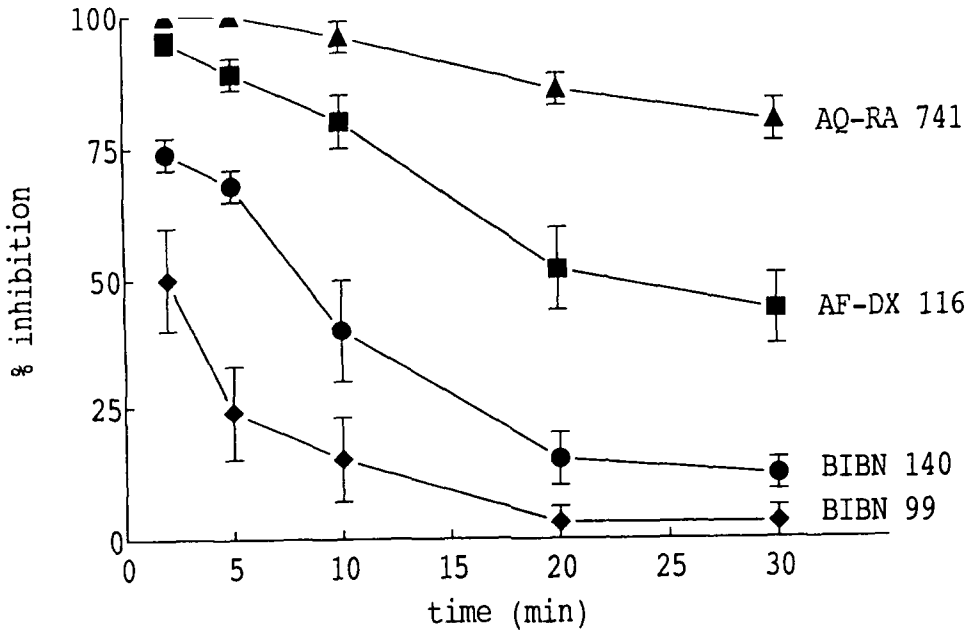


Fig. 4: Time dependent M_2 antagonist effects of 3mg/kg (i.v.) AF-DX 116, AQ-RA 741, BIBN 99 and BIBN 140

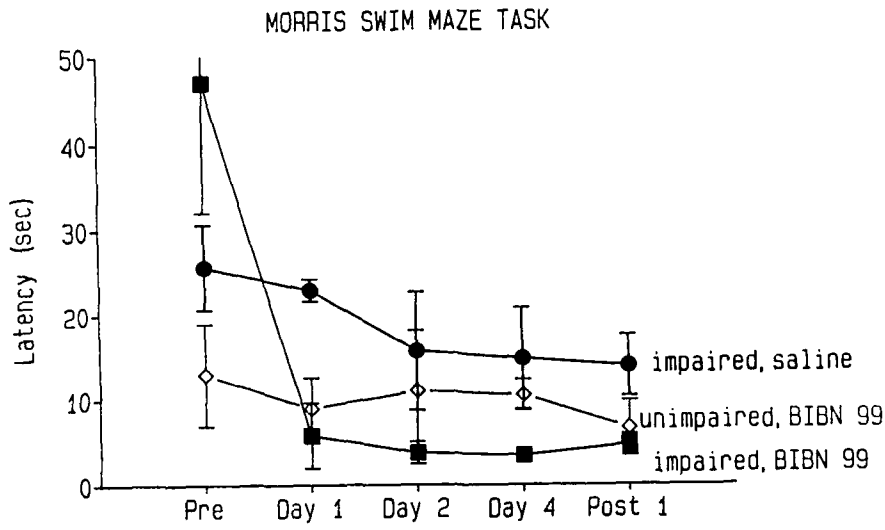


Fig. 5: Effect of BIBN 99 (0.25 mg/kg, sc.) on the learning capacities of 25 month-old age-impaired and unimpaired Long-Evans rats in the Morris Swim Maze task. BIBN markedly improved learning capacities in the aged-impaired animals; a significant effect (vs saline) being evident as early as on day 1. Non-impaired animals were not affected, their performance already being maximal in this task.

Conclusions

It is well established that cholinergic activity is reduced in dementia of the Alzheimer type. A new strategy to improve memory and learning could be the use of lipophilic M_2 antagonists which by blockade of presynaptic M_2 receptors enhance the release of acetylcholine. This concept (see Fig.1) might even show advantages over the classic therapeutic approaches with muscarinic agonists or acetylcholine esterase inhibitors. Choline esterase inhibitors may negate their beneficial effects since acetylcholine will inhibit its own release through stimulation of inhibitory presynaptic receptors. On the other hand, blockade of the M_2 autoreceptors is probably a more physiological approach than using agonists, since agonists will provide a continuous "non-physiological" stimulation of the postsynaptic receptors, whereas M_2 antagonists will only amplify the physiological cholinergic transmission. The lipophilic and highly selective M_2 antagonists synthesized by us represent compounds that penetrate into the brain. The high selectivity for M_2 versus M_1 of these compounds ensures that these drugs do not counteract their presynaptic action by significantly blocking postsynaptic M_1 receptors. The novel antagonist BIBN 99 showed impressive effects in respect to performance in age-impaired rats in the Morris maze task and can be considered as an important tool for future drug research in the field of Alzheimer's disease. Evaluation of this compound in other learning paradigms is thus warranted.

References

1. J. A. GRAY, A. ENZ and R. SPIEGEL, Trends Pharm. Sci. Suppl., 85-88 (1989).
2. W. ENGEL, W. EBERLEIN, G. TRUMMLITZ, G. MIHM, Fed. Proc. 46, 2527, (1989).
3. W. ENGEL, H. DOODS and B. WETZEL, Drugs of the Future Vol 15, 9-14, (1990).
4. W.G. EBERLEIN, W. ENGEL, G. MIHM, K. RUDOLF, B. WETZEL, M. ENTZEROTH, N. MAYER and H.N. DOODS, Trends Pharm. Sci. Suppl. 50-54 (1989)
5. H.N. DOODS, W. ENGEL, C.A.P.F. SU and P. TANSWELL, Cardiovascular Drug Reviews, Vol. 9, 30-40 (1991).
6. W.G. EBERLEIN, W. ENGEL, K.M. HASSELBACH; N. MAYER, G. MIHM, K. RUDOLF and H. DOODS, Trends in Receptor Research, 231-249 (1992).
7. H. DOODS, M. ENTZEROTH and N. MAYER, Europ. J. Pharm., 192, 147-152, (1991).
8. P.A. LAPCHAK, D.M. ARAUJO, R. QUIRION, B. COLLIER, Brain Res. 496, 285-294 (1989).
9. K. GULYA, D. BUDAI and P. KÁSA, Neurochem. Int. Vol. 15, 153-156 (1989).
10. J.W. RICHARD, A. WILSON, R. QUIRION, Soc. Neurosci. Abst. 17, 781, (1991).
11. M.H. RICHARDS, Br. J. Pharmacol. 99, 753-761, (1990).
12. W. HOSS, W.S. MESSER Jr., F.J. MONSMA Jr., M.D. MILLER, B.R. ELLERBROCK, T. SCRANTON, S. GHODSI-HOVSEPIAN, M.A. PRICE, S. BALAN, Z. MAZLOUM and M. BOHNETT, Brain Research, 517, 195-201 (1990).
13. A. WILSON, W. ROWE, J.W. RICHARD, M.J. MEANEY, N.M. WHITE and R. QUIRION; This meeting

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SYNTHESIS AND SAR OF BULKY 1-AZABICYCLO[2.2.1]-3-ONE OXIMES AS MUSCARINIC RECEPTOR SUBTYPE SELECTIVE AGONISTS

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Summary

The synthesis of a series of potent and efficacious 1-azabicyclo[2.2.1]heptan-3-one oxime muscarinic agonists is described. The oximes have extended appendages designed to span the cavity defined by the seven transmembrane helices of the muscarinic receptor. Some members of the series are selective for receptors of the m1 subtype. One such oxime, 31, shows affinity and functional selectivity for m1 over m2, m3, and m4 muscarinic receptor types.

There have been two major impediments in the development of therapeutically useful muscarinic agonists: First, high potency and efficacy seemed to reside only in muscarinic agonists bearing

TABLE I

Muscarinic Standards: Quaternary vs Tertiary amines.

	IC 50 (nM)		
Quaternary	CMD	QNB	QNB/CMD
Carbachol	6.70	33000	4925
CMD	2.90	4200	1448
OXO-M	1.13	1340	1186
Tertiary			
Arecoline	9.60	4000	417
Oxotremorine	1.30	380	292
Pilocarpine	14.00	2900	207
RS-86	29.00	3100	107

a quaternary nitrogen (Table I, for definitions of CMD, QNB and QNB/CMD refer to Materials and Methods). Such agents cannot penetrate the blood brain barrier. They are thus of no use in CNS related diseases. Second, muscarinic agonists tended to be small molecules with very little tolerance for steric bulk (Table II) (1-4). Introduction or extension of one carbon unit to an agonist lead to an antagonist or a partial agonist. The first impediment was overcome with the synthesis of the highly potent and efficacious 1-azabicyclo[2.2.1]heptane oxadiazole muscarinic agonists (2). These compounds are tertiary amines and readily penetrate the CNS.

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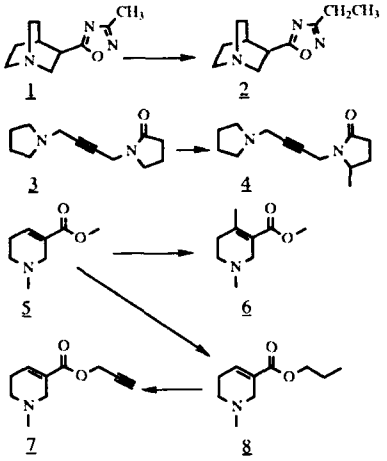
TABLE II

Size Relationship Among Agonists and Functional Antagonists (Partial Agonists)

Agonists	Antagonists
	

The second impediment lingers. Consequently the number of active analogs of a lead compound a medicinal chemist can synthesize is limited and the probability of identifying an analog with a desired pharmacologic profile severely curtailed.

This paper emphasizes the need to make large and elongated muscarinic agonists as a means of preparing pharmaceutically useful m1-subtype selective muscarinic agonists and demonstrates the feasibility of such an approach. The approach is based on the emerging knowledge of the three dimensional structure of the muscarinic receptor subtypes.

Because of the unique distribution of m1 receptors (5-9) in the peripheral and central nervous system, m1 agonists have the potential to enhance cognitive function without inducing unwanted cholinomimetic-mediated side-effects. Muscarinic antagonists can show as much as 20-fold selectivity (e.g. pirenzepine) for the m1 receptor. In contrast, the most m1 selective muscarinic agonist yet described is only twofold selective for m1 over m2 receptor subtypes (McN-A-343 (10), Table IV), a degree of selectivity unlikely to translate into improved pharmacologic profiles. A new approach to the design and synthesis of muscarinic subtype selective agonists is required.

Five muscarinic receptor subtypes (m1-m5) with unique protein structures have been described. These receptor subtypes belong to the G-protein coupled family of receptors (5,11). Receptors of this family are characterized by the proposed existence of seven helices (12, 13) that define a transmembrane cavity. Within the cavity key amino acid residues appear to play a major role in positioning, orienting and binding an agonist to the receptor. While the receptor subtypes differ sufficiently to permit good antagonist selectivity, the differences in amino acid sequence and internal topography is very limited (5, 14) in the regions that are presumed to bind agonists. The design of subtype selective agonists, therefore, is difficult. Nevertheless compounds which are longer and larger than their non-selective counterparts may increase contact between the agonist and the internal surface of the binding cavity and may bring the agonist into proximity with parts of the receptor unique to a particular subtype. Such unique ligand-receptor interactions, introduced by the bulk of the agonists, may achieve greater subtype selectivity.

To this end, the 1-azabicyclo[2.2.1]heptane nucleus was chosen as a starting point and 1-azabicyclo[2.2.1]heptan-3-one oximes with an extended poly-ene/poly-yne appendage were

prepared (Table III and Table IV). Models of the m1 and m2 receptors have been assembled using the 3-dimensional (3D) model proposed for rhodopsin (a G-protein coupled receptor closely related to the muscarinic receptors) and a model of ligand binding was postulated by analogy to the rhodopsin's retinal chromophore (T. Mirzadegan, Life Sci. this issue). Independently, a receptor-point model established possible relationships between the propargyl-containing agonists and m1-selective antagonists (D. Moreland, Life Sci. this issue) . Steric, conformational and electrostatic characteristics suggested by the models were used to assist the design of the novel side chains. It is postulated that the designed ligands span the relatively rigid receptor cavity and interact with sites that differ in the subtype receptors. Both models provide plausible but different explanations for the observed m1 selectivity of the 1-azabicyclo[2.2.1]heptan-3-one oximes .

Materials and Methods

Synthesis of ligands. The target compounds were prepared by treating 1-azabicyclo[2.2.1]heptan-3-one (15) with O-substituted hydroxylamines. Appropriately O-substituted hydroxylamines were prepared from the corresponding alcohols and N-hydroxyphthalimide via the Mitsunobu (16) reaction to give O-substituted phthalimides. Hydrolysis of the latter gave the desired hydroxylamines in good yields. Complex alcohols such as 2,5-hexadiyne-1-ol and 6-substituted 2,5-hexadiyne-1-ol were prepared by Cu mediated coupling (17) of propargyl alcohol and propargyl bromides or terminally substituted propargyl bromides.

Radioreceptor Binding Assays. Muscarinic receptor binding assays were conducted using [^{3}H] quinuclidinyl benzilate to label antagonist sites (QNB) and [^{3}H]-cis-methyldioxolane to label agonist sites (CMD) in the rat neocortex (18, 19). The ratio of QNB/CMD has been shown (20) to predict agonist efficacy at muscarinic receptors. Selectivity for m1 over m2 muscarinic subtypes was determined by estimating agonist affinity for m1 and m2 receptor subtypes labelled by [^{3}H] QNB in CHO cells selectively expressing human m1 and m2 receptors (21).

Second Messenger Activation Assays. The ability of selected agonists to stimulate inositol phosphate accumulation (22) or inhibit forskolin-stimulated accumulation of cAMP in CHO cells selectively expressing human m1 and m2 receptors (21) respectively, was determined.

Results and Discussion

Muscarinic agonists do not tolerate steric bulk. Upon addition of steric bulk, they are transformed into muscarinic antagonists or weak partial agonists (Table II). For example, addition of a methyl group at the four position of arecoline (5) results in the muscarinic antagonist 6. Similarly, lengthening of the O-Me substituent of arecoline to an O-n-propyl subtituent, leads to an antagonist (8). On the other hand, the O-Me substituent may be replaced with O-propargyl moiety without loss of muscarinic agonist activity. Both compounds 7 and 8 are three-carbon extention analogs of arecoline (5). Yet a propargyl extention (7) leads to an agonist analog and an n-propyl extension (8) leads to an antagonist. This observation was first made by Mutchler et. al. in 1973 (4). We have made use of this phenomenon in an iterative manner for designing bulky muscarinic agonists. Starting with the potent, full agonist 1 (discovered in our laboratory), we gradually increased the size of the O-substituent (compounds 9-11, Table III). This lead to a rapid decline in agonist activity (small QNB/CMD ratio and low affinity at CMD binding). When the O-n-propyl substiutent was replaced by an O-propargyl group (11 -> 12), compound 12, an oxime equiactive to 1 was obtained. Surprisingly, 12 was found to tolerate bulk better than 1 (compounds 13 - 16, Table III). Compound 16, an n-butyl analog of 12, is a better agonist than 11 , an n-propyl analog of 1. In a manner similar to the transformation of 11 to 12, the n-propyl portion of 15 was replaced with a propargyl group to give compound 17. Compound 17 bears two serially connected

TABLE III.

The Role of C≡C in Preparing Bulky Muscarinic Agonists

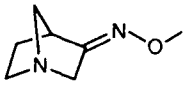
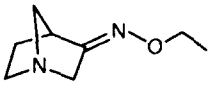
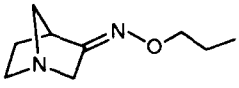
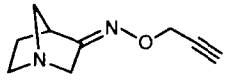
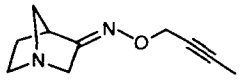
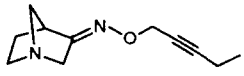
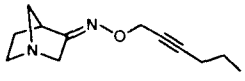
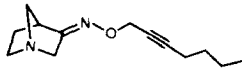
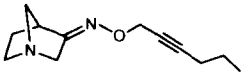
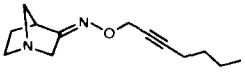
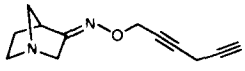
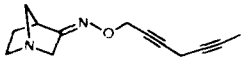
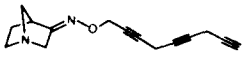
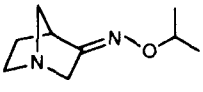
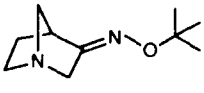
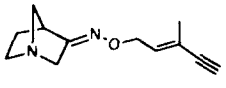
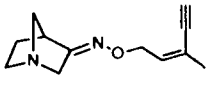
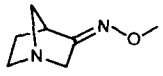
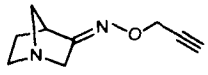
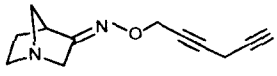
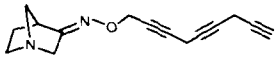
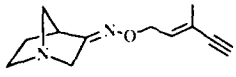
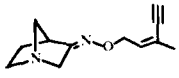
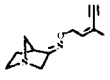
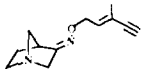
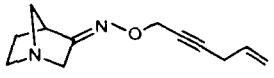
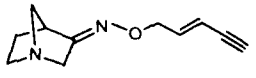
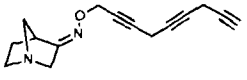
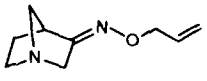
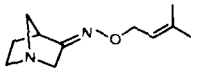
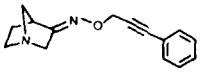
NO	Structure	IC50 nM QNB/CMD
9		24558/18.2 = 1349
10		3112/12.5 = 249
11		3846/120.0 = 32
12		4359/4 = 1090
13		4214/4 = 1053
14		5976/4 = 1494
15		8689/48 = 181
16		6045/44 = 137
15		8689/48 = 181
16		6045/44 = 137
17		8834/22 = 402
18		2914/39 = 75
19		959/8 = 119
20		2030/46.0 = 44
21		3540/91.0 = 39
22		2063/4 = 514
23		807/1 = 807

TABLE IV

Muscarinic Receptor Binding, m1 Selectivity and Stimulation of PI Turnover.

No	Structure	IC50 nM CMD	IC50 nM QNB	QNB CMD	m2 m1	PI %CCh
9		18	24558	1349	0.7	ND
12		4	4359	1089	0.8	104
17		22	8834	402	1.2	79
19		8	958	119	1.1	50
22		4	2063	516	2.3	100
23		1	807	807	1.2	105
24		12	4234	353	1.2	99
25		121	19308	160	0.6	49
26		32	7313	229	1.7	ND
27		35	13850	396	2.4	97
28		25	4680	187	1.5	59
29		34	3467	102	3.0	61
30		7	1279	183	2.4	45
31		46	5012	109	4.8	40
	McN-A-343	25	5304	212	2.6	57
	Carbachol	7	33000	4714	0.03	100

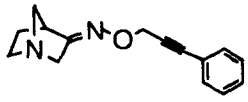
For IC50 determination, each drug was investigated in triplicate at 5-6 concentrations.
ND = not determined

propargyl appendage and is a better agonist than 15. Compound 17, unlike 12, does not tolerate straight alkyl substitution in place of the acetylenic hydrogen (17 -> 18). Replacement of the methyl group in 18 with a propargyl group leads to compound 19. Compound 19 bears three serially connected propargyl appendage and is a better agonist than 18. Like their straight chain analogs, branched alkyl substituents have detrimental effects on the affinity and efficacy of 1 (e.g 20 and 21). On the other hand, branching with E-3-methyl-2-penten-4-ynyl and Z-3-methyl-2-penten-4-ynyl groups render bulky but very potent and efficacious oximes (22 and 23). Analog 23, with 1 nM affinity and a QNB/CMD ratio of 807, may be categorized as a full agonist.

It is of particular interest to note that the four geometric isomers 22 - 25 display binding affinities ranging from 1 nM to120 nM to the agonist state of the receptor (rat cortex).

TABLE IV

Stimulation of PI Turnover and Inhibition of Forsokolin-Stimulated Accumulation of cAM

	PI			cAMP	
	% Carbachol			% Inhibition	
Structure	Hm1	Hm3	Hm5	Hm2	Hm4
	40	8	1	0	0
Carbachol	100	100	100	58	60

The selectivity for the m1 and m2 subtype muscarinic receptors by these isomers is also important. Compound 25 is m2 selective. Compound 22 is slightly m1 selective. Compounds 23 an 24 show no preference for either muscarinic subtypes. The sole difference among compounds 22 - 25 lies in the manner the ene-yne appendage is oriented in space. As a result of this spacial arrangements, once inside the aforementioned cavity, the molecules interact with different parts of the receptor and assume differing properties (m1 vs m2 selectivity, a ten fold change in binding affinities, etc.,). These findings are consistent with our hypothesis that increased size and diversity in the shape of muscarinic agonists would lead to subtype selectivity.

Our results indicate that the synthesis of long/large, efficacious and potent 1-azabicyclo[2.2.1]heptan-3-one oxime muscarinic agonists is possible (Tables III and IV). The 2-propynyl (12), 2,5 hexadiynyl (17) 2,5,8-nonatriynyl (19), and 3-methyl-2-penten-4-ynyl (22 and 23) oximes are potent and efficacious muscarinic agonists (IC_{50} = 4 nM, 22 nM, 8.0 nM, 4 nM, 1 nM, and ratios of QNB/CMD =1090, 402, 119, 514, and 807 respectively). These compounds are longer and larger than previously described muscarinic agonists. As a direct result of this, we believe, enhanced, though modest, selectivity for the m1 receptors is achieved in compound 31, (m2/m1 = 4.8). At the second messenger level, compound 31 demonstrates enhanced m1 selectivity over m2, m3, and m4 muscarinic receptor subtypes (Table V).

In conclusion, it is possible to synthesize large and long muscarinic agonists with high potency and efficacy. This possibility allows medicinal chemists to prepare large number of analogues of a promising muscarinic ligand. As a result of the increased number of analogs and the resulting structural diversity introduced (size, length, electrostatic properties), the modalities of interaction of the ligands with the receptor cavity is increased. This, we believe,

increases the probability of finding subtype selective agonists. The selective agonists in turn will further enhance our understanding of the topological requirements of the receptor subtypes. This is vital information for preparing even better subtype selective ligands which will find wide use in the many disease-state associated with the muscarinic receptor.

References

1. B. RINGDAHL, The Muscarinic Receptors; J. H. Brown (ed), 151-218, The Humana Press, Clifton, New Jersey (1989) and references therein.
2. J. SAUNDERS, S.B. FREEDMAN, Trends Pharmacol. Sci. (suppl. Subtypes Muscarinic Receptors), 10 70-75 (1989)
3. H. TECLE, S. C . BERGMEIER, L. L.COUGHENOUR, R. E. DAVIS, F.M. HERSHENSON, J. A. KESTER, J. S. MCKEE, J. G. MARRIOT, W. H.MOOS, R . D. SCHWARZ, A. J. THOMAS, Abstracts of papers, 198th National Meeting of the American Chemical Society, Abstract MEDI-7. Miami Beach, Fla; American Chemical Society; Washington, DC (1989)
4. E. Mutschler, K. Hultzsch, Arzneim.-Forsch. 23 732-737 (1973)
5. R. BAKER, J. SAUNDERS, Annu. Rep. Med. Chem., 24 31-40 (1989) and references therein.
6. R.K. Goyal, N. Engl. J. Med. 321 1022-1029 (1989)
7. A.I. LEVEY, C. A. KITT, W.F.SIMONDS, D.L. PRICE, M.R. BRANN, J. Neurosci. 11 3218-3226 (1991)
8. V. KUMAR, M. CALACHE, Int. J. Clin. Pharmacol.Ther. Toxicol. 29 23-27 (1991)
9. R. D. SCHWARZ, L. L.COUGHENOUR, R. E. DAVIS, D. T.DUDLEY, W. H. MOOS, M. R. PAVIA, H. TECLE, Cholinergic Basis for Alzheimer Therapy, R. BECKER, E. GIACOBINI, (eds), p. 347-353, Birkhauser: Boston (1991)
10. R. HAMMER, A. GIACHETTI, Life Sci., 31 2991-2998 (1982).
11. N.J . BUCKLEY, Transmembrane Signaling, Intercellular Messengers and Implications for Drug Development, S.R. NAHORSKI (eds), 11-31, John Wiley & Sons (1990)
12. J. C.VENTER, C. M. FRASER, Drug Discovery Technologies, C.R. CLARK, W.H. MOOS, (eds), p 231, Ellis Horwood, Chichester (1990)
13. T.I. BONNER, N.J. BUCKLEY, A.C. YOUNG, M.R. BRANN, Science, 237 527-532 (1987)
14. E.C. HULME, N.J.M.BIRDSALL, N.J. BUCKLEY, Annu. Rev. Pharmacol. Toxicol., 30 633-673 (1990)
15. I.F. COTTRELL, D. HANDS, D.J. KENNEDY, K. J. PAUL, S. H. B. WRIGHT, K. HOOGSTEEM, J. Chem. Soc. Perkin Trans. I, 1091-1097 (1991) and references therein.
16. E. GROCHOWSKI, J. JURCZAK, Synthesis, 682-684 (1976)
17. L. BRANDSMA, Preparative Acetylenic Chemistry, p 225, Elsevier; Amsterdam (1988)
18. T. W. VICKROY, W.R. ROESKE, H.I.YAMAMURA, J. Pharmacol. Exp. Ther., 229 747-755 (1984)
19. M. WATSON, H.T. YAMAMURA, W.R. ROESKE, J. Pharmacol. Exp. Ther., 237 411-418 (1986)
20. H. TECLE, S. BERGMEIER, W. MOOS, F. HERSHENSON, L. COUGHENOUR, R. DAVIS, R.SCHWARZ, D. MORELAND, D. DUDLEY, Abstracts of papers, 197th National Meeting of the American Chemical Society, Dallas, Tex; American Chemical Society; Washington, DC, 1989; Abstract MEDI-47.
21. R.D. SCHWARZ, D.K. BOYD, C.J. SPENCER, G. WOODARD. Abstract of papers, 21st Annual Meeting of the Society for Neuroscience, New Orleans, LA; Society for Neuroscience; Washington, D.C. (1991)
22. L. COUGHENOUR, R. DAVIS, W. MOOS, R. SCHWARZ, Abstracts of papers, 17th Annual Meeting of the Society for Neuroscience, New Orleans, LA; Society for Neuroscience; Washington, D.C. (1987)

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SDZ ENS 163 A NOVEL PILOCARPINE LIKE DRUG: PHARMACOLOGICAL IN VITRO AND IN VIVO PROFILE.

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Summary

The thiolactone analogue of pilocarpine, SDZ ENS 163, acts in vitro and in vivo as a partial agonist at M_1/M_3 and as an antagonist at M_2 muscarinic receptors. In vitro, the properties of SDZ ENS 163 have been investigated in several functional models for muscarinic receptors: it is a full agonist at M_1 (rat superior cervical ganglion, carbachol=100%) and a partial agonist at M_3 receptors (guinea pig ileum). However, the drug shows antagonistic properties at M_2 receptors (rat atria). Radioligand binding studies with ^{3}H-N-methylscopolamine (^{3}H-NMS) using CHO cells expressing m_1 or m_3 receptors indicate that SDZ ENS 163 does not discriminate between m_1 and m_3 receptors (K_i 1.5 and 2.4 μM respectively). Regarding phosphoinositide (PI) turnover in A9L cells, SDZ ENS 163 is a partial agonist at m_1 receptors. In ex vivo neurochemical studies in rats SDZ ENS 163 displays effects characteristic of muscarinic antagonists regarding the turnover of ACh which is increased in the brain. At a similar dose-range SDZ ENS 163 accelerates PI metabolism in the rat brain in vivo and increases the energy of the low frequency band (2-5 Hz) in the rat hippocampal EEG. These effects observed in vivo are consistent with postsynaptic M_1 agonistic and presynaptic M_2 antagonistic activities. Since SDZ ENS 163 at centrally active doses exerts no peripheral cholinergic effects, it may be useful for the symptomatic treatment of Alzheimer's disease.

Pilocarpine, an alkaloid isolated from the leaflets of shrubs of the Pilocarpus species, is a muscarinic agonist which has been known for a long time. The pharmacological actions of this compound on the pupil, the salivary and sweat glands were described at the end of the last century. Like other muscarinic drugs, pilocarpine evokes a characteristic cortical arousal in animals. The spectrum of the central effects of this drug has been reviewed e.g. (1). More recently, Kilbinger et al. (2) found an ACh releasing action of pilocarpine in the periphery. At high doses a similar effect was found in rat brain with pilocarpine (3).

The cholinergic deficit in Alzheimer's disease attracted attention to muscarinic agonists to enhance the decreased central cholinergic transmission and in this way such drugs became candidates for a symptomatic therapy. Many pilocarpine derivatives were synthesized in our laboratories and tested in vitro with regard to their muscarinic activities (4). The thiolactone analogue of pilocarpine, SDZ ENS 163, has been developed as a compound of potential therapeutic value due to its favourable pharmacological properties.

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Materials and Methods.

^{3}H-NMS binding.

^{3}H-N-methylscopolamine binding was performed in chinese hamster oocytes (CHO cells) expressing either m_1, m_2 or m_3 muscarinic receptors (obtained from Marc Brann, University of Vermont). The cells were cultured under standard conditions and the radioligand binding assay using ^{3}H-NMS was performed according to the procedure described by Buckley et al. (5). The K_d and B_{max} values were calculated from Scatchard plots, and the K_i values were estimated using the equation of Cheng and Prusoff (6).

Phosphoinositide metabolism in transfected A9L cells.

The accumulation of myo-inositol-1-phosphate (1-IP) in A9L cells transfected with either m_1 or m_3 muscarinic receptors (obtained from Marc Brann, University of Vermont) was measured after muscarinic stimulation and inhibition of inositol-1-phosphatase by Li^+ using the method described by Berridge et al. (7). The data are expressed as a percentage of the maximal effect induced by muscarine (100%).

Isolated rat superior cervical ganglion.

Rat superior cervical ganglia were taken from male Sprague Dawley rats and muscarinic agonist-induced depolarisations were recorded differentially between the ganglion and its postganglionic trunk using calomel electrodes. After 4-6 min perfusion with agonists a maximal depolarization was obtained which was used as a measure of agonist effect. The data are expressed as a percentage of the maximal depolarization induced by carbachol.

Isolated left rat atria.

Left rat atria were taken from male Sprague-Dawley rats and suspended vertically in an organ bath containing 20 ml oxygenated Krebs solution (36°C) at a resting tension of 7.5 mN. The atria were electrically stimulated by square wave pulses at a frequency of 3 Hz (duration 5 ms, voltage 50% above threshold). Muscarinic agonist-induced inhibition of inotropy was measured with a force displacement transducer. The change in contractility was expressed as a percentage of the maximal effect of carbachol.

Guinea pig ileum.

A segment of the ileum from male guinea-pigs (Ivanovas) was removed. A piece of longitudinal muscle strip was suspended vertically in an organ bath containing 20 ml oxygenated Krebs solution (36°C) at a resting tension of 7.5 mN. Muscarinic agonist-induced contractions were measured with force-displacement transducers. The contractions were expressed as a percentage of the maximal effect induced by carbachol.

ACh levels in rat brain regions.

ACh concentrations in different rat brain regions were determined by gas-chromatography-mass-spectrometry after sacrificing male Sprague Dawley rats (200-250 g) by micro-wave irradiation (Puescher, Mikrowellen Technik, Bremen BR, 2450 MHz, 6kW, 1.7 sec). Following dissection of the brain the tissue was homogenized in 0.1 M perchloric acid containing deuterated ACh-d9 as internal standard. After extraction and demethylation, ACh was determined by GC-mass-fragmentography, according to Jenden et al. (8)

PI metabolism in vivo.

Male Sprague Dawley rats (200-300 g) were used. Twenty-four hours prior to drug administration, the animals received 3mEq/kg LiCl, s.c. For the NMR experiments, the rats were anaesthetized with isoflurane (1.5% in oxygen/nitrous oxide 1:2) and positioned in a

stereotactic holder. The ^{31}P NMR spectra were recorded as described by Sauter and Rudin (10) using an elliptical surface coil with the axes 10mm x 14mm. The NMR signals therefore originate predominantly from cerebral cortex with little contamination from other brain or extracranial structures. Five control spectra were recorded prior to drug administration and the animals were monitored 60 to 80 min following drug administration. For the chromatographic determination of IP concentrations, the rats were killed by micro-wave irradiation and the IP levels measured, as described by Sherman et al. (9).

Hippocampal EEG.

Rhythmical slow wave activity (RSA) in hippocampal EEG was recorded in anaesthetized male Sprague Dawley rats according to the method described by Bevan (11).

Results

In vitro properties.

^{3}H NMS Binding in CHO cells.

In CHO cells expressing either m_1 or m_3 muscarinic receptors, ^{3}H-NMS binds with similar K_d (141 pM and 142 pM respectively) and comparable B_{max} (m_1: 2.78 pmoles/mg protein and m_3: 2.15 pmoles/mg protein). ^{3}H-NMS binds to m_2 receptors expressed in CHO cells with a K_d of 140 pM the B_{max} being 0.67 pmoles/mg protein. SDZ ENS 163 dose-dependently displaced the ligand from m_1/m_3 receptors with similar potencies (K_i 1.5 and 2.4 μM) indicating, no selectivity between these two muscarinic receptors. However, the drug competes with ^{3}H-NMS at the m_2 receptors with a K_i of 0.16 μM (Fig.1).

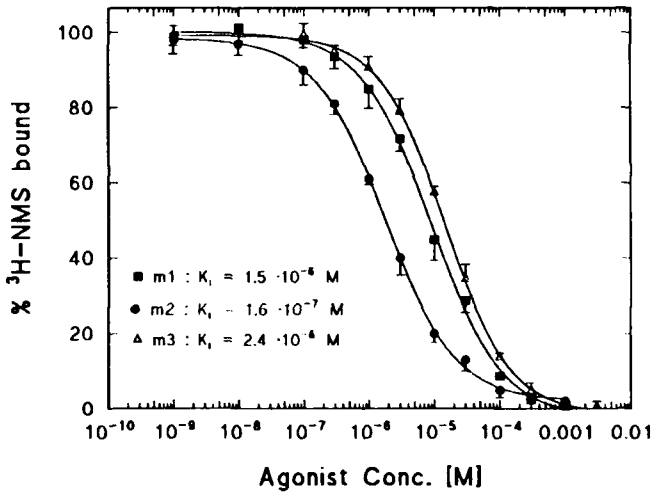

Fig. 1

Competition of ^{3}H-NMS binding in CHO cells expressing m1,m2 and m3 muscarinic receptors by SDZ ENS 163.

Phosphoinositide metabolism in A9L cells transfected with m_1 or m_3 receptors.

SDZ ENS 163 stimulates concentration-dependently the accumulation of 1-IP in A9L cells expressing m_1 muscarinic receptors. The receptor density in this cell line, determined using

^{3}H-NMS as ligand, was 607 fmoles/mh protein. The drug produces a maximal stimulation of only 45% compared to that of muscarine (pD_2 = 5.5), suggesting a partial agonism at this receptor (Fig.2). SDZ ENS 163 has virtually no influence on PI turnover in A9L cells transfected with m_3 receptors (receptor density (^{3}H-NMS): 576 fmoles/mg protein). The maximal stimulation obtained with SDZ ENS 163 was marginal (ca. 7%).

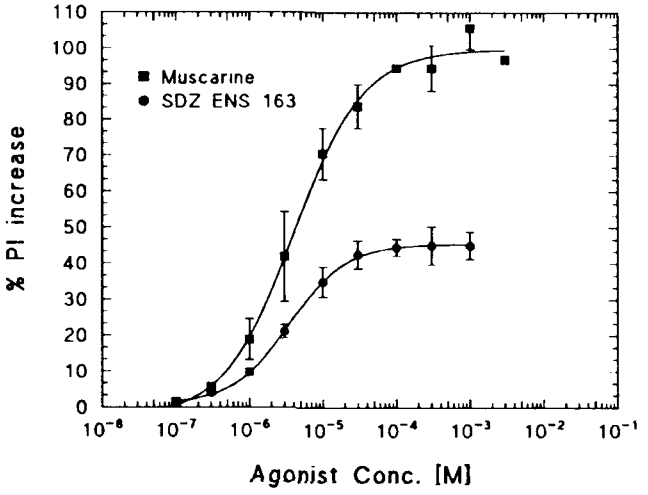

Fig. 2

Partial agonistic effect of SDZ ENS 163 on PI turnover in A9L cells expressing m1 muscarinic receptors. Mean values % of maximal stimulation by muscarine ±SEM, n=4.

Functional muscarinic in vitro tests.

In the rat superior cervical ganglion, a pharmacological model for characterization of M_1 receptors, concentration-dependent depolarization in presence of SDZ ENS 163 was observed. The drug induced a maximal depolarization of 128±4.2% compared to the maximal effect induced by carbachol = 100%. The calculated pD_2 value for SDZ ENS 163 was 6.5±0.3 (Tab.I). After incubation with the M_1 and M_2 muscarinic receptor selective antagonists pirenzepine and AF-DX 116 respectively, the effect of SDZ ENS 163 was antagonized in a competitive manner resulting in pA_2 values of 8.0±0.2 (slope:0.97) and 6.4±0.1 (slope: 1.11), respectively (12).

The induction of negative inotropic activity in left rat atria by SDZ ENS 163 was used as a model for interaction with M_2 receptors. After the application of SDZ ENS 163 a small decrease in contractile force in this preparation was found (maximal effect 14±2.9%). This effect was suppressed by pretreatment with scopolamine (10^{-6}M). The concentration-response curves with respect to the negative inotropic effect induced by carbachol were shifted to the right, indicating competitive antagonistic activity of SDZ ENS 163 at M_2 receptors, and a pA_2 value of 5.8±0.2 was found.

In guinea pig ileum, in which contraction is mediated by M_3 receptors, SDZ ENS 163 acted as a partial muscarinic agonist. The maximal contraction induced by SDZ ENS 163 amounted to 72±4.2% of the maximum effect obtained with carbachol. The calculated pD_2 value in this preparation was 5.3±0.01 (Tab.I).

Table I

Functional muscarinic in vitro tests of SDZ ENS 163.

M_1 Rat cervical Ganglion	M_3 Guinea Pig Ileum	M_2 Rat Left Atria
pD_2 6.5 ± 0.3	pD_2 5.3 ± 0.1	pA_2 5.8 ± 0.2
128 ± 4.2 % of carbachol	72 ± 0.1 % of carbachol	

In vivo data.

Effects of SDZ ENS 163 on central ACh concentrations in rat brain.

The effects of acute oral administration of SDZ ENS 163 on ACh levels in different rat brain regions have been measured and compared to those of scopolamine (Tab.II). Whereas muscarinic agonists increase central ACh concentration (13), SDZ ENS 163 causes a decrease in ACh levels in a dose-dependent manner, similar to that induced by muscarinic antagonists. A reduction of central ACh levels is an indication of an accelerated transmitter turnover. Scopolamine was approximately 10 times more effective in reducing ACH levels in rat brain striatum after intraperitoneal administration compared to SDZ ENS 163 applied orally (Tab. II). This acute effect of SDZ ENS 163 on central ACh turnover was still present following treatment with the drug for 2 weeks (data not shown).

Table II

ACh levels in rat brain regions after administration of SDZ ENS 163 and scopolamine

Dose (μmol/kg)	Cortex (% of controls ±SEM)	Striatum (% of controls ±SEM)
SDZ ENS 163 p.o.		
3	88.6 ± 5.8	90.8 ± 3.2
10	86.4 ± 2.5	86.5 ± 3.3
30	69.9 ± 3.1	77.5 ± 2.6
100	59.7 ± 2.8	71.6 ± 2.9
scopolamine i.p.		
0.33		87.3 ± 4.3
1		79.8 ± 7.0
3		65.5 ± 4.8
10		52.5 ± 6.8
30		46.3 ± 1.4
100		48.6 ± 2.5

ACh was measured 1 hour after drug administration. Control values in pmol/mg fresh tissue ± SD: striatum 72.5 ± 2.5, cortex 16.0 ± 1.3, n = 6

Influence of SDZ ENS 163 on phosphoinositide metabolism in rat brain.

Most muscarinic agonists stimulate the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), which generates the second messenger IP_3. An in vivo influence on this second messenger system can be measured indirectly by determining the accumulation of myo-inositol-1-phosphate following the inhibition of the inositol-1-phosphatase by lithium

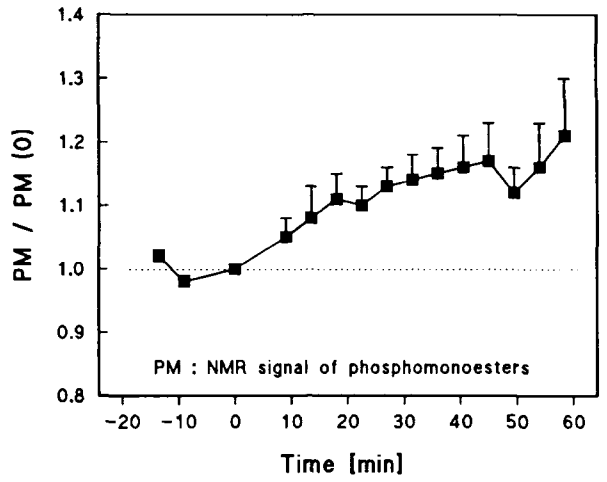

Fig. 3

Effect of SDZ ENS 163 (10 μmol/kg i.p.) on PI turnover in rat cortex measured by in vivo ^{31}P-NMR 8spectroscopy. Mean PM increase % of LiCl pretreated controls, ±SEM, n=5.

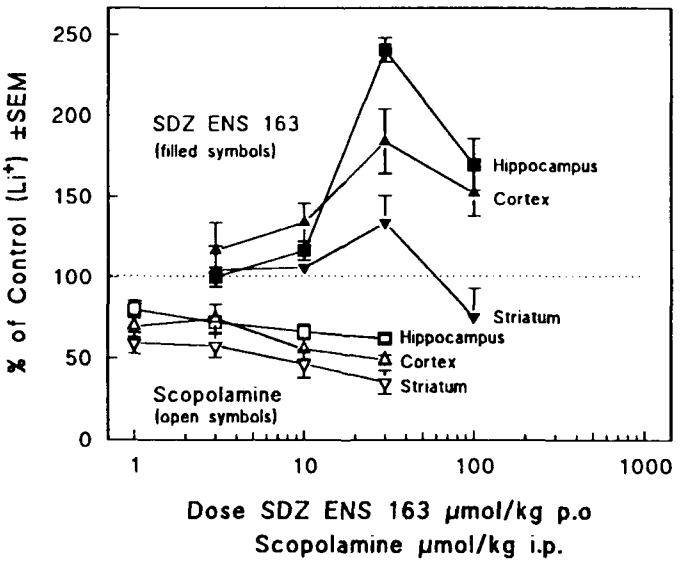

Fig. 4

Influence of SDZ ENS 163 and scopolamine on PI turnover in rat brain. Gas chromatographic measurements ex vivo. Mean % of Li+ treated controls, ±SEM n=7.

administration. The time-dependent formation of IP after administration of SDZ ENS 163 is illustrated in Fig.3. Within 60 min after drug application, SDZ ENS 163 increased the phosphomonoester (PM) NMR signal in Li^+ pretreated rats. The 13 % increase of the PM signal translated to an IP concentration of 0.4 mM 60 min after drug administration. In the ex vivo experiments SDZ ENS 163 accelerated the PI turnover in rat cortex and hippocampus following oral administration, as evidenced by the accumulation of 1-IP in Li^+ pretreated rats (Fig.4). In contrast, scopolamine induced a dose-dependent decrease of 1-IP in the same brain regions, whereas N-methylscopolamine which does not penetrate the blood-brain barrier, had no effect on the levels of 1-IP (data not shown).

Effect of SDZ ENS on hippocampal EEG in the rat.

A characteristic effect of stimulation of central muscarinic receptors is the induction of slow rhythmic activity (RSA) in the nippocampal EEG (11). Specifically a synchronization in the frequency band 1.2 - 4.2 Hz (theta waves) is observed. After i.p. administration of SDZ ENS 163 and pilocarpine in halothane anaesthetized rats, a clear induction of theta waves was observed. SDZ ENS 163 at the dose-range of 0.3 -30 μmol/kg i.p. elicited a 30 to 100% increase in hippocampal RSA, the activity profile being similar to that of pilocarpine (Tab.III).

Table III

Rhythmical slow wave activity (RSA) in rat hippocampal EEG
Maximal Activation of Energy (theta-frequency band 1.2-4.2 Hz)
Values in % above controls, ±SEM, n=6-12

SDZ ENS 163 Dose (μmol/kg i.p.)	%	Pilocarpine8 Dose (μmol/kg i.p.)	%
0.3	27.7±4.0	0.5	30.1±1.6
0.5	49.8±6.5	1	42.3±4.0
1	55.9±1.7	3	57.3±4.7
3	62.5±3.7	5	67.7±6.20
10	53.6±4.4	10	35.7±3.6
30	93.1±12.8	30	51.7±10.8

Discussion

SDZ ENS 163, a muscarinic drug structurally closely realated to pilocarpine, competes with the binding of the muscarinic antagonist N-methylscopolamine (NMS) at muscarinic m_1, m_2 and m_3 receptors in transfected CHO cells. From experiments with cell lines expressing one subtype of muscarinic receptor, it has been established that m_1 and m_3 receptors are involved in the regulation of formation of the second messenger IP_3 (13). SDZ ENS 163 shows a partial agonistic effect in cells transfected with m_1 receptors, but exerts only marginal influence on those containing the m_3 subtype. The receptor densities in both cell lines, measured using ^{3}H-NMS as ligand were similar (m_1 : 607 fmoles/mg protein, m_3 : 576 fmoles/mg protein).

These in vitro findings have been supported in vivo, in experiments in which PI turnover was measured in Li^+ pretreated animals either post-mortem by gas-chromatography or in vivo using the non invasive NMR technique. In these experiments the antagonist scopolamine decreased the accumulation of 1-IP, while N-methylscopolamine, an antagonist not penetrating

the blood-brain-barrier, was without effect, suggesting that the influence on the PI turnover is centrally mediated. These results demonstrate that SDZ ENS 163 is a centrally active muscarinic agonist.

In the rat superior ganglion the drug acts as a muscarinic agonist. The activity was demonstrated to result exclusively from M_1 receptor stimulation, based on experiments with the specific M_1 antagonist pirenzepine and M_2 antagonist AF-DX 116. The selectivity of SDZ ENS 163 over M_3 receptor interactions, measured in guinea pig ileum, is 16 fold. Whereas SDZ ENS 163 is a full agonist at M_1 receptors, it exerts only partial agonistic activity at M_3, which also contributes to the M_1 selectivity of the drug. In vivo muscarinic agonistic activity is demonstrated in experiments in which SDZ ENS 163 increased the RSA activity of rat hippocampal EEG. The direct effect of SDZ ENS 163 in vitro at the M_2 preparation of the left rat atria was as a partial or almost inactive agonist. In this preparation, a competitive antagonistic effect of SDZ ENS 163 was observed. We have demonstrated that SDZ ENS 163 reversed the inhibition of oxotremorine of electrically evoked ACh release in hippocampal slices (12). These results suggest antagonistic activity and are in agreement with the finding that SDZ ENS 163, following oral administration, decreased the levels of ACh in several rat brain regions in a dose range similar to that affecting PI metabolism. Thus, the in vivo results are consistent with the data obtained from in vitro experiments, pointing to greater M_1 agonist selectivity and presynaptic (M_2) receptor antagonistic activity for SDZ ENS 163 than for pilocarpine. With this drug no adverse cholinergic effects in the periphery are expected at doses having clear, cholinergic activities in the CNS.

References.

1. K. KRNJEVIC, Physiol. Rev. 54 418-540, (1974).
2. H. KILBINGER and M. NAFZINGER, Arch.Pharmacol. 328 328-304,(1985).
3. A. ENZ, P.H. KELLY, J.M. PALACIOS, P. SUPAVILAI and K.H. WIEDERHOLD, Int. Symp. Muscarinic Cholinergic Mechanisms. Cohn,S. and Sokolovsky,M. ed. Freund Publishing House London, 120-124, (1987).
4. G. SHAPIRO and A. ENZ, Drugs of the Future, 17 489-501, (1992).
5. N.J.BUCKLEY, T.I. BONNER, C.M. BUCKLEY and M. BRANN, Mol. Pharmacol. 35 469-476, (1989).
6. Y.C. CHENG and W.H. PRUSOF, Biochem. Pharmacol. 22 3099-3108, (1973).
7. M.J. BERRIDGE, C.P. DOWNES and M.R. HANLEY, Biochem.J. 206 587-595, (1982).
8. D.J. JENDEN, L. CHOI, R.W. SILBERMAN, J.A. STEINBORN, M. ROCH and R.A. BOOTH, Life. Sci. 14 55-63, (1974).
9. W.R. SHERMAN, L.Y. MUNSELL, B.G. GISH and M.P. HONCHAR, J. Neurochem. 44 798-807, (1985).
10. A. SAUTER and M. RUDIN, Magn.Reson.Med. 4 1-8, (1987).
11. P. BEVAN, Br. J. Pharmacol. 82 408-417, (1984).
12. A. ENZ, G. SHAPIRO, P. SUPAVILAI and H.W.G.M. BODDEKE, Arch.Pharmacol. 345 282-287, (1992).
13. J.M. PALACIOS, G. BOLLIGER, A. CLOSSE, A. ENZ, G. GMELIN and J. MALANOWSKI, Europ.J.Pharmacol. 125 45-62, (1986).
14. A. ASHKENAZI, E.G. PERALTA, J.W. WINSLOW, J. RAMACHANDRAN and D.J. CAPON, TIPS 10 (Suppl. Subtypes Muscarinic Recept. IV), 16-22, (1989).

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MUSCARINIC RECEPTOR SUBTYPES IN AIRWAYS

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Summary

Muscarinic receptor subtypes in the airways appear to subserve different physiological functions. M_1-receptors facilitate neurotransmission through parasympathetic ganglia and enhance cholinergic reflexes, but are also localized to alveolar walls. M_2-receptors act as autoreceptors on post-ganglionic cholinergic nerves and inhibit acetylcholine release. There is some evidence that they may be defective in asthma (as a consequence of airway inflammation?) and this may enhance cholinergic reflexes and account for ß-blocker-induced asthma. M_2-receptors in airway smooth muscle may also counteract the bronchodilator action of ß-agonists. M_3-receptors mediate contractile responses in airway smooth muscle via phosphoinositide hydrolysis, and are the predominant receptors on submucosal glands and airway vascular endothelium. M_4- and M_5-receptors have not been identified in human airways, but in rabbit lung M_4-receptors are expressed on alveolar walls and smooth muscle. Anticholinergic drugs which selectively block M_3 and M_1-receptors may have an advantage over currently used non-selective antagonists in the treatment of airway obstruction.

Cholinergic antagonists are now widely used in the treatment of obstructive airways diseases. The recognition that there are multiple subtypes of muscarinic receptor in lung has raised important questions about their role in regulation of airway function and creates the prospect of more selective therapy in the future.

Cholinergic nerves are the major bronchoconstrictor neural mechanism in animal and human airways. Cholinergic nerves arise in the brain stem and pass down the vagus nerve to relay in local ganglia situated within the airway walls. From these ganglia short post-ganglionic fibers travel to airway smooth muscle and submucosal glands. Stimulation of the vagus nerve releases acetylcholine (ACh) which activates muscarinic receptors on smooth muscle and submucosal gland cells, which results in bronchoconstriction and mucus secretion, respectively (1). Muscarinic receptors regulate the secretion of mucus from both submucosal glands and airway epithelial goblet cells (2). Autoradiographic mapping studies using [^{3}H]quinuclidinyl benzylate indicate that muscarinic receptors are predominantly localized to airway smooth muscle, vascular endothelium, submucosal gland cells and neuronal structures (3-5), although in some species, including humans, there is also localization to alveolar walls.

Cholinergic neural mechanisms may contribute to the airway narrowing of asthma and chronic obstructive airways disease (COPD). Reflex cholinergic bronchoconstriction may be triggered in asthma by the presence of inflammatory mediators, such as bradykinin, that activate sensitized sensory nerves in the airway. While anticholinergic drugs are less effective as bronchodilators in chronic asthma, they are effective in treating acute exacerbations, suggesting that cholinergic mechanisms become more important during asthma attacks. In COPD cholinergic vagal tone is probably the only reversible element and anticholinergic drugs are the bronchodilators of choice (6).

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Four subtypes of muscarinic receptor (M_1-M_4) have now been recognised in lung both pharmacologically and using specific cDNA probes (7-10)(**Table 1**), but their precise relevance to airway disease and therapy is not yet certain.

TABLE 1

Localization and function of muscarinic receptor subtypes in airways

	Localization	Function
M_1	Parasympathetic ganglia	Facilitation of neurotransmission
	Submucosal glands	Increased secretion?
	Alveolar walls	?
M_2	Postganglionic cholinergic nerves	Inhibit acetylcholine release
	Airway smooth muscle	Antagonism of bronchodilatation
	Sympathetic nerves	Inhibit norepinephrine release
M_3	Airway smooth muscle	Contraction
	Submucosal glands	Increased mucus secretion
	Epithelial cells	Increased ciliary beating?
	Goblet cells?	Increased secretion
	Endothelial cells	Vasodilatation via release of nitric oxide
M_4	Alveolar walls)	?
	Smooth muscle) *rabbit only*	?
	Postganglionic cholinergic nerves?	Inhibit acetylcholine release
m_5	Not yet identified in lung	

M_1-receptors

Binding studies with lung homogenates have indicated that there is a high proportion of pirenzepine-sensitive binding sites, presumed to be M_1-receptors in several species, including humans and rabbits (11-13). In human lung membrane high affinity pirenzepine-sensitive sites make up approximately 70% of total binding and this is confirmed by studies using [^{3}H]pirenzepine as a radioligand (13). Autoradiographic mapping studies indicate that these receptors are localized to the alveolar walls (4). Other species, such as guinea pig and ferret do not appear to have these parenchymal muscarinic receptors (3,4), but their significance is far from clear as there is no evidence for cholinergic innervation of the lung periphery. More recently we have confirmed that these muscarinic receptors are of the m_1-receptor subclass in human lungs using specific cDNA probes. Northern analysis of human lung parenchyma shows a prominent band corresponding to m_1-receptor mRNA and *in situ* hybridization shows that m_1-receptor mRNA is localized to alveolar walls (10).

M_1-receptors are usually localized to neuronal tissue and there is evidence that M_1-receptors are localized to parasympathetic ganglia and to sympathetic nerve terminals in airways (14). In rabbit bronchi low concentrations of pirenzepine inhibit ganglionic transmission, suggesting that M_1-receptors may have a facilitatory effect on transmission through airway ganglia (15). M_1-receptors may also be present in human airway cholinergic pathways. The effects of inhaled pirenzepine and the non-selective antagonist ipratropium bromide were compared on cholinergic reflex bronchoconstriction triggered by the inhalation of the irritant gas, sulfur dioxide, in allergic volunteers (16). A dose of inhaled pirenzepine was found which did not inhibit bronchoconstriction due to an inhaled cholinergic agonist (methacholine), whereas ipratropium bromide blocked its bronchoconstrictor effect as expected. The same dose of pirenzepine, however, was as effective as ipratropium bromide in blocking the cholinergic

reflex bronchoconstriction. Since pirenzepine in this dose could not be acting directly on airway smooth muscle receptors, it might be acting on some peripheral part of the cholinergic pathway, which is most likely to be parasympathetic ganglia in the airways.

The physiological role of the M_1-receptors in ganglia is still not certain. Classically, ganglionic transmission is via nicotinic cholinergic receptors which are blocked by hexamethonium. It is possible that excitatory M_1-receptors are facilitatory to nicotinic receptors and may be involved in "setting" the efficacy of ganglionic transmission (**Figure 1**). Activation of these receptors probably closes K^+ channels, resulting in a slow depolarisation of the ganglion cell (17). Perhaps they might be involved in the chronic regulation of cholinergic tone, whereas nicotinic receptors (which act as "fast" receptors and open ion channels) are more important in rapid signalling, such as occur during reflex activation of the cholinergic pathway. If so, then M_1-antagonists such as pirenzepine and telenzepine might have a useful therapeutic role in asthma and COPD, since they may reduce vagal tone. Since increased vagal tone may play an important role in nocturnal exacerbations of asthma, then pirenzepine might prove to be efficacious in preventing nocturnal wheeze.

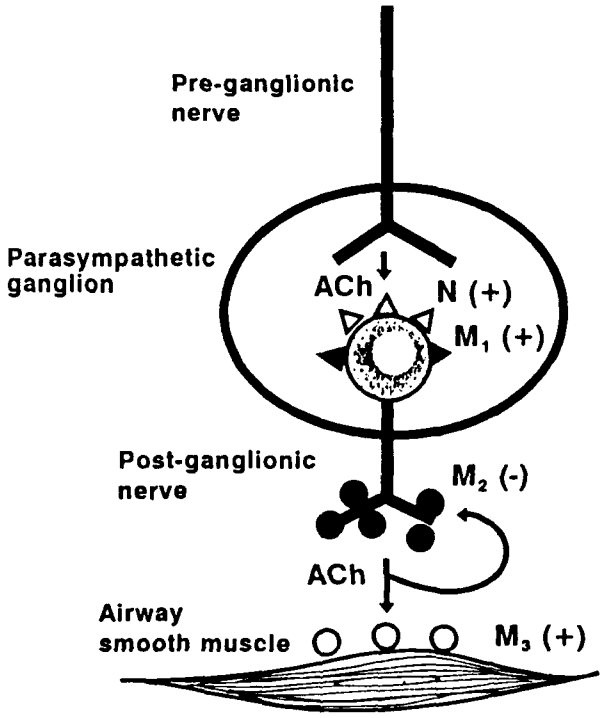

Figure 1. Muscarinic receptor subtypes in airways. Ganglionic transmission is mediated via nicotinic receptors (N), but M_1-receptors may play a facilitatory role. M_2-receptors at the postganglionic terminal may inhibit the release of acetylcholine (ACh), which acts on M_3-receptors on airway smooth muscle.

Pirenzepine is a bronchodilator when given intravenously to human subjects (18), although at the dose used it might be acting non-selectively and blocking smooth muscle muscarinic receptors. Lower doses of intravenous pirenzepine, while having no effect on FEV_1, increases expired flow at low lung volumes, suggesting an effect on more peripheral airways (19). Another study has demonstrated no effect of the longer-acting M_1-selective antagonist telenzepine in patients with COPD however (20).

Receptor mapping studies also indicate that there are M_1-receptors localized to submucosal glands in the larger airways in humans (4), although this has not been confirmed using m_1-receptor cDNA probes. Functional studies of mucus secretion in human airways suggest

that there are no functional M_1-receptors, since pirenzepine at low and selective concentrations has no inhibitory effect on secretion of mucus glycoproteins (21). Whether M_1-receptors may be involved in some other secretory response of submucosal glands or of goblet cells remains to be determined.

M_2-receptors

Binding studies in lung membrane preparations indicate that the population of M_2-receptors is very low (13), although binding to airway smooth muscle indicates that there may be a sizeable proportion of M_2-receptors (22) and m_2-receptor protein has been identified immunologically in peripheral rabbit lung (23). m_2-receptor mRNA has also been detected in cultured human airway smooth muscle cells using Northern analysis (10). M_2-receptors may play a very important physiological role in the regulation of cholinergic neurotransmission (14). In several species, including guinea pig, rat, dog, cat and human there is evidence for pre-junctional muscarinic receptors on post-ganglionic airway cholinergic nerves that inhibit the release of ACh, and therefore function as feedback inhibitory receptors (reviewed in ref 14)(**Figure 1**). These pre-junctional receptors have the characteristic of M_2-receptors and are selectively blocked by methoctramine (24). The presence of these M_2 receptors has recently been confirmed by measurement of ACh release in guinea pig trachea (25). In human airways activation of pre-junctional M_2-receptors has a powerful inhibitory effect on cholinergic nerve-induced contraction of airway smooth muscle *in vitro* (26). In non-asthmatic human subjects inhalation of pilocarpine, which selectively stimulates the pre-junctional receptors, has an inhibitory effect on cholinergic reflex bronchoconstriction induced by SO_2, suggesting that these inhibitory receptors are present *in vivo*, and presumably serve to limit cholinergic bronchoconstriction (27). In asthmatic patients pilocarpine has no such inhibitory action, indicating that there might be some dysfunction of the autoreceptor, which would result in exaggerated cholinergic reflex bronchoconstriction (27). Another study using histamine challenge also supports this conclusion (28). A functional defect in muscarinic autoreceptors may also explain why ß-blockers produce such marked bronchoconstriction in asthmatic patients, since any increase in cholinergic tone due to blockade of inhibitory ß-receptors on cholinergic nerves would normally be switched off by M_2-receptors in the nerves, and a lack of such receptors may lead to increased acetylcholine release, resulting in exaggerated bronchoconstriction (29). Support for this idea is provided by the protective effect of oxitropium bromide against propranolol-induced bronchoconstriction in asthmatic patients (30).

The mechanism by which M_2-autoreceptors on cholinergic nerves may become dysfunctional is not certain. It is possible that chronic inflammation in airways may lead to down-regulation of M_2-receptors which may have an important functional effect if the density of pre-junctional muscarinic receptors is relatively low. Recently experimental studies have demonstrated that influenza virus and major basic protein from eosinophils may inactivate M_2 rather than M_3-receptors (31,32). This may account for an increase in cholinergic reflex bronchoconstriction during an exacerbation of asthma, either due to a virus infection or due to allergen exposure.

Although the bronchoconstrictor responses to cholinergic agonists appear to involve the activation of M_3-receptors leading to phosphoinositide hydrolysis, binding studies have indicated a high proportion of M_2-receptors in airway smooth muscle (22). Receptor mapping studies indicate the presence of M_2-receptors in airway smooth muscle of more peripheral airways (at least in guinea pig) (4), with a relatively low level of gene expression (10). Recently it has been established that these M_2-receptors, by inhibition of adenylyl cyclase, may have a functional role in counteracting the bronchodilator response to ß-agonists due to activation of adenylyl cyclase, both *in vitro* (33) and *in vivo* (34). It is not certain what the physiological role of these airway smooth muscle M_2-receptors might be, however, or whether their function may be altered in airway disease.

Sympathetic nerves interact with airway cholinergic nerves in some species, such as guinea pig. M_2-receptors on sympathetic nerve terminals inhibit the release of norepinephrine from these nerves (35)

M_3-receptors

Binding studies in guinea-pig and human lung membranes indicate the presence of M_3-receptors (13). Autoradiographic studies have demonstrated M_3-receptors in airway smooth muscle of large and small human airways (4), and this has been confirmed by *in situ* hybridization studies with m_3-selective cDNA probes (10) (**Figure 2**). In guinea pig M_3-receptors are localized predominantly to smooth muscle of proximal airways (10), and a similar distribution (of total muscarinic receptors) is seen in ferret airways (36).

In the airways, smooth muscle muscarinic receptor activation results in rapid phosphoinositide hydrolysis (37-39) and the formation of inositol (*1,4,5*) trisphosphate (40) which releases calcium ions from intracellular stores.

M_3-receptors are also localized to submucosal glands in human airways (4) and there is a high concentration of m_3-receptor mRNA in these structures (10). M_3-selective antagonists potently inhibit mucus glycoprotein secretion form human airways *in vitro*, suggesting that M_3-receptors predominate (21). M_3-receptors are only weakly expressed on airway epithelial cells (4), in contrast to the strong *in situ* hybridization signal with a m_3-receptor cDNA probe (10), indicating that there may be a very rapid turnover of receptors. A similar epithelial expression of m_3-receptor mRNA is found in human nasal biopsies (41). M_3-receptors are also localized to endothelial cells of the bronchial circulation and presumably mediate the vasodilator response to cholinergic stimulation of the proximal airways (42). The vasodilator response to ACh in pulmonary vessels is mediated via an M_3-receptor on endothelial cells (43).

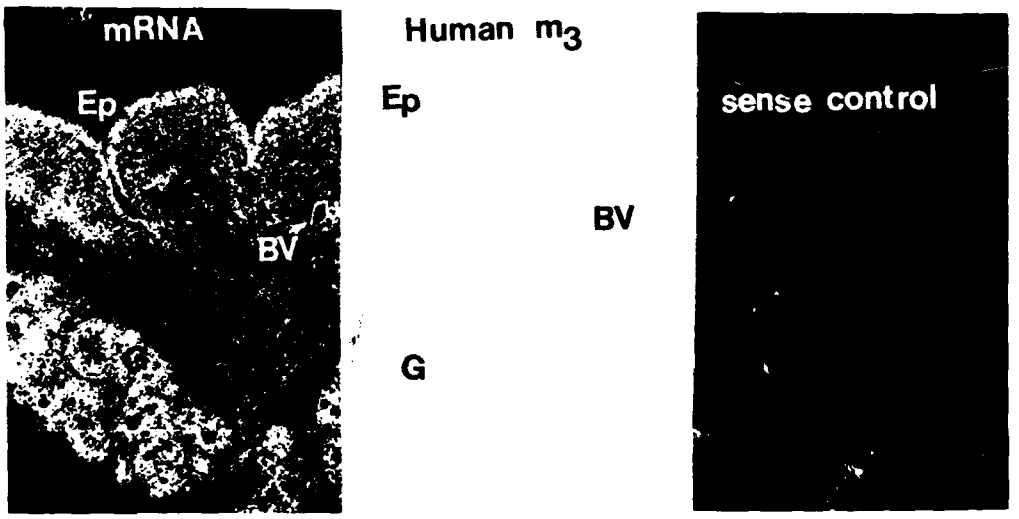

Figure 2. m_3-Receptor expression in human airway. *In situ* hybridization of section of human airway using an antisense cDNA probe of the human m_3-receptor. *Left panel* shows positive hybridization to submucosal gland (G), epithelial cells (Ep) and endothelium of a bronchial vessels (BV). *Right panel* shows a sense control with no positive hybridization.

M_4- and m_5-receptors

In rabbit lung there is evidence from binding studies for the existence of an M_4-receptor and this has been confirmed by the presence of m_4-receptor mRNA on Northern blotting (44) and a preponderance of m_4-receptor protein (23). *In situ* hybridization has demonstrated that this m_4-receptor mRNA is localized to alveolar walls, and vascular and airway smooth muscle

(45). There is preliminary evidence that the muscarinic autoreceptors on post-ganglionic cholinergic nerves in guinea pig trachea may be M_4, rather than M_2-receptors (46). In human lung Northern analysis has not revealed any evidence of either m_4 or m_5-receptor mRNA and *in situ* hybridization has not revealed any evidence for expression of the genes for these receptor subtypes (10).

Clinical Relevance

The discovery of at least three muscarinic subtypes in human lung has important clinical implications, since it raises the possibility of more selective anticholinergic therapy in the future. Atropine, ipratropium bromide and oxitropium bromide are non-selective as anticholinergic drugs and therefore block pre-junctional (M_2) and post-junctional (M_3) receptors. Inhibition of the autoreceptor means that more ACh will be released during cholinergic nerve stimulation and this may overcome post-junctional blockade, thus making these non-selective antagonists less efficient than a selective antagonist of M_3-receptors. Direct evidence for this is the increase in ACh release on nerve stimulation which occurs in the presence of atropine (25,47), and the fact that ipratropium bromide in low doses causes an increase in vagally mediated bronchoconstriction (48). Paradoxical bronchoconstriction has been reported with nebulized anticholinergic drugs and this may be a contributory mechanism. similar analogy exists with α-adrenoceptors and the non-selective antagonist phentolamine, by acting on a pre-junctional α_2-receptor, increases norepinephrine release and is thus far less effective in the treatment of high blood pressure than a selective α_1-antagonist such as prazosin, which acts only on the post-junctional receptor. Unfortunately, muscarinic drugs with the high selectivity shown by prazosin for post-junctional receptors are not yet available for clinical use. Selective anticholinergic drugs which block M_3- or M_3- and M_1-receptors may therefore have an advantage in the treatment of airways obstruction.

References

1. P.J. BARNES, Am. Rev. Respir. Dis. *134* 1289-1314 (1986).
2. K. TOKUYAMA, H-P. KUO, J.A.L. ROHDE, P.J. BARNES and D.F. ROGERS, Am J Physiol *259* L108-L115 (1990).
3. P.J. BARNES, J.A. NADEL, J.M. ROBERTS and C.B. BASBAUM, Eur. J. Pharmacol. *86* 103-106 (1982).
4. J.C.W. MAK and P.J. BARNES, Am. Rev. Respir. Dis. *141* 1559-1568 (1990).
5. C.J. van KOPPEN, W.M. BLANKENSTIEIJN, AR.M. KLAASSEN, J.F. RODRIGUES DE MIRANDA, A.J. BELD and C.A.M. van GINNEKEN, Neursci Lett *83* 237-240 (1989).
6. N.J. GROSS, E. CO and M.S. SKORODIN, Chest *96* 984-987 (1989).
7. P.J. BARNES, J. Appl. Physiol. *68* 1777-1785 (1990).
8. P.A. MINETTE and P.J. BARNES, Am Rev Respir Dis *141* S162-S165 (1990).
9. J. MACLAGAN and P.J. BARNES, Trends Pharmacol. Sci. *10 (Suppl)* 88-92 (1989).
10. J.C.W. MAK, J.N. BARANIUK and P.J. BARNES, Am J Resp Cell Mol Biol *7* 344-348 (1992).
11. J.W. BLOOM, M. HALONEN, L.J. LAWRENCE, E. ROULD, N.A. SEAVER and H.I. YAMAMURA, J. Pharmacol. Exp. Ther. *240* 51-58 (1987).
12. T.B. CASALE and P. ECKLUND, J. Appl. Physiol. *65* 594-600 (1988).
13. J.C.W. MAK and P.J. BARNES, Eur. J. Pharmacol. *164* 223-230 (1989).
14. P.J. BARNES, Physiol Rev *72* 699-729 (1992).
15. J.W. BLOOM, H.I. YAMAMURA, C. BAUMGARTNER and M. HALONEN, Eur J Pharmacol *133* 21-27 (1987).
16. J.-W.J. LAMMERS, P. MINETTE, M. MCCUSKER and P.J. BARNES, Am. Rev. Respir. Dis. *139* 446-449 (1989).
17. J.H. ASHE and C.A. YAROSH, Neuropharmacology *23* 1321-1329 (1984).
18. K. SERTL, S. MERYN, W. GRANINGER, A. LAGNNER, W. SCHLICK and H. RAMERS, Int J Clin Pharmacol Ther Toxicol *24* 655-657 (1986).
19. M. CAZZOLA, S. RANO, D. DE SANTIS, P.J. PRINCIPE and E. MARMO, Int J Clin Pharmacol Ther Toxicol *25* 105-109 (1987).

20. D. UKENA, C. WEHINGER, R. ENGELSTATTER, V. STEINIJANS and G.W. SYBRECHT, Eur Resp J *in press* (1992).
21. C.W. JOHNSON, R.O. RIEVES, C. LOGUN and J.H. SHELHAMER, Am J Resp Cell Mol Biol *in press* (1992).
22. A.F. ROFFEL, C.R.S. ELZINGA, R.G.M. van AMSTERDAM, R.A. DE ZEEUW and J. ZAAGSMA, Eur. J. Pharmacol. *153* 73-82 (1988).
23. F. DÖRJE, A.I. LEVEY and M.R. BRANN, Mol Pharmacol *40* 459-462 (1991).
24. N. WATSON, P.J. BARNES and J. MACLAGAN, Br J Pharmacol *105* 107-112 (1992).
25. H. KILBINGER, R. SCHOREIDER, H. SIEFKEN, D. WOLF and G. D'AGOSTINO, Br J Pharmacol *103* 1757-1763 (1991).
26. P.A. MINETTE and P.J. BARNES, J. Appl. Physiol. *64* 2532-2537 (1988).
27. P.A.H. MINETTE, J. LAMMERS, C.M.S. DIXON, M.T. MCCUSKER and P.J. BARNES, J. Appl. Physiol. *67* 2461-2465 (1989).
28. L.E. AYALA and T. AHMED, Chest *96* 1285-1291 (1991).
29. P.J. BARNES, Thorax *44* 161-167 (1989).
30. P.W. IND, C.M.S. DIXON, R.W. FULLER and P.J. BARNES, Am Rev Respir Dis *139* 1390-1394 (1989).
31. A.D. FRYER and D.B. JACOBY, Br J Pharmacol *102* 267-271 (1991).
32. D.B. JACOBY, G.J. GLEICH and A.D. FRYER, Am Rev Respir Dis *145* A436 (1992).
33. C.M. YANG, S-P. CHOW and T-C. SUNG, Br J Pharmacol *104* 613-618 (1991).
34. L.B. FERNANDES, A.D. FRYER and C.A. HIRSCHMAN, J Pharmacol Exp Ther *262* 119-126 (1992).
35. K. RACKÉ, C. HEY and I. WESSLER, Br J Pharmacol. *107* 3-4 (1992).
36. P.J. BARNES, C.B. BASBAUM and J.A. NADEL, Am. Rev. Respir. Dis. *127* 758-762 (1983).
37. B.M. GRANDORDY, F.M. CUSS, A.S. SAMPSON, J.B. PALMER and P.J. BARNES, J. Pharmacol. Exp. Ther. *238* 273-279 (1986).
38. E.R. CHILVERS, I.H. BATTY, P.J. BARNES and S.R. NAHORSKI, J Pharmacol Exp Ther. *252* 786-791 (1990).
39. A.F. ROFFEL, C.R.S. ELZINGA and J. ZAAGSMA, Pulm. Pharmacol. *3* 47-51 (1990).
40. E.R. CHILVERS, R.A.J. CHALLISS, P.J. BARNES and S.R. NAHORSKI, Eur. J. Pharmacol. *164* 587-590 (1989).
41. J.N. BARANIUK, M.A. KALINER and P.J. BARNES, Am J Rhinol. *6* 145-148 (1992).
42. R. MATRAN, K. ALVING, C-R. MARTLING, J.S. LACROIX and J.M. LUNDBERG, Acta Physiol. Scand. *135* 29-37 (1989).
43. D.G. McCORMACK, J.C. MAK, P. MINETTE and P.J. BARNES, Eur. J. Pharmacol. *158* 293-297 (1988).
44. S. LAZARENO, N.J. BUCKLEY and F.F. ROBERTS, Mol Pharmacol *38* 805-815 (1990).
44. J.C. MAK, N. BUCKLEY and P.J. BARNES, Am Rev Respir Dis *in press* (1993).
45. H. KILBINGER, R.S. von BARBELEBEN and H. SIEFKEN, Life Sci. *in press* (1993).
46. G. D'AGOSTINO, M.C. CHIARI, E. GRANA and H. KILBINGER, Naunyn-Schmiedeberg's Arch. Pharmacol. *342* 141-145 (1990).
47. A.D. FRYER and J. MACLAGAN, Br J Pharmacol *83* 973-978 (1984).

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EFFECT OF INFLAMMATORY CELL MEDIATORS ON M_2 MUSCARINIC RECEPTORS IN THE LUNGS

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Summary

Acetylcholine released from vagal nerve endings constricts airways by stimulating M_3 muscarinic receptors on the airway smooth muscle. At the same time, released acetylcholine feeds back onto inhibitory M_2 muscarinic autoreceptors on the nerve endings, limiting further release of acetylcholine. Loss of function of these M_2 receptors increases vagally-mediated bronchoconstriction after viral airway infections, exposure to ozone, or antigen inhalation. Viral infections may decrease M_2 receptor function by inducing inflammation or via direct damage to the receptors as a result of cleavage of sialic acid residues by viral neuraminidase. Inflammation appears to be critical in the loss of M_2 receptor function after ozone exposure. Antigen-induced loss of M_2 receptor function can be reversed acutely by administering the poly-anionic substances heparin or poly-l-glutamate, possibly by binding and neutralizing positively charged eosinophil proteins. Such positively charged eosinophil proteins, particularly major basic protein, may be acting as endogenous inhibitors at the M_2 receptors, as can be demonstrated in *in vitro* ligand binding studies.

In asthma, as well as in a variety of other pulmonary diseases, an abnormal degree of smooth muscle contraction narrows the airways. This causes the wheezing and shortness of breath characteristic of asthma, and contributes to the symptoms seen after viral infections, exposure to irritant gases such as ozone, and antigen inhalation.

Airway smooth muscle contracts in response to acetylcholine released by parasympathetic nerves which run in the vagus. Increased vagally-mediated reflex bronchoconstriction is a characteristic of asthma as well as the other conditions listed above. While part of this increase may be due to enhanced contraction of the smooth muscle in response to acetylcholine, considerable evidence now supports a role for increased release of acetylcholine from the parasympathetic nerve endings. The

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mechanisms of this increased acetylcholine release will be the topic of this review.

Inhibitory neural muscarinic receptors

The parasympathetic control of airway smooth muscle is determined by the balance of the effects of multiple muscarinic receptor subtypes. Contraction of airway smooth muscle is primarily the result of stimulation of M_3 muscarinic receptors by acetylcholine released from the vagus nerve [1]. At the same time, however, the acetylcholine feeds back onto inhibitory M_2 autoreceptors on the nerve endings, limiting further release of acetylcholine [2] (figure 1).

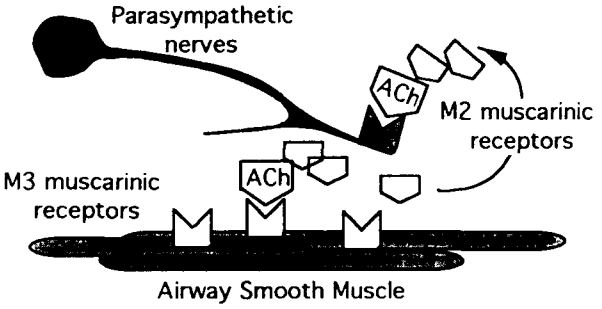

FIG. 1
Parasympathetic innervation of airway smooth muscle, showing excitatory M_3 receptors and inhibitory M_2 receptors.

These inhibitory M_2 autoreceptors, which are present in guinea pigs [2], cats [3], dogs [4], and humans [5], play a major role in the control of vagally-mediated bronchoconstriction. Blockade of these inhibitory autoreceptors by selective M_2 antagonists such as gallamine potentiates airway bronchoconstriction to vagal stimulation by as much as ten-fold. This potentiation is not mediated via the post-junctional M_3 receptors since the airway response to exogenous acetylcholine is not increased [2]. Conversely, stimulating inhibitory M_2 receptors using pilocarpine decreases the airway response to vagal stimulation by as much as 80%.

In asthmatics, neuronal M_2 receptors in the lung do not function normally. Inhaled pilocarpine, which in non-asthmatics attenuates vagally-mediated reflex bronchoconstriction, fails to suppress reflex bronchoconstriction in asthmatics [6]. This loss of inhibitory M_2 receptors may be an important mechanism for the accentuated reflex bronchoconstriction characteristic of asthma.

Animal models of hyperresponsiveness are also characterized by increased vagally-mediated reflex bronchoconstriction. The role of M_2 receptor dysfunction during acute viral infections, and following antigen inhalation and ozone exposure has only recently been elucidated. Similarities between these conditions and asthma may lend insight into the possible mechanisms of M_2 receptor dysfunction in asthma.

Virus-induced M_2 receptor dysfunction and neuraminidase

Viral infections of the airways exacerbate both asthma and chronic obstructive airway disease. Furthermore, in previously normal individuals, viral infections cause temporary airway hyperresponsiveness [7]. The temporary hyperresponsiveness to histamine in virus-infected airways can be blocked by atropine in humans [7] and by vagotomy in guinea pigs [8]. Thus, virus-induced hyperresponsiveness is reflex-mediated via the vagus nerves. The efferent, parasympathetic, limb of the reflex arc is affected since airway smooth muscle contraction in response to electrical stimulation of the vagus is potentiated during viral infections [8].

It now appears that loss of inhibitory M_2 receptor function accounts for the increased airway smooth muscle response to vagal stimulation in virus-infected guinea pigs. As noted above, in normal guinea pigs blocking M_2 receptors with gallamine potentiates vagally-induced bronchoconstriction, while stimulating the M_2 receptors with pilocarpine suppresses vagally-induced bronchoconstriction. When guinea pigs were infected with parainfluenza type 3, the ability of pilocarpine to suppress the response to vagal stimulation was almost entirely lost. Gallamine-induced potentiation of the response to vagal stimulation was also markedly decreased [9] (figure 2).

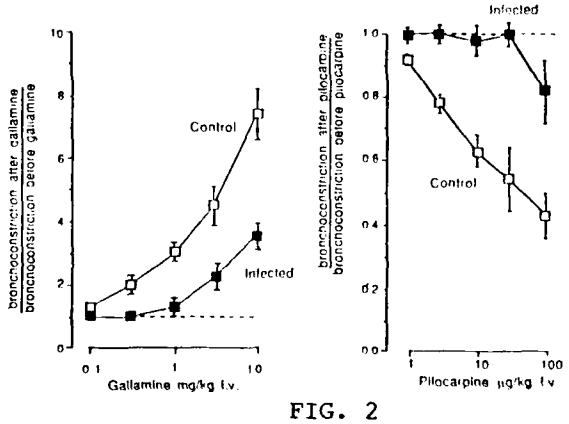

FIG. 2
Viral infection damages M_2 receptors in guinea pig airways. (From Ref. 9).

While the mechanism for loss of virus-induced M_2 receptor function cannot be stated with certainty, one possibility is that it is due to the presence in parainfluenza viruses of neuraminidase. This enzyme cleaves sialic acid residues from glycoproteins and glycolipids. Exposing M_2 receptors to parainfluenza virus *in vitro* has no effect on receptor number or ligand affinity, but markedly decreases agonist affinity (~tenfold increase in K_D for carbachol). This effect can be mimicked by an equivalent concentration of Clostridium perfringens neuraminidase, whereas the neuraminidase inhibitor 2,3-dehydro-2-deoxy-N-acetylneuraminic acid blocks the effect of parainfluenza virus on M_2 receptors [10].

Although the effect of *in vivo* infections with parainfluenza virus on M_2 receptor agonist affinity has not yet been investigated, it is possible that the cleavage of sialic acid residues from the receptor or from other components of the cell membrane decreases the ability of acetylcholine to bind to the receptor and inhibit further release of acetylcholine. This would explain the increased reflex bronchoconstriction characteristic of viral airway infections.

Ozone-induced M_2 receptor dysfunction and inflammatory cells

Exposure to high concentrations of ozone (2-3 parts per million) increases vagally-induced bronchoconstriction *in vitro* [11,12]. This is also associated with loss of M_2 receptor function, since the potentiation and inhibition of vagally-induced bronchoconstriction by gallamine and pilocarpine, respectively, are greatly attenuated following acute ozone exposure [13].

Although it has been recognized for some time that inhalation of ozone causes airway inflammation [14,15], the role of inflammation in causing airway hyperresponsiveness has been debated [16]. In the case of ozone-induced M_2 receptor dysfunction in guinea pigs, the time course of M_2 receptor damage and recovery closely parallels the development and resolution of airway inflammation [13]. M_2 receptor dysfunction develops immediately upon ozone exposure, and resolves (along with inflammation) by 14 weeks after exposure. Furthermore, in guinea pigs that were treated with cyclophosphamide to deplete inflammatory cells, M_2 receptor function was preserved after exposure to ozone [17]. This suggests that inflammatory cells or mediators are important in the pathogenesis of the loss of M_2 receptor function.

Thus airway inflammation, which is present after both ozone exposure and viral infection, may contribute to the pathogenesis of acute M_2 receptor dysfunction. While it is intriguing to note that neuraminidase is present in inflammatory cells as well as in viruses [18,19] another possible mechanism by which inflammation may acutely damage the M_2 receptor is described further below.

Inflammatory cells not only acutely alter neuronal M_2 receptor function, but there is also evidence that they cause long term alterations in receptors. Indomethacin potentiates vagally-induced bronchoconstriction and inhibits M_2 receptor function in guinea pigs. Thus, the function of M_2 receptors in the lung is dependent on cyclooxygenase, and presumably on prostaglandins [20]. However, in pathogen-free guinea pigs the function of M_2 receptors is completely independent of prostaglandin production [21]. The difference between pathogen-free and normal guinea pigs (which have been exposed to environmental pathogens) is probably the occurrence of an inflammatory response in the airways of normal animals. Pathogen-free guinea pigs that were exposed to ozone 8 weeks previously have M_2 receptors that have been converted and are now dependent upon cyclooxygenase. Thus, the presence of an inflammatory response in the lungs has altered the dependence of the M_2 receptors in the lungs on prostaglandins.

Antigen-induced M_2 receptor dysfunction and eosinophil proteins.

Exposure to antigen increases the magnitude of the excitatory post-junctional potentials in the airway smooth muscle [22], as well as acetylcholine release from the parasympathetic nerves in the lungs [23]. This finding prompted studies of the effects of antigen exposure on airway M_2 receptor function. Guinea pigs were sensitized to ovalbumin by intraperitoneal injection. Three weeks later they were exposed to an aerosol of ovalbumin for five minutes daily on four consecutive days. Testing the effects of gallamine and pilocarpine on the airway response to vagal stimulation in these animals showed that, as with viral infection and ozone exposure, the function of the neuronal M_2 receptors was largely lost.[24].

Because antigen inhalation causes airway inflammation that is composed largely of eosinophils, it appeared possible that a product of eosinophils might be responsible for M_2 receptor dysfunction. Such a hypothesis was also attractive because of the presence of eosinophilic inflammation in the airways of asthmatics [25].

It has been noted that M_2 muscarinic receptor antagonists (e.g., gallamine) are frequently positively charged. Several positively charged proteins, including basic histone, protamine, poly-l-arginine, and poly-l-lysine, are also M_2 receptor antagonists [26]. Therefore, the possibility that the positively charged eosinophil proteins, major basic protein, eosinophil peroxidase, and eosinophil cationic protein, might be acting as endogenous M_2 receptor antagonists was investigated. These three eosinophil proteins, as well as the more neutrally-charged eosinophil-derived neurotoxin, were tested for their ability to displace ^{3}H-N-methylscopolamine (^{3}H-NMS) from M_2 and M_3 receptors. Eosinophil major basic protein displaced ^{3}H-NMS from M_2, but not M_3, receptors with a K_I of ~10^{-5}M [27]. This is only a minimally higher concentration than that found in the sputum of patients with acute asthma [28]. Since tissue concentrations would be expected to be higher than sputum concentration, this finding may be physiologically relevant. Eosinophil peroxidase also displaced ^{3}H-NMS from M_2 receptors but not from M_3 receptors, although it was less potent than major basic protein. Neither eosinophil cationic protein nor eosinophil-derived neurotoxin affected ligand binding to either M_2 or M_3 receptors.

Further studies were undertaken to determine the mechanism of the interaction of major basic protein with the M_2 receptor. M_2 receptor preparations were incubated to equilibrium with ^{3}H-NMS, and then allowed to dissociate in the presence of 2 μM atropine for five minutes. This led to dissociation of ~25% of specifically bound ^{3}H-NMS. However, when major basic protein was added at the same time as the atropine, the rate of dissociation was markedly slowed. This ability to decrease the dissociation rate for the ligand at M_2 receptors demonstrates that the interaction of major basic protein with the M_2 receptor is allosteric.

The reversibility of major basic protein binding to the M_2 receptor was also tested. Major basic protein and ^{3}H-NMS were incubated to equilibrium with M_2 receptors. Heparin, which as a poly-anionic polysaccharide binds and neutralizes major basic protein [29], was then added to the incubation mixture. It was possible in this way to reverse completely the effect of major basic protein on ligand binding to M_2 receptors.

Thus eosinophil major basic protein is a selective allosteric antagonist at the M_2 receptor. While the strong positive charge of this protein is likely to be important in its interaction with the M_2 receptor, positive charge alone is not sufficient to explain this interaction, as eosinophil cationic protein is not an M_2 receptor antagonist.

Although it has not been definitively demonstrated that major basic protein causes M_2 receptor dysfunction *in vitro*, several experiments suggest that major basic protein, or another positively charged protein, may be responsible for M_2 receptor dysfunction in antigen challenged guinea pigs. The effects of the negatively charged substances heparin and poly-l-glutamate, which bind and neutralize major basic protein, on the airway response to vagal stimulation were tested. In normal guinea pigs, neither heparin nor poly-l-glutamate affect the response to electrical stimulation of the vagus. In contrast, in antigen-challenged guinea pigs, both heparin and poly-l-glutamate cause a decrease in the airway response to vagal stimulation, so that after 30 minutes the response was only 50% of the original response [30] (figure 3).

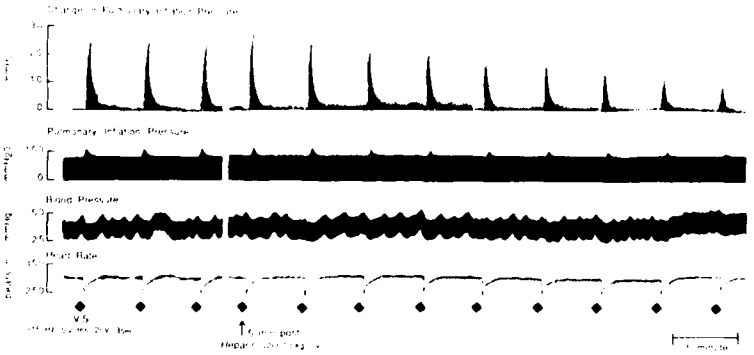

FIG. 3
Heparin decreases vagally-induced bronchoconstriction in antigen-challenged guinea pigs. (From Ref. 30).

After treatment with heparin or poly-l-glutamate, the ability of pilocarpine to suppress the airway response to vagal stimulation, which had been lost in antigen-challenged guinea pigs, was completely restored, as was the ability of gallamine to potentiate vagally-induced bronchoconstriction [30] (figure 4).

Both the decreased bronchoconstriction to vagal stimulation, and the ability of pilocarpine and gallamine to manipulate vagally-induced bronchoconstriction after heparin or poly-l-glutamate, demonstrates recovery of neuronal M_2 receptor function.

These data are consistent with the hypothesis that M_2 receptor dysfunction is due to the interaction of a positively charged substance, such as eosinophil major basic protein, with the neuronal M_2 autoreceptor.

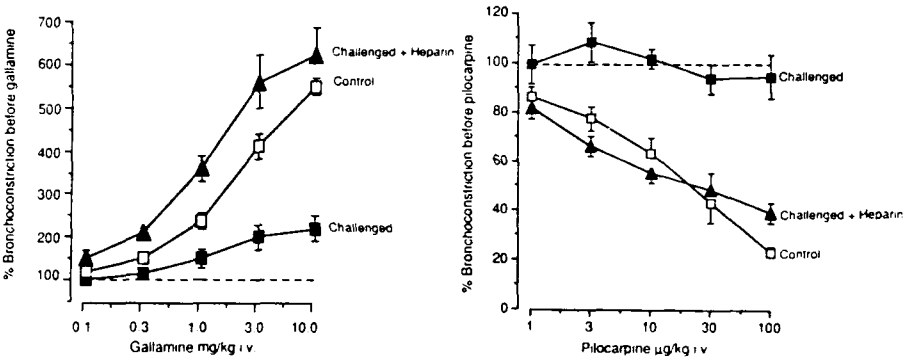

FIG 4
Antigen challenge attenuates M_2 receptors in guinea pig airways. This attenuation is reversed by heparin. (From Ref. 30).

Conclusions

Neuronal muscarinic M_2 receptors exert an important inhibitory control on the parasympathetic nerves supplying airway smooth muscle. Loss of M_2 receptor function leads to increased vagally-mediated bronchoconstriction after viral infections, ozone exposure, and antigen inhalation, and may also be responsible for the enhanced reflex bronchoconstriction characteristic of asthma.

Cleavage of sialic acid residues by viral neuraminidase decreases the agonist affinity of M_2 receptors, and this may lead to M_2 receptor dysfunction after viral infections. Alternatively, airway inflammation, which appears to be a sine qua non for loss of M_2 receptor function after ozone exposure, may also contribute to the effects of viral infections.

Eosinophilic airway inflammation, as seen in asthmatic airways and after antigen challenge, may directly impair the function of neuronal M_2 receptors in the lungs. Positively charged proteins, especially major basic protein, may act as selective allosteric antagonists at the M_2 receptors. This may account for the ability of the negatively charged molecules heparin and poly-l-glutamate to reverse the effects of antigen challenge. Greater understanding of these, and other, mechanisms of M_2 receptor dysfunction may lead to the development of new therapeutic strategies in asthma.

References

1. A.F. ROFFEL, C.R.S. ELZINGA AND J. ZAAGSMA, Pulmon. Pharmacol. 3 47-51. (1990).
2. A.D. FRYER AND J. MACLAGAN, Br. J. Pharmacol. 83 973-978 (1984).

3. L.C. BLABER, A.D. FRYER AND J. MACLAGAN, Br. J. Pharmacol. 86 723-728 (1985).
4. Y. ITO AND T. YOSHITOMI, Br. J. Pharmacol. 93 636-646 (1988).
5. P. MINETTE AND P.J. BARNES, J. Appl. Physiol. 64 2532-2537 (1988).
6. P.J. MINETTE, J.W.J. LAMMERS, C.M.S. DIXON, M.T. MCCUSKER AND P.J. BARNES, J Appl Physiol 67 2461-2465 (1989).
7. D.W. EMPEY, L.A. LAITINEN, L. JACOBS, W.M. GOLD AND J.A. NADEL, Am. Rev. Respir. Dis. 113 523-527 (1976).
8. C.K. BUCKNER, S. V., E.C. DICK AND W.W. BUSSE, Am. Rev. Respir. Dis. 132 305-310 (1985).
9. A.D. FRYER AND D.B. JACOBY, Br J Pharmacol 102 267-271 (1991).
10. A.D. FRYER, E.E. EL-FAKAHANY AND D.B. JACOBY, Eur J Pharmacol 181 51-58 (1990).
11. E.H. WALTERS, P.M. O'BYRNE, P.D. GRAF, L.M. FABBRI AND J.A. NADEL, Clin. Sci. 71 605-611 (1986).
12. G.L. JONES, C.G. LANE, P.J. MANNING AND P.M. O'BYRNE, J. Appl. Physiol. 63 1174-1179 (1987).
13. A. SCHULTHEIS, J.P. BASSETT AND A.D. FRYER, Br J Pharmacol 103 291P (1991).
14. M.J. HOLTZMAN, L.M. FABBRI, P.M. O'BYRNE, B.D. GOLD, H. AIZAWA, E.H. WALTERS, S.E. ALPERT AND J.A. NADEL, Am. Rev. Respir. Dis. 127 686-690 (1983).
15. D.J.P. BASSETT, E. BOWEN-KELLY, E.L. BREWSTER, C.L. ELBON, S.S. REICHENBAUGH, T. BUNTON AND J.S. KERR, Lung 166 355-369. (1988).
16. C.G. MURLAS AND J.H. ROUM, Am. Rev. Respir. Dis. 131 314-320. (1985).
17. L.M. GAMBONE AND A.D. FRYER, Am. Rev. Respir. Dis. 145 A614 (1992).
18. S. MATSUZAKI, T. ITAKURA, T. KADOSKA, H. KAMIGUCHI, M. YAMMAMURA AND T. KATSUNUMA, Alcohol Alcoholism Suppl 1 509-511 (1987).
18. Y. PILATTE, J. BIGNON AND C.R. LAMBRE, Biochem. Biophys. Acta 923 150-155.
20. A.D. FRYER AND O.A. OKANLAMI Am. Rev. Respir. Dis. (1993, In Press).
21. O.A. OKANLAMI, C.A. HIRSHMAN, A.D. FRYER, Am. Rev. Respir. Dis. 145 A437 (1992).
22. D.J. MCCAIG, Br. J. Pharmacol. 92 809-816 (1987).
23. T.M. FAME, H. RENZ, J.E. LOADER, J. GRAVES, E.W. GELFAND AND G.L. LARSEN, Am. Rev. Respir. Dis. 145 A464 (1992).
24. A.D. FRYER AND M. WILLS-KARP, J. Appl. Physiol. 71 2255-2261 (1991).
25. S.T. HOLGATE, J.R. WILSON AND P.H. HOWARTH, Am. Rev. Respir. Dis. 145 S2-S6 (1992).
26. J. HU, S. WANG, C. FORRAY AND E.E. EL-FAKAHANY, Mol. Pharmacol. 42 311-324 (1992).
27. D.B. JACOBY, G.J. GLEICH AND A.D. FRYER, Am. Rev. Respir. Dis. 145 A436 (1992).
28. E. FRIGAS, D.A. LOEGERING, G.O. SOLLEY, G.M. FARROW AND G.J. GLEICH, Mayo Clin Proc 56 345-353 (1981).
29. E. FRIGAS, D.A. LOEGERING AND G.J. GLEICH, Lab Invest 42 35-43 (1980).
30. A.D. FRYER AND D.B. JACOBY, J. Clin. Invest. (1992,In Press).

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BA 679 BR, A NOVEL LONG-ACTING ANTICHOLINERGIC BRONCHODILATOR

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Summary

The use of anticholinergics in antiobstructive therapy is well established in pulmonary medicine. We sought to improve the duration of action of inhaled antimuscarinics. A newly developed compound, Ba 679 BR (abbreviated Ba 679) proved to be a highly potent muscarinic antagonist in guinea pig tracheal rings. Its binding to human receptors (Hm1, Hm2, Hm3) was characterized by K_D-values in the 10^{-10} M concentration range. Assessment of the dissociation rate of complexes of labelled Ba 679 and human muscarinic receptors revealed very slow dissociation in comparison to ipratropium. The half-lives in hours were: Ba 679-Hm3: 34.7, -Hm1: 14.6, -Hm2: 3.6; ipratropium-Hm3: 0.26, -Hm1: 0.11, -Hm2: 0.035. The duration of action in vivo was determined by means of acetylcholine-induced bronchospasms in dogs following inhalation of the drugs. Ba 679 demonstrated a significantly longer duration of protection than an equipotent dose of ipratropium. The plasma levels following inhalation in dogs declined rapidly and are unlikely to reflect the duration of the pharmacological activity.

In summary, Ba 679 represents a novel type of antimuscarinic bronchodilator with a long duration of action, most likely due to its slow dissociation from Hm3-receptors. In addition, the drug showed "kinetic receptor subtype selectivity" by having a more rapid dissociation from Hm2 than from Hm1 and Hm3 receptors.

The role of the vagus in controlling bronchial tone has recently been highlighted by a study in patients with nocturnal asthma which showed the high efficacy of atropine in inducing a significant increase in peak expiratory flow rate (PEFR) in the early morning (1). The introduction of quaternary congeners of atropine and topical administration by inhalation instead of systemic use significantly improved the therapeutic index of antiobstructive antimuscarinic therapy (2, 3). The duration of action of currently available antimuscarinic bronchodilators is 6 h or less (4, 5). Therefore, we sought to find an antimuscarinic bronchodilator for inhalation with a duration of action of 12 h or more.

Materials and Methods

Ba 679 BR, proposed international non-proprietary name tiotropium bromide, [7(S)-(1α,2β,4β,5α,7β)]-7-[(hydroxydi-(2-thie-

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nyl)acetyl)oxy]-9,9-dimethyl-3-oxa-9-azoniatricyclo[3.3.1.$O^{2,4}$]nonane bromide was synthesized in our laboratories by R. Banholzer.

Isolated guinea pig trachea. Tracheal segments were prepared as described (6) with the following modifications: isotonic force measurement, preload of 2 g. Methacholine concentration-effect-curves were determined before and after equilibration for 30 min with the antimuscarinic drug. Individual EC_{50}-values were determined and analysed according to Arunlakshana and Schild (7).

Isolated guinea-pig ciliated cells. The beat frequency of superfused isolated ciliated cells in primary culture was determined by a modification of our photometric method (8). The beat frequency of each cell was expressed in % of its own baseline. The responsiveness to 10^{-5} M methacholine was assessed first (near maximum stimulation). Then the cells were equilibrated with the antimuscarinic agent for 30 min in a concentration known to completely block the response to 10^{-5} M methacholine. The superfusion was switched to cell culture medium containing 10^{-5} M methacholine, but not the antimuscarinic agent and the frequency measured at approximately 15 min intervals.

Human muscarinic receptor studies. Assays were carried out at 23° C. We used membrane preparations from stably transfected chinese hamster ovary-K1 cells (CHO) expressing the genes for the human muscarinic receptors Hm1, Hm2, Hm3.

For determination of K_D values, membrane preparations were suspended in tris-buffer to a final concentration of 80, 200 or 88 µg/ml for the subtypes Hm1 to Hm3, respectively. The membrane suspension was incubated with the tritiated compound for 4 h (Ba 679) or 0.5 h (ipratropium, Hm2) or 1 h (ipratropium, Hm1 and Hm3). These incubation times had been shown in preliminary studies to be necessary for binding to reach near saturation values. After incubation the membrane fraction was separated by filtration and the bound radioactivity determined. Nonspecific binding was determined by addition of 10^{-5} M atropine. The apparent K_D was derived from the law of mass action, describing the corresponding binding equation of receptor and ligand.

The dissociation kinetics were determined in separate parallel studies. After equilibration of the membrane preparations with 5 x 10^{-10} M ^{3}H-Ba 679 for 2 hours or 10^{-9} M ^{3}H-ipratropium for 1 h the dissociation was initiated by the addition of 10^{-5} M unlabelled atropine and the off-kinetics measured for up to 60 h. The dissociation half-life was estimated from the log-linear plot.

Bronchospasmolysis in anaesthetized dogs. The studies were essentially performed as described previously (9). Aqueous solutions of the agents to be tested were nebulized and inhaled by anaesthetized ventilated dogs (10). The bronchial response to intravenous acetylcholine challenge was determined before and after drug administration and the percent change of a measure of pulmonary resistance calculated. Changes of cardiovascular parameters were also calculated. A separate experiment using a recent modification of this method was used to determine the absolute inhaled dose: 15 µl solution of the test substance was nebulized by ultrasonic energy with the piezo-electric nebulizer Respimat® directly into the orotracheal tube during inspiration.

Heart rate in conscious dogs. 4 trained male beagle dogs, 13 to 16 kg, participated in a crossover-study to assess the cardiac effects of 10 µg/kg intravenous ipratropium in comparison to 5 µg/kg Ba 679. The heart rate was monitored by ECG (limb lead II). The time required to reach 50 % of the maximal effect was determined for each animal and expressed as median and range.

Pharmacokinetic studies in dogs. 18 beagle dogs participated in an inhalational 13 week toxicity study. 3 male and 3 female animals were used for each dose group. Blood was drawn after 1, 6 and 13 weeks and analysed by radio receptor assay. The displacement of ^{3}H-N-methylscopolamine from muscarinic receptors in the membranes of rat brain was used to determine "active" Ba 679. By means of a regression model using a logarithmic transformation the predicted mean concentrations were calculated.

Results

In vitro studies. Ba 679 in concentrations ranging from 6×10^{-10} to 6×10^{-9} M shifted the concentration-effect-curve of methacholine in guinea pig trachea dose-dependently to the right. Similar results were obtained for ipratropium, atropine and glycopyrrolate. Fig. 1 and table I show the Schild-analysis for the 4 antagonists and the corresponding calculated parameters. Ba 679 was the most potent compound.

TABLE I

Schild Analysis of the Antagonism against Methacholine in Isolated Tracheal Rings of Guinea-Pigs.

Substance	abscissa intercept	n	slope
Ba 679 BR	9.51 (9.42 - 9.62)	58	-2.53 (-2.79 to -2.27)
glycopyrrolate	9.41 (9.23 - 9.59)	24	-1.21 (-1.45 to -0.97)
ipratropium bromide	9.37 (9.19 - 9.61)	32	-1.32 (-1.53 to -1.11)
atropine	9.17 (9.06 - 9.31)	21	-1.16 (-1.34 to -0.99)

Parameters with their 95 % confidence intervals.

The washout of antagonists from the muscarinic receptor of ciliated cells from guinea pig trachea is shown in fig. 2. Stimulation of the beat frequency was seen at the first measurement 16 min after switching from ipratropium (10^{-6} M) to the washout medium containing methacholine. After incubation with Ba 679 (10^{-7} M) a comparable degree of stimulation by methacholine was not visible until 82 minutes of the washout period had elapsed.

The apparent K_D-values of Ba 679 and ipratropium for the human muscarinic receptor subtypes were similar (table II). Table III summarizes the results of the dissociation experiments. The

dissociation of Ba 679 - muscarinic receptor complexes is more than two orders of magnitude slower than that of ipratropium -complexes. The ranking of dissociation half times for both compounds was Hm3 > Hm1 > Hm2.

TABLE II

Apparent K_D values of Ba 679 and Ipratropium for the Hm1, Hm2 and Hm3 Muscarinic Receptor Subtypes.

	Hm1 [nM]	Hm2 [nM]	Hm3 [nM]
ipratropium	0.43 ± 0.03 (3)	0.54 ± 0.07 (3)	0.69 ± 0.31 (4)
Ba 679	0.27 ± 0.21 (6)	0.12 ± 0.09 (6)	0.33 ± 0.26 (6)

K_D ± standard deviation, ( ) = number of experiments.

TABLE III

Dissociation Half-Lives of Receptor-Drug Complexes.

	Hm1 [h]	Hm2 [h]	Hm3 [h]
^{3}H-ipratropium iodide	0.11± 0.005 (3)	0.035 ± 0.005 (4)	0.26 ± 0.02 (3)
^{3}H-Ba 679 iodide	14.6 ± 2.2 (5)	3.6 ± 0.5 (4)	34.7 ± 2.9 (4)

Half-life ± standard deviation, ( ) = number of triplicate experiments. Association was achieved by equilibration of the membranes with 10^{-9} M ^{3}H-ipratropium for 1 h or 5 x 10^{-10} M ^{3}H-Ba 679 for 2 h. The half-lives are derived from dissociation reactions initiated by addition of 10^{-5} M atropine.

In vivo studies. The effect of inhaled antimuscarinic agents was tested by their ability to protect against acetylcholine induced bronchospasms in anaesthetized dogs. Fig. 3B shows that inhalation of an aqueous aerosol generated from 1.0 g/l Ba 679-solution was 100 % protective. Ipratropium required 3 times higher concentrations to provide a similar degree of protection (efficacy of 1 g/l ipratropium compared to 0.3 g/l Ba 679). The time elapsed for maximal protection to decline to half maximal was dose-dependent. For inhaled ipratropium this value increased from 19 to 107 min with increasing dose; for 0.1 g/l inhaled Ba 679 the value was 236 min. With the two higher concentrations of Ba 679 the duration exceeded the 6 h observation period. The most sensitive cardiovascular parameter, the rate of rise of left ventricular pressure (dp/dt_{max}), did not change following inhalation of Ba 679 or ipratropium (data not shown). An absolute dose for inhaled Ba 679 was determined in a separate protocol. 10 µg of Ba 679 produced 71 ± 3 % (mean ± SEM, n = 6) inhibition of acetylcholine-induced increase in resistance in dogs (mean body weight 27.7 ± 1.12 kg). 20 µg ipratropium was similarly effective (inhibition: 70 ± 7 %, n = 6).

Effects on cardiovascular parameters may only be seen following inhalation of extremely high concentrations. We studied these effects after intravenous administration in conscious dogs. Fig. 4 shows that the heart rate was significantly elevated after injection of 10 µg/kg ipratropium or 5 µg/kg Ba 679. The time elapsed before the maximal chronotropic effect declined to 50 % was 53 min (median, range: 30 - 60 min) for ipratropium and 90 min (75 - 180 min) for Ba 679.

The plasma level of Ba 679 following inhalation was determined at 3 dose levels in 6 dogs each after 1, 6 and 13 weeks of repeated administration. Analysis of variance did not yield statistically significant differences between the phases of the study and between animals of different sexes. Mean values are shown in fig. 5. Following inhalation of 1235 µg/kg the peak plasma levels rose to 23.8 ng/ml, after 100 µg/kg to 1.34 ng/ml, and were below the detection limit following 10 µg/kg. With a half-life in the range of 1 h the plasma concentration decreased to 1.15 ng/ml 6 h after inhalation of the high dose. Dose linearity of the achieved plasma levels could be demonstrated.

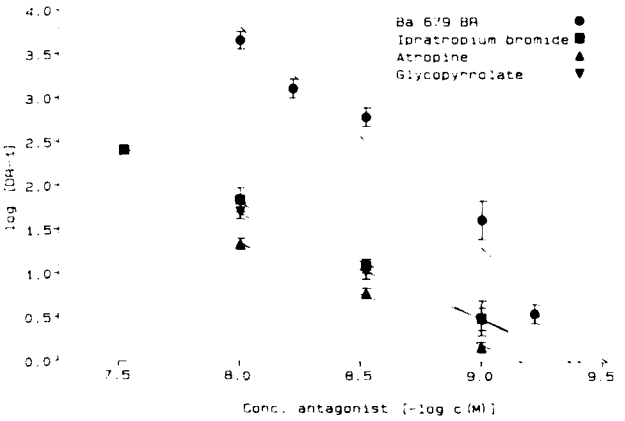

Fig. 1. Schild-plot: antagonism of anti-muscarinic drugs against methacholine in isolated tracheal preparations of guinea pigs.

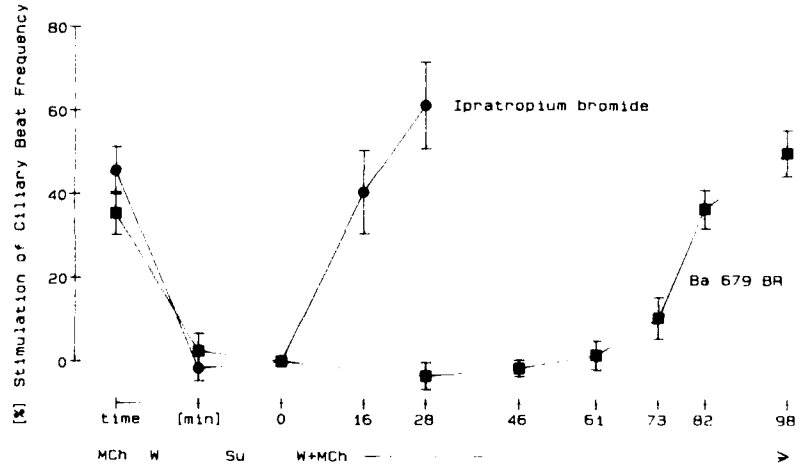

Fig. 2. Washout of ipratropium and Ba 679 from superfused isolated ciliated cells from guinea pig trachea. Means ± SEM, n = 8. MCh = 10^{-5} M methacholine. Su: ● = 10^{-6} M ipratropium, ■ = 10^{-7} M Ba 679. W = washout period.

Discussion

The time/response-curves for bronchodilation in dogs following inhalation of ipratropium (fig. 3A) show that the possibility of prolonging its duration of action by increasing the dose is limited. Increasing the dose by a factor of ten improved the bronchoprotection from 58 to 100 % but only extended the time elapsed for maximal protection to decline to half maximal from 78 to 107 min.

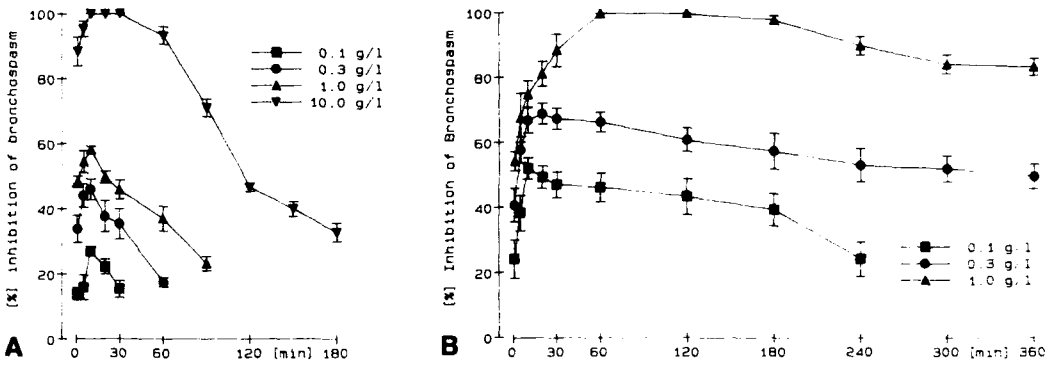

Fig. 3. Time course of the protection against acetylcholine induced bronchospasms by inhaled ipratropium bromide (A) or Ba 679 (B). Means ± SEM, n = 6 dogs for each concentration.

The major structural difference between ipratropium and Ba 679 is the thiophene rings of the latter which allow rapid systemic metabolism. As expected, pharmacokinetic studies in dogs following inhalation showed that the plasma half-life of Ba 679 is in the range of 1 h (fig. 5). The antagonism of Ba 679 or ipratropium against methacholine in guinea-pig isolated tracheal preparations revealed that the slope for Ba 679 was significantly greater than unity (Schild-plot, table I and fig. 1). This finding suggests that equilibrium could not be achieved despite incubation times of 30 min. And indeed slow binding kinetics were found in studies of the receptor off-kinetics (table III): ipratropium dissociated from muscarinic receptors in a few minutes, whereas Ba 679 needed several hours. The washout of Ba 679 from guinea-pig ciliated cells needed 5 times longer than that of ipratropium and confirmed that dissociation kinetics may control the duration of the effect (fig. 2). Nevertheless, the blockade by Ba 679 proved to be reversible.

The duration of antimuscarinic blockade in vivo was investigated in dogs following inhalation of the test compounds. Ba 679 exhibited a long duration of protection against acetylcholine induced bronchospasms (fig. 3B), even at doses below the plateau of the dose-response relationship. This new compound does not need high doses exceeding the plateau of the pharmacological effect to ensure a long duration of action.

The following data and calculation support the view that the long duration of action in vivo may indeed be controlled by receptor dissociation kinetics and not by plasma levels. Six hours after inhalation of the toxicological dose of 1235 µg/kg in dogs, plasma levels of 1.15 ng/ml were found (fig. 5). Assuming dose linearity, an inhaled pharmacological dose of 0.36 µg/kg (calculated from 10 µg effective inhaled dose in dogs of 27.7 kg mean body weight) after 6 h leads to an estimate of the plasma level in the range of 0.3 pg/ml or 7×10^{-13} M. As the K_D for Hm3-receptors (3×10^{-10} M) is 400 times higher, it can be concluded that only a minimal receptor occupancy would result from the plasma levels at 6 h.

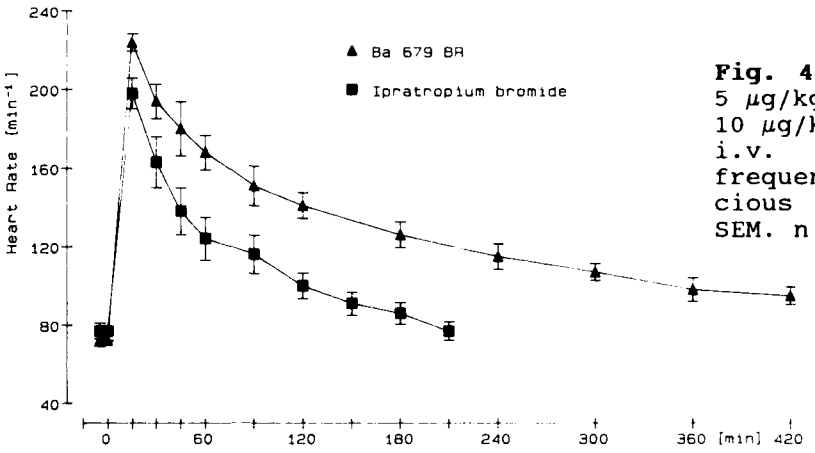

Fig. 4. Influence of 5 µg/kg Ba 679 or 10 µg/kg ipratropium i.v. on the heart frequency of conscious dogs. Means ± SEM. n = 4.

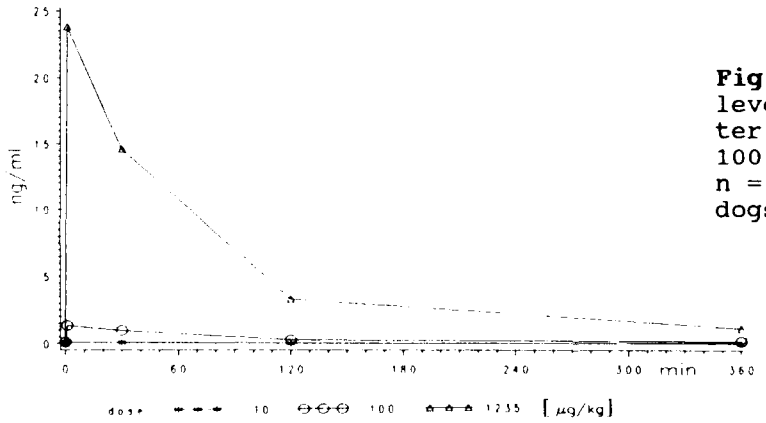

Fig. 5. Mean plasma levels of Ba 679 after inhalation of 10, 100 or 1235 µg/kg/d. n = 3 male + 3 female dogs for each dose.

The apparent K_D-values of Ba 679 and ipratropium for the subtypes of human muscarinic receptors suggest that they are not subtype selective (table II). Interestingly, the dissociation half-lives for the subtypes reveal important differences (table III). For both compounds the dissociation half-life increases in the following order and ratio: Hm2 (1): Hm1 (3.5): Hm3 (8). Because the dissociation rate of ipratropium from all subtypes is in a range of minutes it is unlikely that a kind of "kinetic receptor-subtype selectivity" is found with this compound in vivo. In contrast, the dissociation rate of Ba 679 from muscarinic receptors is sufficiently slow to have a major impact on the in vivo duration of action.

When the durations of the in vivo effects of Ba 679 are compared, it is evident that the decay of the M_2-blockade mediated positive chronotropic effect is faster than the decline of the inhibition of the M_3-mediated bronchoconstriction: in the heart the half-maximal effect level is reached after 1.5 h, in the airways the time needed clearly exceeds 6 h. These findings are in line with the much faster in vitro dissociation rate of Ba 679 from Hm2- than from Hm3-receptors and lend further support to the hypothesis that it is the dissociation process of the Ba 679-receptor complexes which determines the duration of its antagonistic actions in vivo.

Ba 679 was investigated in single and repeated dose toxicity studies and revealed no adverse effects other than the sequelae of systemic antimuscarinic blockade. Healthy human volunteers tolerated the substance well. A clinical pilot study of Maessen and colleagues (11) in 6 patients with chronic obstructive pulmonary disease revealed significant bronchodilation 10 h after dosing 10 - 40 µg by inhalation. The FEV_1-value returned to baseline approximately 19 h after dosing.

In conclusion, Ba 679 represents a novel type of antimuscarinic bronchodilator with a "receptor mediated" long duration of action, very likely controlled by its slow dissociation from Hm3-receptors. In addition the drug may show "kinetic receptor subtype selectivity" Hm3-/Hm1- over Hm2-receptors, a phenomenon which may offer improved efficacy and safety over existing therapy. Ba 679 may prove useful as a therapeutic agent for once or twice-a-day administration.

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References

1. J.F.J. MORRISON, S.B. PEARSON and H.G. DEAN, Br. Med. J. 296 1427-1429 (1988).
2. A. ENGELHARDT and H. KLUPP, Postgrad. Med. J. 51 Suppl. 7 82-85 (1975).
3. H.J. FÖRSTER, I. KRAMER, K.H. POOK and D. WAHL, Arzneim.-Forsch./Drug Res. 26 989-992 (1976).
4. N.J. GROSS, Am. Rev. Respir. Dis. 112 823-828 (1975).
5. G.M. STERLING, Postgrad. Med. J. 63 Suppl. 1 41-46 (1987).
6. G. GAI, J.E. FRANCIS, M. WILLIAMS, R.A. DOTSON, M.F. HOPKINS, D.T. COTE, F.R. GOODMAN and M.B. ZIMMERMAN, J. Pharmacol. Exper. Ther. 242 784-790 (1987).
7. O. ARUNLAKSHANA and O. SCHILD, Brit. J. Pharmacol. 14 48-58 (1959).
8. B. DISSE and H. ZIEGLER, Respiration 51 Supl.1 15-22 (1987).
9. W. TRAUNECKER and G.MUACEVIC, Respiration 50 244-251 (1986).
10. R: BAUER, Arzneim.-Forsch./Drug Res. 26, 531-533 (1976)
11. F.P.V. MAESEN, J.J. SMEETS, M.A.L. COSTONGS, P.J.G. CORNELISSEN and F.D.M. WALD, Europ.Resp. J. 5 Suppl.15 P0671 (1992).

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SUMMARY AND CLOSING COMMENTS

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This volume is dedicated to the late Frank C. (Hank) MacIntosh, whose closing comments at the first Symposium on Subtypes of Muscarinic Receptors in 1983 (1) reviewed with characteristic insight the evolution of this topic during the previous three decades, and pointed to the directions that future research would probably take. His comments about the future have proven to be remarkably accurate.

At that time it was not universally agreed that the muscarinic receptor subtypes were discrete molecular entities and not interchangeable. But Sir Arnold Burgen (2) was optimistic enough to hope that the receptor subtypes would be sequenced within a year or two. It has, of course, been clearly established now that there are at least five subtypes, all of which have been cloned and sequenced; their distribution and coupling mechanisms have been largely worked out, and moderately specific ligands are available for some. Therapeutic applications of this new knowledge have inevitably lagged behind basic research; the great goal of advancing the therapy of Alzheimer's disease seems not much closer, but as MacIntosh pointed out, "the chance of winning at this lottery may be small, but the prize is rich" (1).

The first session was devoted to a review of the molecular biology of muscarinic receptors. The five subtypes appear to be derived from a highly conserved gene family, with maximum homology in the five hydrophobic regions that are presumably transmembrane domains. Brann reviewed data collected using chimeric receptors for the identification of receptor regions involved in defining their selectivity for G-proteins and ligands; identification of amino acid residues involved in ligand interactions using point mutations and covalently binding ligands; and molecular modelling using alignments with bacteriorhodopsin. The regulation and expression of muscarinic receptors were discussed by Haga and by Nathanson. Toxins of natural origin have played a major role in facilitating the isolation and characterization of receptors, but until recently no toxin specific for the muscarinic receptor was known. Following the pioneering work of Karlsson (3), Potter now describes a new toxin from the venom of the African green mamba, *Dendroapsis augusticeps*, which uniquely identifies the extracellular face of genetically defined M_1 receptors, and should provide an invaluable tool for anatomical and functional studies.

The receptor subtypes differ not only in their molecular structure but in the mechanisms coupling them to cellular events, generally (if not always) mediated by G-proteins. These subjects were reviewed in the second session by Lazareno and by Jones. A particularly powerful approach to these questions depends upon the expression of

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individual genes encoding each subtype in isolation, so that their effects and mechanisms of action can be studied. Lazareno measured [^{35}S]-GTPγS binding and [γ^{32}P]-GTP hydrolysis in membranes from CHO cells stably transfected with human muscarinic receptor subtypes. They found that the stimulation induced by a battery of muscarinic agonists was greater in M_2 and M_4 than in M_1 and M_3, and that full agonists differentiated more than partial agonists. It appears that muscarinic receptor subtypes interact with more than one subtype of G-protein, since M_2 and M_4 receptors bind only to G-proteins that are sensitive to pertussis toxin, while M_1 and M_3 receptors bind to both sensitive and insensitive G-proteins. Jones reviewed the literature on coupling mechanisms activated by muscarinic receptor subtypes. M_2 and M_4 produce inhibition of adenyl cyclase, an effect that is mediated by a pertussis toxin-sensitive G-protein, while M_1, M_3 and M_5 activate phospholipase C, causing the release of IP3 and diacylglycerol, which in turn release intracellular Ca^{++} and activate protein kinases respectively, by mechanisms that are generally insensitive to pertussis toxin. Ion conductances can be modulated both directly by G-proteins and indirectly by second messengers. M_1, M_3 and M_5 subtypes often have effects opposite to M_2 and M_4 receptors on K^+ and Ca^+ conductances, but no firm generalizations can be made.

Somewhat less is known about the distribution and functions of the muscarinic receptors. Levey has prepared a panel of subtype-selective antibodies against divergent i3 loop-fusion proteins; each antibody was selective for a single cloned receptor. With these tools it was demonstrated that although the receptor subtypes were differentially distributed, most tissues, including brain, contained a complex mixture, with important differences in their histochemical localization.

The remainder of the symposium was concerned largely with drugs and their selectivity for muscarinic receptor subtypes: methods of characterization, ligand binding and functional effects, and finally their therapeutic applications. The expression of individual genes encoding each subtype in isolation has proven here also to be a valuable tool for the investigation of new compounds. Schwartz used recombinant CHO cells expressing human M_1 - M_5 receptors as well as functional studies on PI turnover (M_1, M_3 and M_5) and forskolin-stimulated cAMP accumulation (M_2 and M_4) to characterize both reference muscarinic agonists and a series of investigational compounds. Both full and partial agonists were found for PI turnover, but all agonists showed similar responses to cAMP. Alkylation studies with propylbenzilylcholine mustard showed that both efficacy and potency were markedly affected in the functional assays by the number of free receptors, indicating that receptor reserve plays a major role in determining agonist subtype selectivity using functional measures.

Ensinger also used CHO cells transfected with human M_1 and M_3 receptor genes to characterize the investigational compound WAL 2014. He reported that this compound is a selective agonist for M_1 receptors, both in transfected CHO cells and in intact tissues. On this basis, and because it produced EEG activation in intact rabbits, he concluded that it was a promising candidate for cholinergic substitution therapy in Alzheimer's disease.

Session V was devoted to selective muscarinic agents. Enhancement of cholinergic function in the central nervous system might be expected either from M_1 agonists, because of their postsynaptic actions, or M_2 antagonists, because it is widely believed that presynaptic autoreceptors inhibiting acetylcholine release are of the M_2 subtype, and antagonists would therefore be expected to increase the normal release of acetylcholine. The most selective compounds were generally partial agonists, i.e. had limited efficacy; there is precedent in

the literature for differential efficacy as a basis for subtype selectivity (4). The principal focus of the session was on drugs with these properties. McN-A-343 was one of the first compounds to point unequivocally to the existence of muscarinic receptor subtypes (5), and appears to be a relatively selective agonist for M_1 receptors. It is, however, a quaternary ammonium compound and therefore has no direct actions on the central nervous system when injected systemically. Lambrecht described the results of a program in which analogs of McN-A-343, including tertiary amines, were investigated to find candidate drugs capable of stimulating M_1 receptors in the central nervous system. Although it remains true that the most active compounds in this series are quaternary ammonium compounds, one tertiary amine (4-F-PyMcN) was described that was active in the micromolar range and might serve as a starting point for future research along these lines.

Freedman first described two very potent azanorbornyl derivatives containing an oxadiazole (L-670548) or a pyrazine (L-680648) group in the side chain. Using competitive binding ratios against NMS and oxotremorine-M as an indicator of efficacy, variations on these molecules were investigated that reduced the efficacy. This approach yielded compounds with greater functional selectivity among muscarinic receptor subtypes, retaining agonist activity on M_1 receptors in the rat superior cervical ganglion, but becoming partial agonists or antagonists on M_2 and M_3 receptors in the ileum and atrium. When tested in vivo, these compounds retained the ability to reverse scopolamine-induced memory deficits, but lacked the cardiovascular side effects associated with more efficacious agents. Tecle also reported on a series of bicyclic compounds, containing substituted oxime residues in the side chain. Using molecular modelling as a guide, it was found that quite large residues could be included in the sidechain without losing agonist activity; this can result in an increase in selectivity for M_1 as opposed to M_2, M_3 and M_4 subtypes when tested in vitro, and improvement in spatial memory without unwanted peripheral cholinergic activation.

Pilocarpine has long been recognized as a non-classical muscarinic agonist which, unlike most potent agonists, is a tertiary amine. Enz described the properties of the thiolactone analog of pilocarpine (SDZ ENS 163), which behaves as an agonist on M_1 receptors but an antagonist at M_2 receptors in the atrium and a partial agonist at M_3 receptors in the ileum. These in vitro properties are reflected in an increase in acetylcholine turnover in the brain which is characteristic of muscarinic antagonists, but an increase in PI turnover that is associated with M_1 agonists. These properties confirm the profile of an agonist at M_1 receptors and an antagonist at M_2 receptors, and make the compound an excellent candidate for the symptomatic treatment of Alzheimer's disease.

The alternative approach of designing a pure centrally active M_2 antagonist was described by Doods; this resulted in the discovery of a lipophilic analog of AQ-RA 741 (BIBN 99), which has a high degree of M_2/M_1 selectivity and as expected, improved performance of aging rats in the Morris maze. In summary, the results presented in this session confirmed the hypothesis that performance in memory tests can be improved in experimental animals by compounds with M_1 agonist properties and/or M_2 antagonist properties, and that these can often be found in the same muscarinic ligand when its efficacy is limited.

In the final session, Barnes described the distribution of muscarinic receptor subtypes in the lung. At least three subtypes play a role in the human lung: M_1 receptors facilitate neurotransmission through parasympathetic ganglia and enhance cholinergic reflexes; M_2 receptors are located on postganglionic cholinergic nerves and inhibit acetylcholine release,

and M_3 receptors mediate contractile responses in airway smooth muscle. The treatment of airway obstructive disease may be improved by agents that selectively block M_1 and M_3 receptors. Fryer focussed on loss of M_2 receptor function as a result of viral infections of the airway, exposure to ozone or antigen inhalation. Viral infections may damage M_2 receptors by neuraminidase cleavage of sialic acid residues, and positively charged proteins may act as allosteric antagonists at M_2 receptors, facilitating acetylcholine release. Finally, Disse described some of the properties of Ba 679 BR, an extremely potent anticholinergic which dissociates more rapidly from M_2 than from M_1 or M_3 receptor subtypes and may therefore represent an improvement over conventional anticholinergics in the treatment of obstructive airway disease.

In the nine years since the first Symposium on Subtypes of Muscarinic Receptors, remarkable advances have been made. Five receptor subtypes have been cloned and sequenced; their coupling mechanisms have been substantially worked out and a number of relatively specific ligands have been discovered, some of which show promise of therapeutic advantages over classical drugs affecting muscarinic receptors. There is no doubt that the biennial Symposia have played a significant catalytic role in the encouragement and dissemination of this research, and we owe a lasting debt of gratitude to Dr. Ruth Levine and the organizers for their continuing efforts.

References

1. F.C. MacIntosh, Trends Pharm. Sci. Suppl. 100-103 (1983).
2. A.S.V. Burgen, Trends Pharm. Sci. Suppl. 1-3 (1983).
3. A. Adem, A. Asblom, G. Johansson, P.M. Mbugua and E. Karlsson, Biochim. Biophys. Acta 968 340-345 (1988).
4. B. Ringdahl and D.J. Jenden, Life Sci. 32 2401-2413 (1983).
5. A.P. Roszkowski, J. Pharmacol. 132 156-170 (1961).

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ABSTRACTS OF POSTER PRESENTATIONS

1

SYNTHESIS AND STRUCTURE-ACTIVITY RELATIONSHIP OF 3-(PYRAZINYL)-1,2,5,6-TETRAHYDRO-1-METHYLPYRIDINES. CONSTRUCTION OF A MOLECULAR MODEL FOR THE M-1 PHARMACOPHORE.

J. S. Ward, L. Merritt, V. J. Klimkowski, M. L. Lamb, C. H. Mitch, F. P. Bymaster, B. Sawyer, H. E. Shannon, P. H. Olesen, T. Honoré, M. J. Sheardown, and P. Sauerberg. Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, IN 46285 and Novo Nordisk CNS Division, Novo Nordisk Park, DK-2760 Måløv, Denmark

A series of 3-(3-substituted-pyrazinyl)-1,2,5,6-tetrahydro-1-methylpyridines were synthesized and found to have high affinity for central muscarinic receptors. M-1 agonist activity was determined in the rabbit vas deferens preparation and found to depend upon the length of the sidechain attached to the pyrazine ring, maximal activity being obtained with the hexyloxy sidechain. A comparison of the M-1 agonist efficacy of these pyrazines and related 1,2,5-thiadiazoles and 1,2,5-oxadiazoles suggests that M-1 efficacy is related to the magnitude of electrostatic potential located over the nitrogens of the heterocycles. The heteroatom directly attached to the 3 position of the pyrazines and 1,2,5-thiadiazoles markedly influences the M-1 efficacy of the compounds by determining the energetically favorable conformers for rotation about the bond connecting the tetrahydropyridyl ring and the heterocycle. Using the model of the muscarinic pharmacophore proposed by Schulman and our own computational studies, we propose a three dimensional model for the M-1 activating pharmacophore.

2

ALKOXY SAR AROUND THE FUNCTIONALLY M_1 SELECTIVE AGONIST HEXYLOXY- TZTP

P. Sauerberg, P.H. Olesen, M.J. Sheardown, P.D. Suzdak, M.D.B. Swedberg, C.H. Mitch, J.S. Ward, D.O. Calligaro, B.D. Sawyer, F.P. Bymaster, H.E. Shannon. Pharmaceuticals Research, Novo Nordisk A/S, DK-2760 Målov, Denmark and Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46285.

Unbranched 3-(3-C_{1-8}Alkoxy-1,2,5-Thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridines (C_{1-8}Alkoxy-TZTP's), were synthesized and tested in vitro and in vivo for muscarinic affinity and efficacy. All the C_{1-8}Alkoxy-TZTP's inhibited ^{3}H-pirenzepine (Pz) binding in rat hippocampus and ^{3}H-oxotremorine-M (Oxo-M) binding in rat cortex with IC_{50} values between 4.0 nM and 148 and 1.4 nM and 30, respectively. Depicting the chain length against Oxo-M binding and against Pz binding, the C_{1-8}alkoxy-TZTP's produced U-shaped curves with the butoxy- and pentyloxy-TZTP's being the optimum chain length, respectively. This U-shaped SAR-curve was also seen in the ability of the compounds to inhibit the twitch height in the isolated electrically stimulated rabbit vas deferens, a model for M_1 efficacy and potency. Pentyloxy- and hexyloxy-TZTP produced greater than 90% inhibition of the twitch height with IC_{50} values in the low picomolar range. In cloned BHK cells expressing human m_1 receptors, the SAR for the ability of the Alkoxy-TZTP's to increase phosphoinositide turnover was similar. Again the hexyloxy-TZTP had the highest efficacy of the series (70% as compared to the stimulation produced by carbachol at the same concentration - 100 μM). However, at the M_2 receptors in the spontaneously beating isolated guinea pig atria the SAR was quite different. Propoxy-TZTP was a full agonist (IC_{50} = 90 nM), but then the potency and efficacy declined with increasing chain length. In vivo none of the Alkoxy-TZTP's produced tremor or salivation in mice at the screening dose 10 mg/kg i.p. At the same dose only propoxy-, butoxy- and heptyloxy-TZTP produced more than a 3°C drop in body temperature.

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3

HEXYLOXY-TZTP: A POTENT AND SELECTIVE M1 AGONIST *IN-VITRO*.

C.H. Mitch, F.P. Bymaster, D.O. Calligaro, S. J. Quimby, B. D. Sawyer, H.E. Shannon, J.S. Ward, P.H. Olesen, P. Sauerberg, M.J. Sheardown, P.D. Suzdak. Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN, USA 46285 and Novo Nordisk, CNS Division, MålØv, Denmark.

Hexyloxy-TZTP (3-(4-hexyloxy-1,2,5-thiadiazole-3-yl)-1,2,5,6-tetrahydro-1-methylpyridine) inhibited ^{3}H-pirenzepine binding and ^{3}H-oxotremorine-M binding with IC_{50} values of 10 nM and 3 nM, respectively, in rat brain membranes. In cloned cells expressing human m1 receptors, hexyloxy-TZTP increased phosphoinositide turnover, and the potency and efficacy differed dependent upon the cell type in which the receptors were expressed (CHO>A9L>BHK). In cloned cells expressing m2, m3, m4 or m5 receptors, hexyloxy-TZTP was considerably less active. Hexyloxy-TZTP also was selective for M1 receptors in isolated tissues. At M1 receptors in rabbit vas deferens, it inhibited twitch height with an IC_{50} of 8 pM. Pirenzepine blocked the effects of hexyloxy-TZTP in rabbit vas deferens and had a Kb value of 10 nM. At M2 receptors in guinea pig atria, hexyloxy-TZTP had an IC_{50} of 3 µM. Hexyloxy-TZTP was a partial agonist in guinea pig ileum. In guinea pig bladder, it was neither an agonist nor an antagonist. The present results demonstrate that hexyloxy-TZTP is a potent, efficacious and selective M1 muscarinic agonist in-vitro.

4

NEW FUNCTIONALLY SELECTIVE M1 AGONISTS RELATED TO McN-A-343

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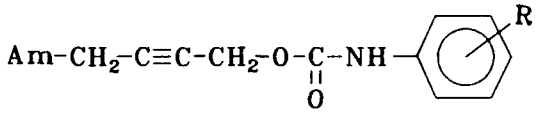
4-(3-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride (McN-A-343) is widely used to study muscarinic receptor mechanisms. The unique feature of McN-A-343 is its relatively high potency in stimulating M1 receptors in autonomic ganglia and rabbit vas deferens (RVD) and its weaker muscarinic actions at M2 and M3 receptors, as for example in guinea-pig atria (GPA) and ileum (GPI).



The aim of the present study was to develop muscarinic agonists with improved M1 selectivity using McN-A-343 as lead structure.

Variations were made in the ammonium group and the phenylring substitution pattern of the McN-A-343 molecule as shown above. Muscarinic activities of the new compounds were determined at M1 heteroreceptors in RVD, cardiac M2 receptors present in GPA and smooth muscle M3 receptors present in GPI. All compounds retained the functional selectivity for M1 receptors shown by the parent drug McN-A-343. Most favourable results have been obtained with the p-fluoro derivative **5** which was a potent full agonist at M1 receptors in RVD, but a competitive antagonist at M2 receptors in GPA and a weak partial agonist at M3 receptors in GPI.

Agonist Potencies (-log EC 50 [a]) at Muscarinic Receptor Subtypes.

no.	Am	R	RVD-M1	GPA-M2	GPI-M3
1[b]	$^{+}N(CH_3)_3$	m-Cl	6.57 ± 0.04	4.87 ± 0.21[c]	5.51 ± 0.06[d]
2	$^{+}N(CH_3)_3$	p-Cl	7.12 ± 0.06	5.26 ± 0.07[e]	5.71 ± 0.06
3	$^{+}N(CH_3)_3$	p-Br	7.12 ± 0.02	5.06 ± 0.07[f]	5.62 ± 0.03
4	$^{+}N(CH_3)_3$	p-F	6.94 ± 0.05	4.64 ± 0.10[g]	5.06 ± 0.02
5	$^{+}N(c\text{-}C_4H_8)CH_3$	p-F	6.89 ± 0.03	(pA_2 = 5.51 ± 0.04)	5.40 ± 0.05[h]

[a] *Negative log molar concentrations producing 50 % of the maximum response. Values are means ± S.E.M. (n = 3 - 14).* [b] *McN-A-343.* *Partial agonism: intrinsic activity = 0.49*[c]*; 0.83*[d]*; 0.77*[e]*; 0.66*[f]*; 0.5*[g]*; 0.26*[h].

In conclusion, the results obtained demonstrate that the structural variations made in the McN-A-343 molecule lead to new compounds with greater discriminatory properties.

5

LIPOPHILIC M_2 ANTAGONISTS AS POTENTIAL THERAPEUTIC IN ALZHEIMER'S DISEASE

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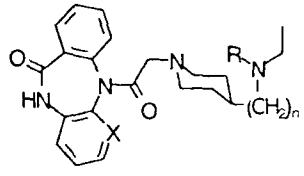
Department of Pharma Research, Dr. Karl Thomae GmbH, Biberach an der Riss, Germany

In recent years evidence has accumulated that selective anticholinergic drugs may play an important role in the field of Alzheimer's disease. Investigations with M_1 and M_2 selective radioligands in cortex and hippocampus have shown that both M_1 and M_2 receptors are present in these brain regions. Moreover, it has been shown that M_2 receptors are predominantly located presynaptically and modulate the release of acetylcholine. Stimulation of these presynaptic autoreceptors will reduce and blockade will enhance the acetylcholine release. Since cholinergic activity is reduced in dementia of Alzheimer type, the use of selective M_2 antagonists offers a new strategy to improve memory and learning. The target compounds should possess a good penetration into the CNS and a high selectivity for M_2 versus M_1 receptors, since the drug should not counteract its presynaptic action by blocking postsynaptic M_1 receptors.

In this communication we present a new class of lipophilic M_2 antagonists that possess according to their binding affinities the required selectivity profile

$$M_2 >> M_1, M_3$$

The leads in this series exhibit M_2 affinity with K_i-values in the range of 10 - 30 nM and M_2/M_1-ratios between 20 - 30. Such compounds could be interesting tools to elucidate the role of M_2 receptors in cholinergic transmission in the CNS and might be candidates of further investigation in Alzheimer's disease.



I: R = Acyl
X = N,CH
n = 3,4

6

FUNCTIONAL CHARACTERIZATION OF MUSCARINIC RECEPTORS IN RABBIT PERIPHERAL LUNG

E.K. Vockert, H.P. Egerer, R. Tacke*, G. Lambrecht and E. Mutschler; Dept. of Pharmacology, Univ. of Frankfurt, D-6000 Frankfurt/M., and *Dept. of Inorganic Chemistry, Univ. of Karlsruhe, D-7500 Karlsruhe, Germany

In rabbit peripheral lung muscarinic M2 and M4 receptors (mAChRs) have been identified as the predominant subtypes by the use of radioligand binding studies (Lazareno et al., Mol. Pharmacol. *38*, 805, 1990) and immunoprecipitation assays (Dörje et al., Mol. Pharmacol. *40*, 459, 1991). However, their function and cellular location remain to be determined.
The aim of this study was to characterize the postjunctional mAChRs in rabbit peripheral lung strips (PLS) studying the contractile response to muscarinic stimulation. In addition, the muscarinic modulation of noradrenaline (NA) overflow evoked by electrical field stimulation (EFS) was investigated.
(±)-Methacholine (EC_{50} = 7.55 (±0.61) μM ; n=81)-induced contractions of PLS were competitively inhibited by five subtype-selective muscarinic antagonists. The calculated pA_2 values and slopes of Schild plots are shown in the table. Our results suggest that M3 mAChRs located postjunctionally are mediating contraction of rabbit PLS.

Antagonist	pA_2 value	slope**	n
Pirenzepine	6.43 ± 0.02	-1.01 ± 0.03	15
AF-DX 384	6.96 ± 0.02	-1.12 ± 0.04	16
Himbacine	6.90 ± 0.02	-0.86 ± 0.03	15
(R)-HHD*	7.46 ± 0.02	-1.01 ± 0.03	15
(S)-HHD*	6.04 ± 0.01	-1.03 ± 0.02	13

*Hexahydro-difenidol.
**Not significantly different from unity.

The NA overflow of rabbit PLS evoked by EFS was determined by HPLC-ED-analysis. EFS (3 Hz, 1 ms, for 3 min; S1 - S6) was applied every 30 min. S1 (reference stimulation) increased NA overflow 23.39 (±4.02)-fold above basal level (n=5). The potent and selective muscarinic agonist arecaidine propargyl ester (0.01 - 10μM) suppressed the evoked overflow of NA in a dose-dependent and reversible manner. These results demonstrate that prejunctional muscarinic heteroreceptors inhibit the release of NA from sympathetic nerves of the rabbit PLS. Characterization of the subtype involved is under current study.

7

ELECTRONIC REQUIREMENTS IN A SERIES OF ARECOLINE DERIVATIVES FOR MUSCARINIC RECEPTOR-STIMULATED PHOSPHOINOSITIDE METABOLISM IN THE RAT BRAIN

W.P. Hess, D.O. Ngut, S. Roknich, C.H. Mitch, S.J. Quimby, J.S. Ward, L. Merritt, P. Sauerberg and W.S. Messer, Jr.; The University of Toledo, Toledo, OH, U.S.A.; Lilly Research Laboratories, Indianapolis, IN, U.S.A.; Novo-Nordisk, Måløv, Denmark

A series of arecoline derivatives was utilized to assess steric and electronic effects important for activating muscarinic receptors coupled to phosphoinositide (PI) metabolism in the rat CNS. Arecoline derivatives, in which the methyl ester moiety was replaced by hexyloxy-1,2,5-oxadiazole, hexyloxythiophene, or hexyloxypyrazine, were compared with the hexyloxy-1,2,5-thiadiazole compound, known from previous work to be an active muscarinic agonist.

Computational calculations showed that the N-S bonds of the alkyloxythiadiazole ring were highly polarized with the nitrogens having the ability to receive hydrogen bonds. The smaller oxadiazole had lower polarities in the N-S bonds and reduced hydrogen bonding capacity. The thiophene was of size comparable to the thiadiazole and had large C-S polarities without the hydrogen bond capacity while the pyrazine had limited ability to form hydrogen bonds.

The compounds were compared for their abilities to stimulate PI turnover in rat hippocampal slices. The 1,2,5-thiadiazole derivative was more active than the 1,2,5-oxadiazole, thiophene, or pyrazine derivatives. The data suggest that the ability to form hydrogen bonds is an important factor for the stimulation of M_1 receptors in the CNS by the 1,2,5-thiadiazole derivative of arecoline.

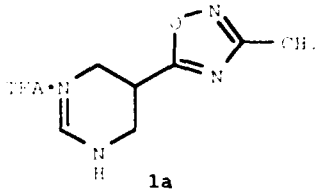
Work was supported by Lilly Research Laboratories and HHW grants NS 25765, NS 27926 and NS 31444.

8

5-(3-ALKYL-1,2,4-OXADIAZOL-5-YL)-1,4,5,6-TETRAHYDROPYRIMIDINES AS AGONISTS FOR M_1 MUSCARINIC RECEPTORS

E.G. Dunbar, G.J. Durant*, C. Fang, Y.F. Abdh, A. El-Assadi, D.O. Nnur, S. Periyasamy, W.P. Hess and W.S. Messer, Jr.; The University of Toledo, Toledo, OH, U.S.A.; Washington State University, Spokane, WA, U.S.A.; Cambridge Neuroscience, Inc., Cambridge, MA, U.S.A.

A series of 5-(3-alkyl-1,2,4-oxadiazol-5-yl)-1,4,5,6-tetrahydropyrimidines was synthesized for biological evaluation as M_1 agonists. Each ligand displayed high affinity for muscarinic receptors from rat brain as measured by inhibition of [^{3}H]-(R)-QNB binding. 5-(3-Methyl-1,2,4-oxadiazol-5-yl)-1,4,5,6-tetrahydropyrimidine trifluoroacetate (CDD-0098-5; **1a**) displayed high affinity (IC_{50} 2.7 ± 0.69 μM) and was a full agonist at muscarinic receptors coupled to PI metabolism in the rat cortex (700 ± 99 % above basal).



1a

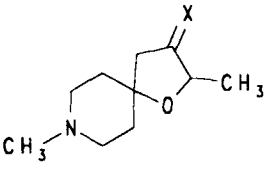
Increasing the length of the alkyl substituent increased affinity for muscarinic receptors, yet decreased activity for stimulating PI metabolism. The data indicate that the 1,2,4-oxadiazole moiety is useful as an ester bioisostere in the 1,4,5,6-tetrahydropyrimidine series of muscarinic agonists.

Work was supported by Lilly Research Laboratories and HHW grants NS 25765, NS 27926 and NS 31444.

9

M_1 RECEPTOR ACTIVITIES OF SUBSTITUTED AZASPIRODECANONES

G Mullen, J Blosser, A Kover, J Loch, S McCreedy, A Machulskis, and ESC Wu. Fisons Pharmaceuticals, Rochester, NY 14603.

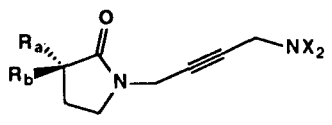


1

Structure-activity relationships at the M_1 muscarinic receptor were explored by modification of the potent muscarinic agonist 2,8-dimethyl-1-oxa-8-azaspiro[4,5]decan-3-one (1, X = O). The effect of replacement of the 3-keto group by a variety of polar and lipophilic groups on affinity and efficacy at muscarinic receptors was investigated. Affinity was determined by competitive binding with [^{3}H]Oxo-M using a rat brain crude membrane preparation. Efficacy at M_1 receptors was assessed by measuring the stimulation of rate of inositol phosphate formation from phosphatidyl inositol in rat hippocampal prisms coincubated with [^{3}H]inositol. Reduction of the 3-keto group to the corresponding alcohol 1, X = H, OH, led to a dramatic decrease in affinity (7 nM vs 2700 nM, resp). The olefin 1, X = CH_2, also exhibited reduced binding (460 nM) as well as efficacy (9% vs 50% for 1, X = O), possibly due to its inability to form a hydrogen bond to the postulated serine residue of the muscarinic receptor; the oxime derivative 1, X = NOH, however, exhibited only about a 4-fold reduction in affinity (to 30 nM), while retaining modest efficacy (15%). Small alkyl groups, such as methyl, on the oxime oxygen atom were also tolerated with only minor differences in affinity and efficacy. The introduction of hydrogen bond acceptors such as oxygen and nitrogen at the 3-position of the tetrahydrofuran ring of 1 should lie in the plane of the ring for optimal affinity and efficacy.

10

CHOLINERGIC AGONISTS: POTENTIAL THERAPEUTICS FOR ALZHEIMER'S DISEASE AND PROBES FOR MAPPING THE MUSCARINIC ACETYLCHOLINE RECEPTOR



11

2-ALKYLTHIO-DIPHENYLACETIC ACID ESTERS: A NEW CLASS OF POTENT AND SELECTIVE MUSCARINIC ANTAGONIST

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Tricyclic derivatives like Pirenzepine and AFDX 116 show high selectivity versus some of the known subtypes of muscarinic receptors [1, 2]. Although apparently very different in molecular structure, they can be considered as restricted analogs of classical antimuscarinic antagonists that usually are devoid of selectivity. Based on this observation we have designed and synthesized a series of compounds where the structure of classical muscarinic antagonists such as Adiphenine is sterically hindered by a large group (-X-R; X=O,S; R=CH_3, C_2H_5, $CH(CH_3)_2$, C_3H_7...) inserted on the carbon atom carrying the bulky lipophilic substituents that drive the interaction with the receptors.

The pharmacological results have indeed shown that, when X=S and R is larger than CH_3, the compounds show high potency and selectivity on g.p. heart (M_2) and r. vas deferens (M_1) respect to the g.p. ileum (M_3) receptors. The selectivity ratios are in the range : M_1/M_2=1-2; M_2/M_3=50-400; M_1/M_3=20-500 depending on R and on the nature of the alcohol. Therefore this new class of muscarinic antagonists shows a unique pharmacological profile that can be useful in receptor classification.

Stucture activity relationships regarding the role of the hetero atom and that of conformation will be discussed.

References

[1] Eberlein, W. G., Engel, W. W., Trumlitz, G., Schmidt, G., Hammer, R. *J. Med. Chem.*, 1988, 31, 1169.

[2] Engel, W. W., Eberlein, W. G., Mihm, G., Hammer, R., Trumlitz, G. *J. Med. Chem.*, 1989, 32, 1718

12

MUSCARINIC RECEPTOR SUBTYPES REGULATING ACETYLCHOLINE RELEASE IN HUMAN AND RAT CORTEX

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Different muscarinic receptor subtypes have been suggested to be involved in the regulation of acetylcholine (ACh) release in the brain. In rat brain, the M_2 and M_3 receptor subtypes might have an autoinhibitory function, while the M_1 receptor subtype has been suggested to increase the release of ACh. Human postmortem control and Alzheimer cortical brain slices were preincubated with [^{3}H] choline ([^{3}H]Ch) and the efflux of [^{3}H]Ch were investigated during potassium stimulation. In both control and Alzheimer cortex, muscarinic antagonists increase the efflux of [^{3}H]Ch indicating a preserved autoinhibitory function. In Alzheimer cortex a defict in the release regulating mechanism has been observed, since acetylcholinesterase (AChE) inhibitors increase the efflux of [^{3}H]Ch, which is decreased in control brain tissue. To further investigate the underlying receptor mechanisms regulating ACh release in Alzheimer cortex different muscarinic antagonists were added together with the AChE inhibitor. The results indicate that the M_1 receptor subtype in Alzheimer cortex is involved in the AChE-induced efflux of [^{3}H]Ch. In a similar experimental condition using rat cortical slices it was shown when studying an AChE inhibitor, tetrahydroaminoacridine (THA) and some of its analogues, that the AChE inhibiting effect is necessary for its action on [^{3}H]Ch efflux in rat cortex. In an additional experimental set up, nerve endings (synaptosomes) from rat cortex were preloaded with [^{3}H]Ch and depolarized in superfusion with 35 mM K^+ for 5 minutes at 70 and 100 minutes after having initiated the release process. McN-A 343, an M_1 receptor agonist was added for 5 minutes during S_2 and enhanced the efflux of [^{3}H]Ch from the synaptosomal preparation in a dose-range of (10^{-6}-10^{-4} M). The effect of different muscarinic antagonists, such as pirenzepine, AF-DX 116 and p-fluoro-hexahydrosila-diphenidol, on the McN-A-343 induced efflux of [^{3}H]Ch from rat cortex was investigated. These results indicate that M_1 receptor subtype may have a facilitatory effect on the outflow of [^{3}H]Ch both in Alzheimer and rat cortex.

13

DIFFERENTIAL REGULATION OF HUMAN CNS m1-m5 MUSCARINE ACETYLCHOLINE RECEPTOR GENE EXPRESSION IN ALZHEIMER'S DISEASED AND AGE-MATCHED POST-MORTEM CONTROL TISSUES.

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A consistent relationship between Alzheimer's Disease (AD) and central nervous system (CNS) neurotransmitter function has emerged in the cholinergic synapse. Yet the precise nature and impact of this dysfunction remains to be defined. Binding, biochemical and autoradiographic studies of post-mortem brains of AD and control (C) human tissues reveal alterations in muscarinic acetylcholine receptor (mAChR) subtypes, both on pre- and post- synaptic neurons. Tritiated (-)quinuclidinylbenzilate (QNB), (+)cismethyldioxolane (CD), pirenzepine (PZ), AF-DX 384 and hemicholinium-3 (HC-3) autoradiograms and in situ hybridization histochemistry (ISHH) data were quantified, as was described previously. No significant alterations in Kd (affinity) were observed. While no significant differences in QNB binding were determined, other mAChR ligands including PZ and CD showed significant alterations. HC-3 binding site density in the hippocampal formation of AD patients was only 53% of C values, a significant finding which is consistent with values showing low levels of choline acetyltransferase activity in AD brains. ISHH using five human 3'-end labeled (^{35}S-dATP; SA~0.8mCi/ug) oligodeoxyribonucleotide probes complementary to three regions of 4-48/4-49 base sequences of the m1-m5 mAChR was done after verifying specificity via Northern blots. Sections were incubated (36h; 25°C), washed (Tm=46°-53°C; 0.5x SSC), dried and Hyperfilm-ßmax apposed (1w; 0°-3°C). AD brains show low hippocampal m3 expression ($p<.05$) and higher m5 mRNA levels in temporal cortex ($p<.05$). Other changes were insignificant. Altered modulation of mAChR gene expression may underlie lower cognition seen in AD. (MH-43024).

14

MUSCARINIC AND NICOTINIC RECEPTOR CHANGES IN THE CORTEX AND THALAMUS OF BRAINS OF CHRONIC ALCOHOLICS.

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The cholinergic system in the cortex and thalamus was studied in postmortem brain tissue from 21 chronic alcholics and 20 age matched controls. A significant decrease in the M_1 and M_2 receptors with age was observed in the frontal cortex of both controls and chronic alcoholics. An age related increase in number of M_1 and M_2 receptors was observed in the thalamus of controls but not in chronic alcoholics. When the subjects were divided into two age groups (19-57ys and 59-84ys) a significant loss in total number of muscarinic receptors as well as M_1 and M_2 receptor subtypes was found in the thalamus of the older group of alcoholics compared to controls. Guanylylimidodiphosphate (Gpp(NH)p) resulted in a steepening and rightward shift of the agonist curves obtained from measurements of carbachol displacement of ^{3}H-QNB bindning to membrane preparations from thalamus of controls and alcoholics indicating no change in muscarinic receptor coupling in chronic alcoholics. The number of high affinity nicotinic bindning sites and the activity of choline acetyltransferase in frontal cortex and thalamus was not significant different in alcoholics compared to age matched controls.

15

CHARACTERIZATION OF MUSCARINIC RECEPTOR SUBTYPES IN NORMAL AND ALZHEIMER BRAIN TISSUE BY USING SELECTIVE MUSCARINIC ANTAGONISTS

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Subtypes of muscarinic receptors were characterized in the human frontal cortices of control as well as Alzheimer brain tissue, using competition binding experiments with [^{3}H]-quinuclidinyl benzilate (QNB) and pirenzepine, AF-DX 116, hexahydro-sila-diphenidol (HHSiD), p-fluoro-hexahydro-sila-diphenidol (p-F-HHSiD) and himbacine respectively. Two binding sites was observed for both pirenzepine and AF-DX 116 in the frontal cortices. The majority of the pirenzepine binding sites (76%) showed high affinity to the determined receptors, corresponding to the M_1 site, while the rest of the binding sites had an affinity that was 40 times less. The AF-DX 116 high affinity binding site suggested the presence of a component of M_2 sites (26%), whereas the major low affinity component indicated non-M_2 binding.A single class of binding sites was observed for HHSiD, p-F-HHSiD and himbacine in human frontal cortices. HHSiD showed an affinity comparable to that of the pirenzepine high affinity binding site, suggesting binding mainly to the M_1 sites. A relative low affinity was found for p-F-HHSiD representing binding to M_1 and/or M_2 sites. The affinity for himbacine was similar to that for p-F-HHSiD and lower than for HHSiD, possibly illustrating difficulties in detecting M_4 sites in the human frontal cortices by competition experiments. In Alzheimer brain tissue a significant increase was observed in the Bmax and affinity of the pirenzepine high affinity site (M_1), as well as for HHSiD. A significant increase was also observed in the Bmax for the AF-DX 116 low affinity site (non-M_2).
The results indicate a heterogenous population of muscarinic binding sites in human frontal cortices, with a major population of M_1 sites and a preservation of the muscarinic receptors in Alzheimer's disease with a relative increase in the number of M_1 receptor sites.

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VISUALIZATION OF MUSCARINIC RECEPTORS IN ALZHEIMER BRAINS IN VIVO BY POSITRON EMISSION TOMOGRAPHY

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It is of utmost importance to find early diagnostic markers for Alzheimer's disease (AD). The positron emission tomography (PET) technique provides unique possibilities to study functional neuronal activity in human brain.We have developed a multi-tracer system of ligands which enables us to measure cerebral blood flow (^{11}C-butanol, ^{15}O-H_2O), glucose metabolism (^{18}F-FDG), nicotinic receptors (^{11}C-nicotine) as wells as muscarinic receptors (^{11}C-benztropine) in brain of normal individual and patients with dementia disorders.
Receptor binding studies in autopsy brain tissue material indicate preserved muscarinic receptors in Alzheimer patients which is opposite to the marked reduction of nicotinic receptors found in both post-mortem brain studies and by PET. In order visualize the muscarinic receptors in brain by PET we have used benztropine to label the receptors. [N-^{11}C-methyl]-benztropine (^{11}C-Cogentin^R) was synthesized from norbenztropine.The labelled compound (300 MBq) was injected intravenously and the PET camera (AB Scanditronix, Uppsala) was immediately started and the scanning was performed with eyes open for 80 mins. Arterial blood samples were repeatedly drawn during the investigation. ^{11}C-benztropine showed a different regional distribution in brain compared to ^{11}C-nicotine with high uptake in the basal ganglia, visual cortex and lower in the thalamus and cerebellum. In order to study whether long-term treatment with the cholinesterase inhibitors such as tacrine can induces changes in muscarinic receptors Alzheimer patients were investigated by ^{11}C-benztropine and PET after various lenghts of tacrine treatment.

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FURTHER CHARACTERIZATION OF THE DEFECT IN M1 MUSCARINIC RECEPTOR-G PROTEIN COUPLING IN ALZHEIMER'S DISEASE.

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Cortical M1 receptors are a current target for the development of potent and selective cholinomimetic drugs to improve memory and alleviate some of the cognitive impairments associated with Alzheimer's disease (AD). We have demonstrated a defect in the ability of the M_1 muscarinic receptor subtype to form high affinity agonist receptor - G protein complexes in AD frontal cortex (Flynn et al. Ann. Neurol. 29: 256, 1991). The regional defects in M_1 receptor coupling to its G protein have been mapped using quantitative autoradiography. In AD cases with severe neocortical pathology, carbachol (10 μM) failed to displace [^{3}H]-pirenzepine binding in frontal and temporal association areas, while high affinity agonist binding was spared in primary sensory and motor cortices. In brain areas where high affinity carbachol binding was spared, and in those brains where there was a partial loss of high-affinity agonist binding, guanine nucleotides retained the ability to completely uncouple the receptor from its G protein. Also, immunoblotting demonstrated no apparent alteration in G protein number in AD. Taken together, these results suggest that the loss of high affinity agonist binding to cortical M_1 receptors is not at the level of the G protein and may reflect a disease-related modification of the receptor which renders it incapable of interacting with its G protein. Muscarinic receptor desensitization has been shown to be a consequence of receptor phosphorylation, resulting in a decrease in high affinity agonist binding and an 'uncoupling' of the receptor from its G protein (Richardson et al, Mol.Pharmacol. 40: 908, 1991). In addition, significant alterations in the activity of protein kinases have been suggested to play a role in the processing of the amyloid precursor protein (APP) to β/A4 amyloid and in the 'tangling' of the tau protein. The possibility that the M_1 muscarinic receptor is abnormally phosphorylated by specific kinases is being investigated. (Supported by NS19065, NS25785, and the American Health Assistance Foundation.)

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MUTATIONAL ANALYSIS OF THE m3 MUSCARINIC RECEPTOR: FUNCTIONAL ROLE OF PROLINE RESIDUES THAT ARE HIGHLY CONSERVED AMONG ALL G PROTEIN-COUPLED RECEPTORS

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All G protein-coupled receptors share a considerable degree of sequence similarity which is greatest within the seven transmembrane domains (TMD I-VII). Particularly well conserved are a series of four proline residues (located in TMD IV, V, VI, and VII, respectively) which are likely to introduce bends into the transmembrane helices. To study their importance for ligand binding and receptor function, these proline residues were individually replaced by alanine (a good helix former that should result in a "straightening" of the TM helices), using the rat m3 muscarinic receptor as a model system. The ligand binding and functional properties of the resultant mutant receptors were studied following their transient expression in COS-7 cells. [^{3}H]NMS saturation binding assays showed that Pro201->Ala (TMD IV) was expressed at similarly high levels as the wild-type receptor. In contrast, Pro242->Ala (TMD V), Pro505->Ala (TMD VI), and Pro540->Ala (TMD VII) were expressed at ca. 30-100-fold lower levels than wild-type m3. Most strikingly, Pro201->Ala displayed up to 450-fold lower binding affinities for both muscarinic agonists (acetylcholine, carbachol) and antagonists (NMS, 4-DAMP). However, this mutant receptor retained full functional activity stimulating carbachol-induced phosphatidyl inositol (PI) hydrolysis to a similar maximum extent as the wild-type m3 receptor. Pro242->Ala, Pro505->Ala, and Pro540->Ala displayed "m3-like" antagonist binding properties, but clearly differed in their agonist binding profiles. Pro242->Ala and Pro505->Ala displayed 3-30-fold lower agonist binding affinities than wild-type m3, but retained strong functional activity (70-100 % of the "wild-type E-max"). Interestingly, Pro540->Ala bound agonists with ca. 10-fold increased affinity (compared with wild-type m3), but was severely impaired in its ability to mediate carbachol-induced stimulation of PI metabolism (E-max ca. 25 % of the "wild-type response"). Our data indicate that the proline residues that are highly conserved across the entire family of G protein-coupled receptors play important roles in receptor expression, ligand binding, and receptor activation.

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COEXPRESSION OF N- AND C- TERMINAL MUSCARINIC RECEPTOR DOMAINS RESULTS IN FUNCTIONAL RECEPTORS

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Truncated forms of the human m2 (m2-*trunc*) and the rat m3 muscarinic receptor (m3-*trunc*) containing transmembrane domains (TMD) I-V and the N-terminal portion of the third cytoplasmic loop (i3) were created by linker-insertion mutagenesis. The truncated receptors were coexpressed in COS-7 cells with cDNA fragments encoding the C-terminal receptor domains (referred to as m2- or m3-*tail*), comprising TMD VI and VII and the adjacent intra- and extracellular sequences. Expression of the truncated receptors or m2- or m3-*tail* alone did not yield any detectable [^{3}H]NMS binding activity. However, coexpression of m2-*trunc* with m2-*tail* (m2-t/t), and m3-*trunc* with m3-*tail* (m3-t/t) resulted in "reconstituted" receptors that displayed agonist and antagonist binding affinities similar to those found with the wild-type (wt) receptors:

	[^{3}H]NMS K_D(pM)	*4-DAMP K_i(nM)	*Acetylcholine IC_{50}(μM)	*Carbachol IC_{50}(μM)
m2 (wt)	62 ± 7	3.3 ± 0.3	0.87 ± 0.17	2.1 ± 0.6
m2-t/t	79 ± 10	3.7 ± 0.2	0.31 ± 0.10	0.65 ± 0.20
m3 (wt)	29 ± 2	0.40 ± 0.03	12.3 ± 1.3	59.3 ± 8.7
m3-t/t	23 ± 2	0.40 ± 0.02	8.3 ± 1.9	47.7 ± 4.8

*Data were obtained in competition binding assays using 200 pM of [^{3}H]NMS

The "reconstituted m3-t/t receptor" was able to stimulate carbachol-dependent phosphatidyl inositol hydrolysis in a fashion similar to the wild-type m3 receptor (carbachol EC_{50}, μM: m3 (wt), 6.1 ± 2.5; m3-t/t, 0.96 ± 0.32; maximum increase in inositol monophosphate above basal levels: m3 (wt), 141 ± 7 %; m3-t/t, 107 ± 12 %). These data raise the possibility that muscarinic receptors may behave in a fashion analogous to multiple-subunit receptors, with the i3 loop serving as a linker sequence between two independently folding receptor domains.

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CONSERVED THREONINE AND TYROSINE RESIDUES PLAY IMPORTANT ROLES IN ACETYLCHOLINE BINDING AND MUSCARINIC RECEPTOR ACTIVATION

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All muscarinic receptors contain within their hydrophobic transmembrane domains a series of conserved threonine and tyrosine residues most of which do not occur in other G protein-coupled receptors. To test the hypothesis that the hydroxyl groups present in these residues can interact with muscarinic ligands by means of hydrogen bonding, single point mutations eliminating the various hydroxyl moieties (Thr->Ala, Tyr->Phe) were introduced into the rat m3 muscarinic receptor. Six mutant m3 receptors were identified which bound antagonists with affinities similar to the wild-type receptor, but displayed 10-60-fold reduced binding affinities (compared with wild-type m3) for the agonists acetylcholine (ACh) and carbachol (Wess et al., *EMBO J.* 10, 3729-3734, 1991). In [^{3}H]NMS competition binding studies, ACh derivatives lacking the ACh ester bond bound to most of these mutant receptors with affinities that were similar to or differed only slightly from the corresponding "wild-type affinities". This finding supports the notion that the conserved threonine and tyrosine residues interact with the ACh ester moiety (e.g. by hydrogen bonding) to induce high affinity binding of ACh to the muscarinic receptor. The two mutant m3 receptors, Thr234->Ala and Tyr506->Phe, which showed the most pronounced decreases in ACh binding affinities (ca. 40-60-fold as compared with wild-type m3), were stably expressed in CHO cells for further functional analysis. Both mutant receptors were found to be severely impaired in their ability to stimulate agonist-dependent phosphatidyl inositol hydrolysis. Consistent with this observation, ACh binding to the two mutant receptors was not significantly affected by addition of the GTP analog, Gpp(NH)p (100 μM). In contrast, addition of Gpp(NH)p to membrane preparations obtained from cells expressing the wild-type m3 receptor resulted in a small but significant rightward shift and a steepening of the ACh competition binding curves. Thr234 and Tyr506 are located on transmembrane domains V and VI, respectively, which are connected by the third intracellular loop (i3). Since this region is known to play a pivotal role in G protein activation, one may speculate that these two amino acids participate in the agonist-induced changes in receptor conformation that trigger the functional activation of the i3 domain and the interaction with specific G proteins.

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A MODEL FOR LIGAND BINDING AT THE MUSCARINIC CHOLINERGIC RECEPTOR

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An analysis of agonist and antagonist hydrogen-bonding capabilities has allowed the construction of a model for a hypothetical ligand-binding cavity in the muscarinic cholinergic receptor. Defined by five inferred points of interaction which determine ligand orientation, the cavity contains two adjacent agonist binding sites which overlap an antagonist binding site. These sites relate agonist and antagonist structures while providing a rationale for differences in activity and receptor subtype selectivity.

The relationship between the hypothetical sites suggests a dynamic scheme in which agonists travel from a recognition site to an active site by a series of hydrogen-bond exchanges that antagonists are unable to perform. The scheme provides a rationale for designing unsaturated agonist sidechains that incorporate diverse, bulky substituents while retaining full agonist activity. Sidechains that impart m1 subtype selectivity for agonists introduce steric bulk in space shared by m1-selective antagonists.

As a working model, the hypothetical ligand-binding cavity and its associated binding scheme have provided a useful conceptual framework for the development and optimization of m1-selective agonists.

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HM1 RECEPTOR INTERNALIZATION INDUCED BY CARBACHOL REQUIRES A SHORT S/T RICH DOMAIN OF THE i3 LOOP.

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Stimulation of many cell membrane receptors with agonists causes rapid internalization/sequestration, followed by recycling or down-regulation. Whereas the cognate receptor domains required for receptor internalization have been identified for some receptor classes (e.g. LDL, growth factors), no such domains had been described for any of the numerous G protein coupled receptors. We have previously shown that mutational deletions of the i3 loop of Hm1 diminished or abrogated carbachol induced Hm1 internalization in transiently and stably transfected cell lines (Maeda et al. *FEBS Lett.* **269**, 386, 1990; Lameh et al., *J. Biol. Chem.* **267**, 13406-13412, 1992). The putative target domain was located in the middle of the i3 loop (G283-E292). Further multiple point mutations revealed that domain SLTSS is required for internalization. This function appears to be associated with the multiple S and T residues, as a SLTSS → ALAAA mutation was also strongly defective in internalization. The presence of S and T residues implies possible phosphorylation as a regulatory mechanism. Similar S and T rich domains are found in several other G protein coupled receptors, including m2-5. Therefore, this regulatory domain may be relevant to receptors other than Hm1. Supported by GM 43102.

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MUTATION OF ASP TO GLU IN THE THIRD TRANSMEMBRANE HELIX OF THE MUSCARINIC RECEPTORS

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The muscarinic acetylcholine receptors (mAChRs) have a conserved aspartic acid in the third transmembrane helix (Asp105, m1 mAChR) which is thought to form an ionic bond with the positively charged headgroup of agonists and antagonists. We have probed the function of this residue by mutating it to glutamic acid in both the m1 and m2 mAChRs. Binding studies carried out on the receptors expressed in COS7 cells were analysed using a ternary complex model of binding to distinguish the formation of the high-affinity agonist-receptor-G-protein (ARG) ternary complex, from that of the binary agonist-receptor (AR) complex. The affinity for the antagonist ^{3}H-N-methylscopolamine, was only reduced by 3-fold in the mutant. In contrast, binding of ACh was greatly affected; the corrected IC_{50} values increasing by 30-fold (m1) to 100-fold (m2). The most striking and consistent effect of the Asp/Glu mutation was to disfavour the ARG complex with respect to the AR complex. This effect was seen with all the agonists studied. The effect of the mutation on formation of the binary complex was variable, depending both on the structure of the agonist and on the receptor subtype; the largest reduction was seen using ACh itself and large effects, particularly on the m1 subtype, were also evident using L660,863. In contrast, formation of the oxoM-m1-receptor complex was unaffected. These results suggest that the charge interaction between the agonist headgroup and the Asp sidechain makes its most important contribution in the catalytically-active ARG complex, but makes a smaller, more variable contribution to formation of the initial binary AR complex.

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SITE-DIRECTED MUTAGENESIS OF A CONSERVED ARGININE IN THE RAT M1 RECEPTOR.

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Using oligonucleotide-directed mutagenesis, we have substituted a conserved arginine (R122) at the cytoplasmic interface of transmembrane helix 3 of the rat M1 receptor, by a lysine. Following expression in COS-7 cells, this conservative mutation did not appear to have a direct effect on the ligand binding site, since binding of the antagonist [^{3}H]-*N*-methylscopolamine was not affected (K_D = 0.1nM for both wild type and mutant). Further analysis of ligand binding was performed using the ternary complex model of agonist binding, which indicated a four-fold increase in the low affinity binding of acetylcholine to the K122 receptor.

This mutation produced effects reminiscent of those of divalent cations, which are known to promote agonist-receptor-G-protein interaction. In the presence of 1mM EDTA, the flattened K122 binding curve indicated the formation of the ternary complex, although the calculated affinity of the G-protein for the agonist-receptor complex was not greatly affected. Addition of 3mM Mn^{2+} had little further effect. However, the wild type receptor, which couples to G-protein very poorly in EDTA containing buffer, in the presence of divalent cations gave a binding curve reminiscent of the K122 EDTA curve. The effect of Mn^{2+} on the binary complex remains unclear.

A preliminary interpretation of these results is that the mutation does not directly affect interactions at the ligand binding site, but promotes high affinity binding by facilitating the conformational changes which occur on receptor activation, thus enabling G-protein recruitment.

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STUDIES ON MUSCARINIC RECEPTORS USING NITROGEN MUSTARDS.

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Labelling the acetylcholine binding site of the muscarinic receptor using tritiated acetylcholine mustard. Acetylcholine mustard, (AChM), an irreversible analogue of acetylcholine, (ACh), binds to muscarinic receptors reversibly as an agonist, with a similar dose response curve to ACh itself. The reversibly bound AChM reacts with the receptor, to produce a complex with no activity. ^{3}H-AChM covalently bound to purified, solublized cortex receptors was shown to label asp 105, (m1 sequence), by specific cleavage of the receptor by enzymes or cyanogen bromide, followed by repetitive Edman degradation of the fragments. This same residue is labelled by the irreversible antagonist, propylbenzilylcholine mustard[1]. These data suggest that AChM, and by analogy ACh, interacts with asp 105 when it binds to the muscarinic receptor.
4-DAMP mustard selectivity. The aziridinium ion of 4-diphenylacetoxy-N-(2-chloroethyl)-piperidine, (4-DAMP mustard), has recently been shown to exhibit M_3/M_2 selectivity in tissue preparations[2,3]. The selectivity of this compound has been examined in CHO cells bearing single receptor subtypes. In contrast to PrBCM which exhibits little subtype selectivity, 4-DAMP mustard is selective for m3 over m1, m2, m4 and m5 receptors. This is due to 4-DAMP mustard having both a higher affinity for m3 receptors, and a faster alkylation rate. 4-DAMP mustard may be of use in selectively alkylating m3/M_3 receptors in tissues which contain other subtypes, thereby allowing the other subtypes to be characterised more readily.
References: 1. Kurtenbach et al, J. Biol. Chem. (1990) 265:13702-08. 2. Barlow et al, J. Pharm. Pharmacol. (1990)42:412-18. 3. Thomas et al, Mol. Pharmacol. (1992) 41:718-26

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MOLECULAR MODELING STUDIES OF THE m1 AND m2 MUSCARINIC RECEPTORS LEADING TO THE DESIGN OF SELECTIVE m1 AGONISTS.

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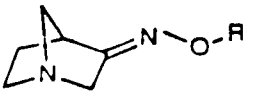
The primary sequence of various muscarinic receptor subtypes (m1-m5) as well as other G-protein coupled receptors (GPCR's) have been aligned. Sequence analyses demonstrated a high degree of residue conservation (over 75% identity in the transmembrane regions) between the m1 and m2 receptor subtype sequences.

Given the difficulty of generating suitable crystals for membrane bound receptors, no crystallographic data are available today to describe the 3-dimensional topography of these receptors. However, a model of m1 and m2 receptors has been assembled using the 3-dimensional (3D) model proposed for rhodopsin[1]. Experimental results and structural knowledge available for retinal analogs and the opsin binding site have been used as a template to model and design a class of m1 selective muscarinic agonist ligands. These selective agonists present an elongated side chain when compared to previously studied ligands. Steric and electrostatic properties observed in the receptor binding site model were used to assist the design of novel side chains that incorporate a conjugated moiety resembling the retinal chromophore. It is postulated that the designed ligands span the relatively rigid receptor cavity and interact with sites that differ in m1/m2 receptor subtypes.



R = Poly-ene and/or -yne

1. T. Mirzadegan, R. S. H. Liu, "Probing the Visual Pigment Rhodopsin and Its Analogs by Molecular Modeling Analysis and Computer Graphics", *Progress in Retinal Research*, **1991**, 110, 57-74.

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POST-TRANSCRIPTIONAL REGULATION OF THE m1 MUSCARINIC ACETYLCHOLINE RECEPTOR

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Agonist promoted down-regulation of the m1 muscarinic acetylcholine receptor (m1 mAChR) mRNA via message destabilization was investigated in a permanent cell line. Chinese hamster ovary (CHO) cells were stably transfected with a 1.64 kb fragment of the m1 mAChR gene containing an open reading frame of 1.38 kb and a .26 kb 3' flanking untranslated region (3'UTR). Carbamylcholine (CBC), at 1mM, down-regulated the m1 mAChR in a time-dependent fashion from 2 to 12 hours with maximal decreases (50-66%) occuring after 12 hours. Correspondingly, steady state levels of m1 mAChR mRNA decreased after 6 hours of CBC pretreatment with maximal decrements (40-70 %) occurring between 12 to 18 hours before approaching control levels at 24 hours (67% of control). No changes were observed in the steady state levels of glyceraldehyde-3-phosphate dehydrogenase mRNA with CBC pretreatment. Since the rate of mRNA degradation represents an important potential regulatory mechanism to control the level of gene expression, we investigated the effects of CBC pretreatment on m1 mAChR mRNA stability. The stability of m1 mAChR mRNA in control and 12 hour CBC-pretreated cells was measured by incubating cells with actinomycin D (5 μg/ml) to block transcription. Total cellular RNA was prepared after various times of incubation and an RNase protection assay was performed. The half-life of m1 mAChR mRNA in untreated control cells was 14 ± 1.4 hours. In contrast, receptor transcript half-life declined to 3.0 ± 0.5 hours in cells treated with CBC.

A construct of the m1 mAChR gene missing its 3'UTR was made and stably transfected into CHO cells. Deletion of this region abolished agonist-induced destabilization of the resulting receptor transcript. These results demonstrate that agonist-induced destabilization of the m1 mAChR mRNA represents a viable mechanism to regulate receptor expression. Furthermore, genetic elements responsible for message destabilization reside in the 0.26 kb 3'UTR.

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MUTATIONS OF ASPARTATE 103 IN HUMAN MUSCARINIC RECEPTOR SUBTYPE m2 EXPRESSED IN COS-7 CELLS: EFFECTS ON RECEPTOR BINDING AND SIGNAL TRANSDUCTION.

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Based upon molecular biological characterization, five subtypes of the human muscarinic receptor have been cloned and expressed in mammalian cells in culture. Recent results using rat m1 receptors have shown that the Aspartate (Asp) residue at position 105 was crucial for both agonist and antagonist muscarinic ligand binding. To gain a greater understanding of the involvement of this Asp in agonist/antagonist binding, Asp 103 in the Hm2 receptor (corresponding to Asp 105 in m1) was mutated to Asn, Ala, and Glu. Hm2 was chosen since both agonist (3H-CMD) and antagonist (3H-NMS) binding could be performed as well as measurement of alterations in signal transduction, e.g. adenylate cyclase activity. Mutagenesis was achieved by a modification of the Kunkle method and mutant and wild type receptors were expressed in COS-7 cells by transient transfection. Similar to the rat m1 receptor, changing Asp to Asn in Hm2 markedly inhibited 3H-NMS binding. Comparisons to this effect will be made for the Ala and Glu mutations. Thus, our results confirm the key role Asp 103 plays in binding of agonists and antagonists to the Hm2 receptor.

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COMPARISONS BETWEEN HUMAN MUSCARINIC RECEPTOR SUBTYPES COUPLED TO PHOSPHOLIPASE C AND THOSE COUPLED TO ADENYLYL CYCLASE: EFFECT OF RECEPTOR RESERVE.

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Muscarinic receptors have been shown to exist as five distinct proteins which are functionally coupled through G proteins to phospholipase C (m1, m3, and m5) and adenylyl cyclase (m2 and m4). Stable expression of human receptors in CHO cells yielded cell lines with B_{max} values ranging from 210-2450 fmoles/mg protein and K_d values of 0.11 - 0.63nM (whole cell [^{3}H]-NMS binding). The use of these lines has allowed the pharmacological selectivity of various muscarinic agonists and antagonists to be determined using receptor binding and second messenger assays. Previous results obtained measuring phosphatidylinositol (PI) hydrolysis, showed that both efficacy and potency of muscarinic agonists were markedly affected by receptor number, while antagonist results were not. However, it was not known whether similar effects occurred in cyclase linked receptors. In the present study, alkylation of Hm2 and Hm4 receptors was performed in order to determine the effect of receptor number on agonist/antagonist-induced changes in cAMP formation. These results are compared to those obtained measuring PI turnover under similar conditions in Hm1, Hm3, and Hm5 cells.

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MUSCARINIC INHIBITION OF NORADRENALINE RELEASE EVOKED BY FIELD STIMULATION ON RABBIT ISOLATED VAS DEFERENS

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Field stimulation of the rabbit vas deferens (RVD) results in a biphasic contraction. ATP triggers the initial twitch response, while noradrenaline (NA) is responsible for the slow second phase (G. J. Trachte, EJP 164: 425, 1989). This study describes the muscarinic modulation of NA overflow evoked by field stimulation (HPLC-ED analysis). Vasa deferentia were incubated in Krebs solution (1.8 mM Ca^{2+}) containing (μM) cocaine (10), corticosterone (10) and rauwolscine (1).

At 1 Hz (1 ms, for 30 s), the A1 receptor antagonist 8-cyclopentyl-1,3-dipropyl-xanthine (DPCPX, 0.03 μM) and the ATP-blocking agent pyridoxal-phosphate-6-azo-phenyl-2',4'-disulfonic acid (PPADS, 10 μM; in the presence of 0.03 μM DPCPX) increased the evoked NA overflow by 150.5 ± 52.6 % and up to 12.4-fold, respectively. Indomethacin (10 μM; in the presence of 0.03 μM DPCPX and 10 μM PPADS) enhanced the evoked NA overflow by 16.8 ± 2.1 %. Thus, the release of NA was under a tonic inhibition by endogenous adenosine, ATP and prostanoids. In further experiments the Krebs solution additionally contained DPCPX (0.03μM), PPADS (10 μM) and indomethacin (10 μM).

The muscarinic agonists (±)-methacholine (MCH, 1 - 10 μM) and arecaidine propargyl ester (APE, 0.1 - 1 μM) suppressed the evoked release of NA (EC_{50} = 0.86 and 0.054 μM, respectively). Atropine (0.03 μM), pirenzepine (0.1, 0.3 and 1 μM), methoctramine (0.5 μM) and himbacine (0.2 μM), when present alone, failed to affect the evoked overflow, but shifted the MCH (atropine as antagonist) and APE concentration-inhibition curve to the right without suppressing the maximum. These results clearly indicate the existence of presynaptic inhibitory muscarinic receptors in RVD. On the basis of the pA2 values, no clear-cut conclusions can be drawn on the receptor subtype involved. However, the M_2, M_3, and M_5 receptor subtypes may be excluded.

	Apparent pA2 values
Atropine (n = 4)	9.47 ± 0.13
Pirenzepine (n = 12)[1]	7.90 ± 0.21
Methoctramine (n = 4)	6.90 ± 0.10
Himbacine (n = 4)	8.30 ± 0.08

[1] Slope = 0.84; not significantly different from unity.

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GLYCOPYRRONIUM BROMIDE, AN ULTRAPOTENT M_1-SELECTIVE MUSCARINIC RECEPTOR ANTAGONIST *IN VITRO*.

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Glycopyrrolate is a muscarinic receptor antagonist widely used in anesthesia instead of atropine, but little is known about its selective blockade of muscarinic receptor subtypes. We therefore determined equilibrium dissociation constants of glycopyrronium bromide under *in vitro* conditions for M_1 (inhibition of twitch response on rabbit vas deferens), M_2 (inhibition of force of contraction of paced rat left atria), and M_3 (contraction of guinea pig ileum), and an atypical muscarinic receptor (contraction of rabbit iris sphincter; see Bognar et al. Naunyn-Schmiedeberg's Arch. Pharmacol. 1992, 345:611-618) which neither corresponds to M_1-M_3 nor to M_4 or m_5. (±)-Methacholine served as agonist in all models except in rabbit vas deferens where McN-A-343 was used. The affinity of glycopyrronium bromide was high for the M_1 receptor (apparent -log K_B value of 11.4±0.08, n=14). The drug blocked M_2 receptors in rat atria (n=14) with considerably lower affinity (-log K_B 9.1±0.08) compared to M_1 and M_3, and the atypical receptors, and possessed about equal potencies at the M_3 (-log K_B 10.3±0.03, n=10) and at the iris receptor (-log K_B 10.2 ±0.08, n=10). It is concluded that glycopyrronium bromide is *in vitro* an M_1-selective antimuscarinic drug with an about 100 or 10 fold higher affinity for M_1 or M_3 and iris receptors, respectively, compared to atropine. In contrast, at the M_2 receptor it is equipotent with atropine.

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ON THE OVER-ADDITIVE ANTIMUSCARINIC ACTION WITH ATROPINE OF POTENT ALLOSTERIC STABILIZERS OF ANTAGONIST BINDING TO M_2-RECEPTORS

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W84 (hexamethylene-bis-[dimethyl-{3-phthalimidopropyl} ammonium bromide]) stabilizes antagonist binding to M_2-receptors by an allosteric mechanism and acts in combination with atropine over-additively antimuscarinic.

Two derivatives of W84 were synthesized in which the phthalimide groups were replaced by 2-phenyl-2,3-dihydro-1H-quinazolin-4-one (Chin3/6) and in which the central bisquaternary moiety was changed to give 4,4'-bis-(phthalimidomethoxyiminomethyl)-1,1'-propane-1,3-diyl-bis-pyridinium dibromide (W-DUO). The stabilizing effect on [^{3}H]N-methylscopolamine binding was studied in guinea pig cardiac membranes (3mM $MgHPO_4$, 50mM Tris, pH 7.3, 37°C) and in intact left atria (3Hz, Tyrode's solution). The antimuscarinic action of the compounds was measured in left atria with oxotremorine as agonist. In cardiac membranes, the [^{3}H]N-methylscopolamine dissociation rate was reduced to half of the control value by the three compounds at EC_{50}~1μM. In intact atria, the allosteric activity was less pronounced (EC_{50}~10μM). The compounds had similar antimuscarinic potencies (pA_2~6). When combined with 1μM atropine, W84 and W-DUO exhibited (≥10μM) an over-additive antimuscarinic action. In contrast, Chin3/6 did not induce an overadditive effect. Thus, the structural modifications did not attenuate allosteric activity. However, the over-additive effect was lost when the phthalimide group was replaced by the quinazolinone moiety.

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FUNCTIONAL ACTIVITIES OF THE NOVEL CHOLINOMIMETICS RU 35926 (CI 979) AND RU 35963 WITH RESPECT TO MUSCARINIC RECEPTORS.

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RU 35926 and RU 35963 are potent cholinomimetic compounds, structurally related to arecoline. Their agonist profiles with respect to different muscarinic receptor subtypes in the rat have been evaluated in binding and functional models in vitro.

The ratios of the low affinity (K_l) and high affinity (K_h) components of agonist competition curves were determined for M_1, M_2 and M_3 receptors using antagonist ligands. With respect to M_1 and M_3, both RU 35963 and RU 35926 show low K_l/K_h ratios, indicative of partial agonists. At the M_2 receptor the K_l/K_h ratio for RU 35963 is nearer to that of a full agonist, while that of RU 35926 is significantly lower.

Phosphatidyl-inositol (PI) hydrolysis (M_1 / M_3) was measured in both hippocampal slices and in primary cultures of cerebellar neurons. In the former system, RU 35963 like carbachol, strongly stimulates the formation of inositol phosphates (EC_{50} values = 0.4 and 1.9 mM, respectively) while RU 35926 (10^{-2}M), only produces ~30% of the maximal effect of carbachol. In the latter system where only the M_3 receptor is involved, both compounds show partial PI responses.

Presynaptically (probably M_2), RU 35963 causes a complete and dose dependent inhibition of acetylcholine release from electrically stimulated hippocampal slices (EC_{50} = 1.9 mM), while RU 35926 partially inhibits (~60%). At the M_2 receptor in cardiac membrane homogenates however, both compounds (EC_{50} = 0.88 and 3.5 μM respectively), like carbachol (EC_{50} = 6.1 μM), are efficient inhibitors of adenylate cyclase.

These results confirm the muscarinic agonist properties of both RU 35926 and RU 35963 but also show that their efficacy varies according to the tissue preparation and the receptor subtype involved. Functionally, RU 35926 more often shows partial agonist character while RU 35963 tends to behave more as a full agonist.

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TRANSMEMBRANE SIGNALING OF M1 MUSCARINIC RECEPTORS IN THE RAT BRAIN AND CELL CULTURES.

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Full muscarinic agonists increase phosphoinositide (PI) hydrolysis and cAMP levels in mammalian cells transfected with m1 muscarinic receptors (mAChRs). However, it is still not clear whether the artificial combination of the transfected receptors and the endogenous G-proteins in such cells reflects the situation in neuronal tissues. The rat brain contains a mixture of m1-m4 AChR subtypes, and their respective signal transduction mechanisms in their native environment. In this study, we used AF102B, a relatively selective M1 agonist (Fisher et al., JPET 257: 392, 1991), in order to elucidate the nature of signal transductions by the M1 AChRs in rat brain. Synaptosomes, slices and homogenates from rat forebrain (rich in m1 and m3 AChRs) were used to determine PI hydrolysis and adenylyl cyclase (AC) activity. AF102B (1 μM-10 mM) neither inhibited AC activity, nor did it potentiate PI hydrolysis. Yet, coincubation of AF102B and 1 mM carbachol (CCh) inhibited CCh-induced PI hydrolysis with an IC_{50}=75 μM without affecting CCh-induced inhibition of AC. Preincubation (30 min) of synaptosomes, slices or membranes with AF102B (100 μM-10 mM), followed by extensive washing, also inhibited CCh (0.1 or 1 mM)-induced PI hydrolysis, without any effect on mAChR binding sites as determined by [^{3}H]QNB binding. In Chinese hamster ovary cells transfected with human m1 AChR preincubation (120 min) with AF102B (1 μM-10 mM) potentiated (1-100 μM) or inhibited (1-10 mM) CCh-induced PI hydrolysis. Notably, AF102B acts as a partial agonist on m1 AChRs in these cells, stimulating PI hydrolysis yet, unlike CCh, lacking an effect on cAMP levels in the same cell preparation (Fisher et al., Biorg. Med. Chem. Lett., in press). We suggested that AF102B is selective at the level of both m1 AChR and distincts signal transduction pathway. The present results further support the notion that an agonist like AF102B can modulate indirectly the interaction of mAChRs with discrete signal transduction processes.

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MODULATION OF MUSCARINIC AGONIST BINDING BY CATIONS AND GUANINE NUCLEOTIDES DURING AGING

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Responsiveness to cholinergic muscarinic receptor stimulation is decreased in lung tissues during senescence (Wills and Douglas, Br. J. Pharmacol. 93: 918-924, 1988). We have previously shown that there are no changes in the muscarinic receptor affinity or density, however, the ability of guanine nucleotides to decrease agonist affinity for the receptor is decreased during aging (Wills and Hirshman, Am. Rev. Resp. Dis. 193:A470,1989). To further explore the mechanisms of this age-related effect, we evaluated the regulation of agonist-receptor-G protein interactions by divalent cations and guanine nucleotides. Guinea pig lung membranes were incubated with 50 mM Tris-HCL (pH 7.4), 0.5 nM [^{3}H]QNB, 1μM carbachol, and varying concentrations of Ca^{2+} with or without 100μM Gpp(nh)p for 30 min at 25° C. Non-specific binding was defined in the presence of 1μM atropine. Ca^{+2} concentrations from 1nM to 0.1 μM had no effect on agonist displacement of [^{3}H]QNB in young or old tissues, however, 10 mM Ca^{2+} caused a 33% increase in agonist displacement of [^{3}H]QNB. Gpp(nh)p inhibited agonist displacement of [^{3}H]QNB by 35% at concentrations of Ca^{2+} between 1nM and 0.1μM. At 10mM Ca^{2+} the effect of guanine nucleotides was abolished. In contrast, guanine nucleotides had no effect on agonist displacement in the old tissues at low concentrations of Ca^{2+}. The effects of 10mM Ca^{2+} on old tissues was similiar to that observed in the young tissues. Addition of 10mM Mg^{2+} to the assays restored the abililty of Gpp(nh)p to decrease agonist affinity in the old tissues. These studies suggest that altered coupling of the muscarinc receptor to G proteins during aging may be due to altered Ca^{2+}-Mg^{2+} modulation of receptor-G protein interactions. Supported by Grant from NIH HLB 43312.

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COMPARISON OF MUSCARINIC RECEPTOR-MEDIATED ACTIVATION OF CYCLIC GMP SYNTHESIS AND NITRIC OXIDE RELEASE IN A NEURONAL CLONE.

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Muscarinic receptor-mediated cyclic GMP (cGMP) synthesis and nitric oxide (NO) release were compared in mouse neuroblastoma N1E-115 cells. [^{3}H]cGMP was assayed in cells prelabeled with [^{3}H]guanine. Release of NO upon the addition of muscarinic agonists to neuroblastoma cells (donor cells) was quantitated indirectly by its ability to increase cGMP level in cells whose muscarinic receptors were inactivated by irreversible alkylation (detector cells). Carbamylcholine increased NO release in a concentration-dependent manner, with an EC_{50} of 170 μM (as compared to 95 μM for direct activation of cGMP synthesis). This effect was markedly blocked by hemoglobin. The maximal effect of carbamylcholine was 4-fold lower in stimulating NO release than in elevating cGMP directly. There was a good correlation in the ability of a series of muscarinic agonists (at 1 mM each) to release NO or to activate cGMP formation. Furthermore, preincubation of cells with 1 mM carbamylcholine for 30 min resulted in a similar magnitude of desensitization of both responses. These results support an important role of diffusion of NO from one neuronal cell to another in the regulation of intracellular cGMP levels by muscarinic receptors.

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EFFECTS OF Mg^{2+} ON THE INTERACTION OF ATRIAL MUSCARINIC RECEPTORS AND G PROTEINS.

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Effects of Mg^{2+} on the interaction of muscarinic receptors (mAChRs) and GTP-binding regulatory proteins (G proteins; Gi or Go) were examined in a reconstituted system of purified components. Approximately 70 % of the mAChRs showed high-affinity for carbamylcholine in the absence of guanine nucleotides and the affinity increased with the increase of Mg^{2+} concentration (Kd=0.06 μM in the presence of Mg^{2+} (10 mM) compared to Kd=1.6 μM in the absence of Mg^{2+} (< 0.1 μM)). mAChRs showed only low-affinity (Kd=100 μM) binding in the presence of GTP or GDP, irrespective of the presence or absence of Mg^{2+}. These results indicate that mAChRs may assume at least three different conformations, and that Mg^{2+} is not necessary for the formation of the mAChR-G protein complex but can induce a conformational change in the complex. The affinity for GDP was decreased 20-fold by the addition of carbamylcholine in the presence of 10 mM Mg^{2+} (apparent Kds =2.8 and 0.15 μM). No such effect of carbamylcholine was observed in the absence of Mg^{2+}. These results suggest that Mg^{2+} is necessary for the action of mAChR on G proteins and that the agonist-mAChR-G protein complex formed in the presence of Mg^{2+} is an intermediate for the action of mAChR on G proteins.

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MUSCARINIC AUTORECEPTORS ON CHOLINERGIC NERVES INNERVATING HORSE TRACHEA ARE NOT OF THE M_1, M_2, OR M_3 SUBTYPES

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To determine the subtype of the prejunctional muscarinic autoreceptors on horse airway cholinergic nerves, we studied the influence of competitive muscarinic antagonists [atropine: nonspecific; pirenzepine: M_1-selective; AF-DX116: M_2-selective, and hexahydrosiladifenidol (HHSiD): M_3-selective] on ACh release induced by electrical field stimulation (EFS) of tracheal cholinergic nerves. Trachealis strip bundles were suspended in 2-ml tissue baths. After incubation with 10^{-5} M neostigmine and guanethidine for 60 minutes, six 20-min EFS (20 V, 2 ms, 4 Hz) periods were applied in the presence of increasing concentrations of each muscarinic antagonist. Bath solution was collected after each EFS period for acetylcholine (ACh) analysis by HPLC and electrochemical detection. All muscarinic antagonists augmented ACh release in a concentration-dependent manner. However, the potency and maximal effect of atropine were much greater than those of any of the three selective muscarinic antagonists. The ED_{50} for atropine, pirenzepine, and AF-DX116 were (6.0 ± 1.3) X 10^{-8} M, (3.9 ± 0.6) X 10^{-6} M, and (2.8 ± 0.8) X 10^{-6} M, respectively. The variable effect of HHSiD did not allow calculation of ED_{50}. The maximal ACh release rate after atropine (72.5 ± 11.7 pm/g/min) was significantly higher than those after the three selective antagonists (44.5 ± 10.5, 46.7 ± 12.6, and 31.6 ± 10.2 pm/g/min for pirenzepine, AF-DX116, and HHSiD, respectively). These results suggest that the muscarinic autoreceptors on cholinergic nerves innervating horse trachea are not of the M_1, M_2, or M_3 subtypes.

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CYCLIC AMP POTENTIATES MUSCARINIC RECEPTOR-STIMULATED PHOSPHOINOSITIDE HYDROLYSIS IN HUMAN NEUROEPITHELIOMA CELLS

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A stimulatory role for cAMP in the regulation of muscarinic receptor (mAChR)-stimulated phosphoinositide (PPI) hydrolysis has been examined in human neuroepithelioma cells. These cells elicit an increase in PPI hydrolysis in the presence of agonists specific for muscarinic, α_1-adrenergic, endothelin and ATP receptors. All combinations of agonists resulted in a release of inositol phosphates that was at least additive. However, the addition of optimal concentrations of oxotremorine-M and norepinephrine to SK-N-MCIXC or SK-N-MCIIE cells resulted in a PPI hydrolysis that was 30% greater than additive. This potentiation of inositol lipid hydrolysis resulted from an increased activity of the mAChR after the addition of norepinephrine and persisted after α_1-adrenergic receptor blockade. The enhancement of mAChR-stimulated inositol phosphate release could be quantitatively mimicked by inclusion of the β-adrenergic agonist, isoproterenol, but not by α_1 or α_2-adrenergic agonists. Potentiation of oxotremorine-M stimulated inositol lipid hydrolysis observed in the presence of either norepinephrine or isoproterenol was reduced in the absence of added Ca^{2+}. Addition of either norepinephrine or isoproterenol to SK-N-MCIXC cells also resulted in a 16-fold increase in cAMP concentration. Inclusion of the cell permeant 8-chloro-4-phenylthio-cAMP resulted in a 30% enhancement of mAChR-stimulated inositol phosphate release. We conclude that in SK-N-MCIXC cells, an increase in cAMP concentration potentiates mAChR-stimulated phosphoinositide hydrolysis.
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AFFINITY PROFILE OF S(-)-HYOSCYAMINE FOR MUSCARINIC RECEPTOR SUBTYPES

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Ghelardini et al. (1992) reported that S-(-)-hyoscyamine, contrary to R-(+)-hyoscyamine, is not able either to induce analgesia in rodents or to potentiate electrically evoked guinea-pig ileum contractions. In order to clarify the reason for the lack of S-(-)-hyoscyamine in vivo and in vitro effects, we investigated its affinity profile for three muscarinic receptor subtypes. Antagonist potencies were determined by functional studies performed using field-stimulated rabbit vas deferens (M_1), rat ileum (M_3) and guinea pig uterus (M_4). Concentration-response curves were constructed by the addition respectively of McN-A-343, ACh and carbachol in the absence or presence of antagonist. pA_2 values, obtained by using Arunlakshana-Schild plots are listed below.

Preparation	Subtype	pA_2	Slope
Rabbit vas deferens	(M_1)	9.28 ± 0.21	1.21 ± 0.52
Rat ileum	(M_3)	9.01 ± 0.01	0.95 ± 0.01
Guinea-pig uterus	(M_4)	8.71 ± 0.02	0.91 ± 0.02

S-(-)-hyoscyamine affinity for guinea-pig atrium (M_2) has been already determined by Barlow et al.(1986). pA_2 values revealed that S-(-)-hyoscyamine does not discriminate between M_1 and M_3 receptors and that it shows less affinity for the M_4 receptor subtype than R-(+)-hyoscyamine (Ghelardini et al. this meeting). The above reported results suggest that the lack of analgesic effect of S-(-)-hyoscyamine compared to its enantiomer R-(+)-hyoscyamine reflects their different affinities for both M_1 and M_4 receptors.
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R-(+)-HYOSCYAMINE: THE FIRST SELECTIVE ANTAGONIST FOR GUINEA-PIG UTERUS MUSCARINIC RECEPTOR SUBTYPE

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Recent studies (Ghelardini et al., 1992) showed that R-(+)-hyoscyamine at very low doses (0.5 - 5 μg/kg s.c.) exerted a central antinociceptive effect in mice and rats by increasing ACh release through the antagonism of central muscarinic presynaptic autoreceptors.
In order to elucidate which muscarinic receptor subtype was responsible for this effect, the affinity profile of R-(+)-hyoscyamine for four muscarinic receptor subtypes was investigated. Antagonist potencies were determined by functional studies performed using field-stimulated rabbit vas deferens (M_1), rat electrically-driven left atrium (M_2), rat ileum (M_3) and guinea pig uterus (M_4) (Dörje et al. 1990). The agonists used were: McN-A-343 for vas deferens, ACh for atrium, ACh for ileum and carbachol for uterus. pA_2 values of R-(+)-hyoscyamine obtained in the above mentioned preparations, by using Arunlakshana-Schild plots are listed below.

Preparation	Subtype	pA_2	Slope
Rabbit vas deferens	(M_1)	7.13±0.19	0.81±0.21
Rat left atrium	(M_2)	7.11±0.06	1.13±0.06
Rat ileum	(M_3)	6.99±0.17	0.92±0.17
Guinea-pig uterus	(M_4)	9.56±0.03	0.99±0.05

pA_2 values revealed high selectivity of R-(+)-hyoscyamine for the muscarinic subtype present in guinea-pig uterus (selectivity-ratio M_4/M_1=355; M_4/M_2=219; M_4/M_3=427). Since Dörje et al. (1990) reported that M_4 receptors are predominantly present in guinea-pig uterus it appears possible that R-(+)-hyoscyamine may be the first selective M_4 antagonist.

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CARBACHOL INHIBITS L-TYPE CALCIUM CURRENT IN GUINEA PIG VENTRICULAR MYOCYTES: POSSIBLE ROLE OF cGMP

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Muscarinic inhibition of L-type calcium current $I_{Ca(L)}$ in mammalian ventricular myocytes is evident when $I_{Ca(L)}$ has been augmented by drugs that increase intracellular cAMP content. The mechanism(s) for muscarinic inhibition of cAMP-elevated $I_{Ca(L)}$ are poorly understood. We studied the effect of carbachol (CCh) on $I_{Ca(L)}$ in guinea pig ventricular myocytes with the whole-cell voltage-clamp technique at 22-24 °C. The phosphodiesterase (PDE) inhibitors, isobutylmethylxanthine (IBMX) or papaverine, augmented $I_{Ca(L)}$ as much as 5-10 fold in a manner similar to isoproterenol, forskolin or intracellular cAMP. As expected, CCh (100 μM) suppressed the increase of $I_{Ca(L)}$ produced by either IBMX or papaverine. However, $I_{Ca(L)}$ was not restored to its original amplitude when CCh was washed out in the continued presence of the PDE inhibitors. IBMX also increased $I_{Ca(L)}$ in cells that had been pretreated with pertussis toxin or in the presence of LY-83583, a drug that inhibits the formation of cGMP. Under these conditions, CCh was not able to inhibit $I_{Ca(L)}$. Assuming that the principal mechanism to raise intracellular cAMP by IBMX and papaverine is PDE inhibition, it is difficult to explain the effect of CCh by an action that either inhibits adenylyl cyclase or stimulates PDE activity. We propose that CCh suppression of the effect of PDE inhibitors on $I_{Ca(L)}$ involves elevation of cGMP levels by muscarinic agonist.

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DESENSITIZATION AND DEINHIBITION OF ACH-INDUCED EXOCRINE SECRETION AT THE RECEPTOR LEVEL.

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Ach, 4 μM, induces a maximal Cl^- secretion in the tracheal epithelium of hen, equal to a short circuit current (I_{sc}) of ≈ 90 $\mu A \cdot cm^{-2}$ and a concomitant conductance increase ≈ 4 $mS \cdot cm^{-2}$. At 500 μM ACh the I_{sc} induced by ACh is completely eliminated (desensitization) whereas the conductance is nearly unaffected. Desensitization of ACh-induced I_{sc} is rapidly ($t_{1/2}$ = 1-2 min) reversed by atropine (deinhibition). Thus ACh-desensitization of ACh-induced I_{sc} is not due to a down-regulation of surface receptors.

In order to explain the dissociation in desensitization of I_{sc} and conductance we have constructed a model consisting of two separate ACh receptors. One receptor induces the opening of basolateral Ca^{2+} and K^+ channels and of a luminal Cl^- channel which leads to a Cl^--secretion via a constitutively operating Na^+,Cl^--cotransporter. The other muscarinic receptor is thought to inhibit the cotransporter through a pertussis toxin-insensitive G protein.

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FORSKOLIN INHIBITS MUSCARINIC RECEPTOR-MEDIATED PHOSPHOINOSITIDE HYDROLYSIS IN THE RAT PAROTID GLAND.

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Previous studies in our lab have provided evidence for an interaction between two of the major signal transduction pathways in the rat parotid gland, namely control of cyclic AMP levels and hydrolysis of phosphoinositides. Further studies have extended these preliminary results. Oxotremorine-M (OXO-M), a highly efficacious, non-selective muscarinic agonist, stimulated the accumulation of inositol monophosphates in slices of the rat parotid gland with an EC_{50} of 5.66 μM, and a maximal stimulation of 6.32-fold increase over basal. The effect of the adenylate cyclase-stimulator, forskolin (75 μM) was to increase OXO-M's EC_{50} to 22.14 μM, a 4-fold increase, while having no effect on the maximal response. We next investigated the response to pilocarpine, a partial agonist in the rat parotid gland. Pilocarpine stimulated the accumulation of inositol monophosphates in this tissue preparation with an EC_{50} of 6.55 μM and a maximal stimulation of 2-fold over basal. In this instance, forskolin (75 μM) did not affect the EC_{50} (7.0 μM) but the maximal response to pilocarpine ws reduced to only a 1.5-fold increase over basal, which represents a decrease of 49.4%. In preliminary experiments, forskolin dose-dependently inhibited the accumulation of inositol monophosphates produced by OXO-M (4 μM) with an IC_{50} of 19.01 μM. The preceding results support our hypothesis that there is an interaction between these two second messenger systems in the rat parotid gland.

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HUMAN M1 RECEPTOR-TRANSFECTED RBL CELLS: A NOVEL TEST SYSTEM FOR THE CHARACTERIZATION OF MUSCARINIC AGONISTS

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RBL cells transfected with the human m1 receptor (RBL-hm1 cells) have previously been shown to respond to carbachol with the exocytosis of granules containing mediators and enzymes (Jones et al. 1991, FEBS Lett. 289:47-50). To establish whether this response could be utilized for the functional characterization of muscarinic agonists, we determined the quantitative relationship between receptor binding, phosphoinositide (PI) hydrolysis, rise in cytosolic free calcium, and exocytotic release of the enzyme ß-hexosaminidase. There was a close correspondence between the concentration-response curves for all four parameters. Carbachol stimulated the enzyme release up to 83-fold over basal, as compared to only 7-fold stimulation of the PI turnover. The enzyme release induced by several partial agonists reached only 3-26 % of the carbachol (full agonist) effect, thus allowing the safe discrimination between full and partial agonists. The muscarinic agonist-induced enzyme release was markedly synergized by the concomitant stimulation of endogenous adenosine receptors. Under these conditions the response to partial agonists was increased up to 19-fold. The order of potency of muscarinic agonists was not altered upon stimulation of the adenosine receptors. The pertussis toxin-sensitive effect of adenosine receptor agonists was, to some extent, reflected in an increased PI turnover and rise in cytosolic free calcium. Our results reveal that the agonist-induced enzyme release in RBL-hm1 cells can be utilized as a very sensitive and convenient measure of the efficacy and potency of agonists at the m1 receptor.

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MUSCARINIC RECEPTOR PROTECTION STUDIES IN ISOLATED, FUNCTIONAL PREPARATIONS.

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The role of heterogeneous muscarinic receptor populations in some smooth muscle is not yet defined. Selective inactivation of one population can facilitate studies on the roles of the respective subtypes. To date, selective alkylating agents for M_1 and M_2 receptors are unavailable although 4-diphenyl-N-(2-chloro ethyl)-piperidine has been suggested as a selective M_3 receptor alkylating probe (Barlow et al., 1990, J. Pharm. Pharmacol., 42:412-418). In the present study, we have used the technique of receptor protection against the non-selective alkylating agent, phenoxybenzamine, to prepare functional preparations with homogeneous muscarinic receptor populations.

M_1, M_2 and M_3 receptor function were studied, in vitro, using canine saphenous vein, guinea-pig paced left atria and ileum, respectively. Concentration-response curves to (+)cis dioxolane were constructed in the absence and presence of the alkylating agent phenoxybenzamine (0.1-3 µM). In separate studies, the tissues were equilibrated with atropine (0.3 µM), para-fluoro hexahydrosiladifenidol (p-F-HHSiD, 0.1 µM), methoctramine (0.1 µM) or pirenzepine (0.3 µM) and then exposed to phenoxybenzamine for 20 min. All tissues were then washed and a second curve established. In all studies, parallel control studies were undertaken to correct for changes in tissue sensitivity.

Phenoxylbenzamine alone, resulted in dextral shift and depression in the maxima of the concentration response curves in all preparations. In all tissues, atropine completely protected against alkylation. Of the selective antagonists, in atria, only methoctramine gave complete protection, in ileum this was seen only with p-F-HHSiD and finally, in saphenous vein, only pirenzepine, rather than p-F-HHSiD or methoctramine protected against alkylation.

These data are consistent with M_1, M_2 and M_3 receptors mediating responses in saphenous vein, left atria and ileum, respectively. This suggests that functional inactivation of the major M_2 population in ileal smooth muscle did not directly affect contraction and failed to expose a direct functional role for the majority M_2 population.

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CHARACTERIZATION OF MUSCARINIC RECEPTORS IN GUINEA-PIG SEMINAL VESICLE

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Muscarinic receptor subtypes in smooth muscle from various species are heterogeneous (see Eglen et al., 1991, Mol. Neuropharmacol. 1:117-127 for references). In these tissues, contraction is mediated by a minor M_3 receptor population and the role of the major M_2 receptor population is unclear. We have evaluated the guinea-pig seminal vesicle, a secretory tissue which also contracts in response to muscarinic receptor stimulation (see Gonzales, 1989, Arch. Androl. 22: 1-13 for review).

Contraction of the tissue, which is concentration dependent to (+)cis-dioxolane (-log EC_{50} = 6.0), was phasic and rapidly desensitized. The antagonists, atropine, pirenzepine, methoctramine, para-fluoro-hexahydro siladifenidol (p-F-HHSiD), 4-diphenylacetoxy-N-methyl piperidine methiodide (4-DAMP), himbacine and imperialine surmountably antagonized responses to (+)cis-dioxolane with the following affinities (-log K_B) 10.3 ± 0.2; 7.2 ± 0.1, 6.0 ± 0.1, 8.1 ± 0.1, 9.6 ± 0.1, 7.2 ± 0.1 and 6.8 ± 0.1 (mean ± s.e. mean, n=4-8). (+)cis-Dioxolane, carbachol and (±)L-660,863 also elicited a concentration-dependent increase (31 ± 5 fold over basal) in total accumulation of inositol phosphates (IPs; -log EC_{50} = 6.0, 5.3 and 6.5, respectively). Pilocarpine (0.1-1.0 mM) was inactive. These responses were inhibited by the following antagonists (mean -log K_B values in parenthesis) atropine (9.8), pirenzepine (7.2), methoctramine (5.9), HHSiD (7.9), p-F-HHSiD (7.4), 4-DAMP (10.6), Benzyl-4-DAPine (7.3), himbacine (6.8), imperialine (6.8) and DAC 5945 (8.7). These data are consistent with M_3 receptors mediated contraction due to enhanced phosphoinositide metabolism. Northern blot analysis revealed the expression of only M_3 mRNA, with no expression of M_1, M_2 or M_4 mRNA.

It is concluded that this tissue represents a contractile tissue which expresses only functional M_3 receptors. This contrasts with other guinea-pig smooth muscles, such as ileum or trachea, in which M_2 receptors predominate. As such, it provides a useful preparation to study M_3 function in smooth muscle.

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DISTRIBUTION OF THE MUSCARINIC RECEPTOR SUBTYPES IN RAT OLFACTORY BULB AS DETERMINED WITH THREE SELECTIVE SECOND GENERATION ANTAGONISTS DAU 6202, AQ-RA 741 AND DAG 5600

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The distribution of muscarinic receptor subtypes (mAChRs) in the rat olfactory bulb (OB) was determined with the aid of antagonists DAU 6202 [4-hydroxy-3-(tropyl)oxycarbonyl-3,4-dihydro-1H-quinazoline 2-one] (M_1/M_3 selective); AQ-RA 741 [11-[[4-[4-(diethylamino)butyl]-1-piperidinylacetyl]5,11-dihydro-6H-pyrido [2,3b] [1,4]benzodiazepine-6-one] (M_2/M_4 selective); and DAG 5600 (1-chloro-6,11-dihydro-11-[(1-methyl-4-piperidinyl)-carbonyl]5H-dibenz[b,e]-azepin-6-one) (M_1 selective), each of which gave the proportion of a single site within the mixed receptor population in OB. DAG 5600 bound to pure mAChRs with K_D's of (nM): 40 (cortex, M_1), 6667 (heart, M_2), 2050 (subm. glands, M_3), 452 (NG 108-15 cells, m4) and 112 (hm5) in competition experiments vs (^{3}H)NMS (or (^{3}H)PZ, cortex) and gave a flat inhibition curve in the OB. Computer analysis showed that DAG 5600 bound 43% of total OB sites with a K_D of 40 nM (i.e. M_1 sites) and 57% with a K_D of 935 nM (representing all other sites). DAU 6202 bound to mAChRs with K_D's of (nM): 1.9, M_1; 234, M_2; 5.2, M_3; 26, m4; 5.2, m5 and bound 51% of OB sites with a K_D of 1 nM (M_1+M_3 sites) and 49% with a K_D of 25 nM (apparently M_4/m4 sites). None of the affinity constants suggested the presence of an M_2 binding site. Finally, AQ-RA 741 bound mAChRs with K_D's of (nM): 23, M_1; 5, M_2; 200, M_3; 6.3, m4; 1423, m5 and bound 26% of OB sites with 2.3 nM affinity and 74% with 34 nM affinity. The 2.3 nM site has approximately the same affinity as the m4 site. The OB thus appers to contain about 40% M_1, 10% M_3, 25% M_4/m4 and 25% of an as yet unidentified, Mx, site.

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SELECTIVE BLOCKADE OF M3 RECEPTOR-MEDIATED RESPONSES BY 4-DAMP MUSTARD.

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The ability of 4-DAMP mustard to discriminate between M_2 and M_3 receptor mediated responses was investigated in the longitudinal muscle of the rat ileum. Pretreatment with 4-DAMP mustard (10 nM; 1 hr) caused a significant blockade of phosphoinositide (PI) hydrolysis, mediated via the M_3 muscarinic receptor, resulting in a 6.6 fold increase in the EC_{50} value and an accompanying 65% decrease in the maximal response. This effect corresponded to a 96% alkylation of the muscarinic receptors as estimated by the method of Furchgott. Similar treatment with 4-DAMP mustard, however, only blocked the M_2 receptor mediated inhibition of adenylate cyclase by a 2.1-fold increase in the EC_{50} value with no change in the maximal inhibition. The fraction of receptors occupied by 4-DAMP mustard under these conditions was calculated to be 52%, according to the relationship described by Paton. In order to inactivate the M_3-mediated responses more selectively, ileal slices were pretreated with 4-DAMP mustard (30 nM; 1 hr) in the presence of the reversible M_2 selective antagonist AF-DX-116 (1 μM) and then washed extensively. This treatment caused a 2.5 fold increase in the EC_{50} value for the stimulation of PI hydrolysis with an 80% reduction in the maximum response, and corresponded to an 88% alkylation of the muscarinic receptors. In contrast, no change was observed in the ability of oxo-M to inhibit adenylate cyclase under these conditions. These results demonstrate that 4-DAMP mustard can selectively block M_3 receptor mediated responses.

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MUSCARINIC RECEPTORS IN HUMAN CILIARY MUSCLE IDENTIFIED BY POLYMERASE CHAIN REACTION (PCR) AND IMMUNOPRECIPITATION

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Contraction of the ciliary muscle enhances the outflow of aqueous humor from the anterior chamber of the eye. This is thought to be the mechanism by which muscarinic agonists, such as pilocarpine, lower the intraocular pressure of glaucoma patients. Pharmacological studies with subtype-selective muscarinic receptor antagonists have suggested that the M_3 subtype mediates the muscle contraction, but drugs do not distinguish between all five receptor subtypes. Reverse transcription PCR was carried out on RNA isolated from human ciliary muscle using primer pairs complementary to regions of unique sequence in each of the five muscarinic receptor genes. Following 30-40 cycles of amplification, a fragment encoding the human m3 receptor, with predicted size and restriction enzyme sites, was the major product. A low level of the m2 subtype was also detectable. Because PCR is not a quantitative technique, we also used subtype-selective antisera, generated in the lab of B. Wolfe, to immunoprecipitate solubilized ^{3}H-QNB labeled receptors. Almost all (95%) the muscarinic receptors were recognized by the m3 antisera. The remaining receptors were precipitated by the m2 antisera. These results contrast to data on rat cortex generated in parallel experiments in which significant amounts of m1-4 subtypes were detected. Thus, the immunoprecipitation studies confirm pharmacological and PCR results that the muscarinic receptors in the human ciliary muscle are almost entirely of the m3 subtype.

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HIMBACINE DISCRIMINATES BETWEEN TWO M_1 RECEPTOR-MEDIATED RESPONSES

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Neuronal muscarinic receptors mediating the depolarization of rat superior cervical ganglion (RSCG)[1] and the inhibition of electrically-induced twitch contraction of rabbit vas deferens (RVD)[2] are classified as M_1 sites. We have investigated the antagonistic properties of the muscarinic antagonist himbacine[3] (pK_i at human m_1 receptor 6.97)[4] in comparison with those of pirenzepine (pK_i 8.20)[4] in these two preparations. RSCG and RVD were set up as previously described[1,2]. In the RSCG, 5-methylfurmethide caused concentration-related depolarization, (pEC_{50} 7.31±0.02), while McNeil-A-343 concentration-dependently reduced RVD twitch contractions (pEC_{50} 5.81±0.07). Affinity estimates for pirenzepine (30-300 nM) in the two models were similar; conversely, himbacine discriminated between RSCG and RVD M_1 receptors, antagonizing the M_1-mediated depolarization in RSCG (0.1-3 μM) with a 9-fold lower affinity (**=P<0.01) compared with the RVD M_1 site (10-300 nM).

COMPOUND	pA_2	RSCG slope	pA_2	RVD slope
Pirenzepine	7.78 ± 0.08	1.0 ± 0.10	8.13 ± 0.25	1.0 ± 0.21
Himbacine	7.14 ± 0.06**	1.0 ± 0.06	8.08 ± 0.08	1.2 ± 0.09

The affinity profile shown by himbacine suggests that the M_1 receptors of RSCG and RVD may be heterogeneous.

1. Newberry, N.R., & Priestley, T. (1987). Br. J. Pharmacol. 92, 817-826.
2. Eltze, M. (1988). Eur. J. Pharmacol. 151, 205-221.
3. Gilani, S.A.H., & Cobbin, L.B. (1986). Naunyn-Schmied. Arch. Pharmacol. 332, 16-20.
4. Dörje, F., et al. (1991). J. Pharmacol. Exp. Ther. 256, 727-733.

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VARIATIONS IN THE DEGREE OF ALLOSTERIC INTERACTION WITH MUSCARINE RECEPTOR ANTAGONISTS.

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The bisquaternary muscarine receptor antagonist heptane-1,7-bis-(dimethyl-3'-phthalimido propyl ammonium bromide) (C_7/3-phth) was evaluated against [^{3}H]pirenzepine in rat cortex and [^{3}H]quinuclidinyl benzilate in rat heart and rabbit lung. The K_i values (nM) (95% confidence limits) obtained for interaction at putative M_1 (cortex), M_2 (heart) and M_4 (lung) receptors were 313 (179 - 545), 70.1 (23.0 - 213) and 114 nM (74.2 - 176) respectively. C_7/3-phth is known to interact allosterically at muscarine receptors in the heart and this was also found in the rat cerebral cortex. C_7/3-phth (10 µM) slowed the offset rate of [^{3}H]quinuclidinyl benzilate by a factor of 2.1 ± 0.1 and of [^{3}H]N-methyl scopolamine by a factor of 2.1 ± 0.3. The offset rate of [^{3}H]pirenzepine was however unaffected.

Studies on M_2 receptors in the guinea pig isolated left atrium and on M_1 receptors in the rabbit isolated vas deferens showed that C_7/3-phth also interacted allosterically with pirenzepine at these sites to a lesser extent than with either atropine or N-methylscopolamine. In these experiments allosteric interaction was assessed from the degree of supra-additivity occurring with combinations of C_7/3-phth and the other antagonists using carbachol as the agonist in atrium and McN-A-343 in the rabbit vas deferens.

These findings suggest that the nature of the competing ligand is important in determining the magnitude of the allosteric effect with C_7/3-phth.

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DIFFERENCE IN THE AFFINITY OF McN-A-343 FOR MUSCARINE RECEPTORS IN SMOOTH AND CARDIAC MUSCLE.

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Studies with antagonists and receptor antibodies have suggested that the majority of the muscarine receptors in intestinal smooth muscle are of the M_2 subtype similar to those in cardiac muscle.

Investigations were undertaken with a partial agonist at muscarine receptors, McN-A-343, to compare its affinity for muscarine receptors in the smooth muscle of the guinea pig taenia caecum with those in the atrium.

In the taenia, suspended in McEwen's solution, McN-A-343 produced only 72.3% of the maximal response to carbachol with an EC_{50} of 1.2 μM. The K_A (dissociation constant) for McN-A-343 determined by comparing equiactive concentrations of the two agonists was 4.6 μM. Displacement binding studies with McN-A-343 versus [^{3}H]QNB in McEwen's solution containing Gpp(NH)p (50 μM) gave a K_I value of 5.6 μM for binding to a single site and this value was not altered significantly ($P > 0.05$) when binding was performed in 50 mM phosphate buffer. McN-A-343 in concentrations up to 0.3 mM failed to affect oxotremorine-induced inhibition of the increase in cAMP produced by isoprenaline in the taenia.

In guinea pig isolated left atrium set up in McEwen's solution McN-A-343 (50 to 300 μM) was used as an antagonist of the negative inotropic response to carbachol. The K_B for McN-A-343 estimated by the method of Kaumann and Blinks (1) was 13.1 μM. Binding studies with [^{3}H]QNB and McN-A-343 in McEwen's solution containing Gpp(NH)p (50 μM) gave a K_I value of 14.8 μM. In phosphate buffer this value was 7.0 μM. Both the atrial K_B value and K_I estimate in McEwen's solution were significantly different ($P < 0.05$) from the K_A and K_I values obtained in the taenia.

It is concluded that McN-A-343 exhibits a different affinity for muscarine receptors in the two tissues and that it is unable to detect a binding site in the taenia similar to that observed in cardiac muscle.

1. A.J. KAUMANN and J.R. BLINKS. Naunyn-Schmiedeberg's Arch. Pharmacol. 311 241-248 (1980).

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GTP-γ-S MODULATION OF AGONIST AFFINITY AT m1 RECEPTORS EXPRESSED IN A9 L CELLS

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The receptor binding properties of muscarinic agonists with varying efficacy were examined in A9 L cells expressing m1 receptors.

Agonist affinity was determined by the inhibition of [^{3}H]-(R)-QNB binding. Activity was assessed by examining the effects of GTP-γ-S on agonist affinity (Potter & Ferrendelli. *J. Pharmacol. Exp. Therap. 248:* 974-978, 1989). IC_{50} values and Hill slopes were determined from Hill plots of the inhibition data. GTP-γ-S lowered the affinity of carbachol by four fold from 17 ± 9.8 μM to 51 ± 15 μM. GTP-γ-S was less effective in lowering the affinity of arecoline from 5.6 ± 1.2 μM to 8.3 ± 1.0 μM. The 3-methyl-1,2,4-oxadiazole derivative of arecoline was synthesized and showed higher affinity than arecoline, yet the magnitude of the GTP-γ-S effect was comparable (IC_{50} shift from 0.71 ± 0.14 μM to 1.6 ± 0.67 μM).

The data indicate that GTP-γ-S decreases agonist affinity for m1 receptors, and that the magnitude of the shift may be related to efficacy. The partial agonist character of arecoline is consistent with PI metabolism data obtained in the CNS and in measures of cAMP formation in A9 L cells expressing m1 receptors (Novotny & Brann, *TIPS supplement*, 116, 1989). Furthermore, the data indicate that substituting the ester with a 1,2,4-oxadiazole moiety increases ligand affinity but not efficacy at m1 receptors. In summary, GTP-γ-S modulation of agonist affinity provides a useful measure of agonist activity in cells expressing single subtypes of muscarinic receptors.

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ACTIVATION BY FULL AND PARTIAL AGONISTS OF M1 TO M5 HUMAN MUSCARINIC RECEPTORS EXPRESSED IN A9L OR CHO CELLS.

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Interactions of muscarinic agonists with muscarinic receptors were studied in A9L or CHO cells transfected with human genes for one of the five receptor subtypes. Receptor densities were determined by Scatchard analysis using [3H]NMS as ligand. Bmax values (fmols/mg protein) were: A9Lm1 328, CHOm2 560, A9Lm3 623, CHOm4 531, CHOm5 415. Receptor activation was assayed by determining the formation of [3H]IP1 at m1, m3 and m5 receptors or changes in levels of cAMP at m2 and m4 receptors. Oxo-M (oxotremorine-M), carbachol, and muscarine were full agonists at all five receptor subtypes. McN-A-343, RS 86, arecoline, bethanechol and pilocarpine distinquished among the receptor subtypes, displaying full, partial or no agonism. All agonists tested were full agonists for the inhibition of forskolin-stimulated cAMP formation in CHOm4 cells. At high agonist concentrations (μM to mM), some agonists stimulated the formation of cAMP at CHOm4 receptors. For this response, the agonists fell into three categories: Oxo-M was significantly more active than carbachol, muscarine and oxotremorine whereas McN-A-343, arecoline, pilocarpine, RS 86 and bethanechol were weakly active or inactive. Both the inhibitory and stimulatory responses were fully antagonized by 100 nM atropine. The inhibitory response desensitized after 30 min preincubation with 0.3 μM carbachol whereas the stimulatory reponse to 1 mM carbachol did not appear to desensitize during this period. The stimulation of cAMP formation by 1 μM to 1 mM Oxo-M was abolished by 15 min incubation with 10 nM propylbenzilylcholine mustard (PrBCM). In contrast, the inhibitory response elicited by 0.1 to 100 nM Oxo-M was shifted 15 fold by 30 nM PrBCM with no change in the maximum response. Although m2 and m4 receptors were expressed in the same cell line and at similar densities, Oxo-M could differentiate between them. It was about 10 times less active in eliciting the inhibitory response and did not stimulate cAMP formation in CHOm2 cells. The high degree of homology between m2 and m4 receptors does not preclude differences in receptor-effector coupling or efficiency.

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BINDING TO AN ALLOSTERIC SITE ON MUSCARINIC RECEPTORS.

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All the muscarinic antagonists that we have tested to date were able - at concentrations close to 1 mM - to slow the (^{3}H)NMS dissociation rate. We therefore thought that another test was needed to distinguish drugs interacting with the muscarinic binding site as opposed to the allosteric site.

d-Tubocurarine could prevent (^{3}H)NMS dissociation from rat cardiac muscarinic receptors (EC_{50} 10 μM) but increased only 2-3-fold their (^{3}H)NMS Kd value. We therefore assumed that it might inhibit competitively the effects of other drugs which bind to the allosteric site, thereby preventing their effect on (^{3}H)NMS binding.

The gallamine competition curve was indeed shifted to higher concentrations in the presence of d-tubocurarine, indicating that these two compounds share the same (allosteric) site on muscarinic receptors. In contrast, the AF-DX 116, atropine, dexetimide and levetimide competition curves were barely affected in the presence of d-tubocurarine : these compounds do not recognize the allosteric site at low concentrations. We are applying the same test to other competitive and putative allosteric drugs.

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IS THE PRESYNAPTIC MUSCARINIC AUTORECEPTOR IN THE GUINEA-PIG TRACHEA AN M2 RECEPTOR ?

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Pre- and postsynaptic muscarinic receptors of guinea-pig trachea have been suggested to belong to the M2 and M3 subtypes, respectively (see 1). However, the M3-selective antagonist HHSiD was nearly equipotent in increasing the electrically evoked release of acetylcholine (ACh) (trains of 100 pulses at 20 Hz every 30 sec) and in inhibiting the smooth muscle contractions (1). We have therefore determined presynaptic pA_2 values of some antagonists in an isolated trachea preparation described recently (1). Inhibition by oxotremorine (OT) of the electrically evoked release of [^{3}H]ACh was used as a parameter of presynaptic activity. Stimulation conditions were such (trains of 10 pulses delivered at 100 Hz every 10 sec for 5 min) that pA_2 values were not distorted by endogenous autoinhibition. Scopolamine (0.01-0.1 μM) and HHSiD (0.1-1 μM) alone did not affect the evoked [^{3}H]ACH release. OT concentration-dependently inhibited the evoked release (-log EC_{50} 8.0). Dicyclomine (DIC) and HHSiD (both 0.1, 1.0 and 3.0 μM) caused parallel shifts of the concentration response curve for OT without affecting the maxima. The slopes in the Schild plots for HHSiD (0.91±0.08) and DIC (1.09±0.13) did not differ from unity. HHSiD (pA_2 7.3) and DIC (pA_2 7.0) had higher affinities to presynaptic autoreceptors than to M2 receptors of guinea-pig atria (pA_2 for both HHSiD and DIC 6.3). This suggests that the presynaptic autoreceptor does not belong to the M2 subtype but is rather similar to the M4 sites found in rabbit lung (2).

(1) Kilbinger et al., Br. J. Pharmac. 103, 1757, 1991. (2) Lazareno et al., Mol. Pharmacol. 38, 805, 1990.

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PHARMACOLQGICAL COMPARISON OF THE CLONED RAT AND HUMAN M2 MUSCARINIC RECEPTOR GENES EXPRESSED IN THE MURINE FIBROBLAST (B82) CELL LINE.

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Amplification of the coding sequence of the human m2 receptor gene was achieved by polymerase chain reaction (PCR), stably transfected into the murine fibroblast B82 cell line via the eukaryotic expression vector pHßAPr-1-neo. We have compared the human clonal cell line HM2-B10 to the previously cloned rat m2 M2LKB2-2 cells in order to assess drug specificity, drug selectivity and effector coupling. The human and rat clones showed high level of specific, saturable [^{3}H](-)MQNB binding, with K_d values 243±(155-352)pM and 345±(234-593)pM and B_{max} values 96.9±3.8 fmol/10^6 cells and 338±16 fmol/10^6 cells, respectively. Inhibition of [^{3}H](-)MQNB binding to HM2-B10 cells and to M2LKB2-2 cells showed the same rank order potency of the antagonists atropine > dexetimide > 4-DAMP > himbacine > methoctramine > AF-DX 116 > HHsID > PZ. Correlation analysis of the pK_i values indicate that the expressed human and rat M2 receptors have nearly identical ligand-binding characteristics. Carbachol, at 10 μM, suppressed the forskolin stimulated cAMP formation in both cell lines. In the M2LKB2-2 cells carbachol slightly stimulated the [^{3}H]inositol monophosphate (IP_1) formation, but in the HM2-B10 cells carbachol had no significant effect, probably due to the lower level of receptor expression. *In conclusion, the human and rat m2 receptors expressed in* B82 cell line have very similar binding properties and effector coupling mechanisms.

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ALLOSTERIC REGULATION OF [^{3}H]ACETYLCHOLINE BINDING TO m2 MUSCARINIC RECEPTORS.

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Many studies have established that a number of ligands are capable of interacting at an allosteric site on muscarinic receptors, thereby affecting the primary binding site. Relatively few of these studies have investigated the interactions between allosteric modulators and muscarinic *agonists*, and, to our knowledge, none has investigated the endogenous agonist, acetylcholine (ACh). This is due, in part, to the advantages of using high affinity antagonists that are not easily degraded in binding assays. For the study of competitive ligands, the nature of the labeled ligand is not critical, since competitive interactions will generalize to other labeled ligands. However, this is not the case for allosteric interactions, wherein the degree of cooperativity between two simultaneously bound ligands depends on the natures of both ligands. Ultimately, interactions between putative allosteric modulators and the endogenous agonist are the most important. Therefore, we labeled m2 muscarinic receptors expressed in CHO cells with [^{3}H]ACh and observed its rate of dissociation in the presence and absence of compounds known to exert allosteric effects on labeled antagonist binding. At 25°C, [^{3}H]ACh dissociated with a half-time of about 2 min. As expected, disruption of receptor-G protein complexes with guanine nucleotides led to the immediate (< 5 sec) dissociation of [^{3}H]ACh. On the other hand, inclusion of gallamine, tetrahydroaminoacridine, physostigmine, or obidoxime resulted in a concentration-dependent slowing of the rate of dissociation of [^{3}H]ACh. Thus, the binding of ACh to m2 muscarinic receptors is sensitive to allosteric actions of these modulators. [Supported in part by PHS R01 05214]

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DETECTION OF TWO MUSCARINIC RECEPTORS ON TRACHEOCYTES FROM THE HEN

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Employing the muscarinic antagonist ^{3}H-N-methyl-scopolamine (NMS) in radio-ligand displacement and saturation assays we find two dissociation constants, K_1 = 0.92 nM and K_2 = 16.9 nM, for NMS. The high affinity receptor represents 25.4 fmol/mg protein with a Hill coefficient of 1.54 while the low affinity receptor consists of a maximal binding capacity of 60.5 fmol/mg protein with a Hill coefficient of 0.92. The ratio between dissociation constants around 20 and the values for Hill coefficients are equal to vaules of these parameters found for atropine in functional studies of ACh-induced chloride secretion.

In another study we have found evidence for two separate muscarinic receptors in hen tracheal epithelium with dissociation constants and Hill coefficients comparable to the present .

This study supports the notion of two separate muscarinic cholinoceptors on tracheocytes regulating exocrine Cl^--secretion.

Winding B. & Bindslev N. Am J Physiol. 258: C982, 1990

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CHARACTERIZATION OF THE VASODILATORY MUSCARINIC RECEPTOR IN PERFUSED RAT KIDNEY BY THE USE OF AGONISTS AND ANTAGONISTS

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The muscarinic receptor mediating vasodilation in constant-pressure perfused (preconstriction by 0.1 μM cirazoline) rat kidney (RK) was characterized by subtype-prefering agonists and selective antagonists. The agonists produced vasodilation (rank order of potency: APE > 5-methyl-furtrethonium = methacholine = oxotremorine > (S)-aceclidine > bis-arecaidine-2-butyne-1,4-diol ester > 4-Cl-McN-A-343 = (R)-NAEE > NEN-APE = (R)-aceclidine = (S)-NAEE > McN-A-343) which disappeared after destroying the endothelium with detergent. -log ED_{50}'s of 16 agonists in RK significantly correlated with pD_2 values at M_3-receptors in guinea-pig ileum (GPI, r = 0.95). Potencies (pIC_{50}) of subtype-selective antagonists to attenuate vasodilation to 5 nmol APE significantly correlated with affinities (pA_2) derived from functional studies at M_3-receptors in GPI (r = 0.94), but differed from those at M_1- (r = 0.74) and M_2-receptors (r = 0.38) in rabbit vas deferens.

	RK: pIC_{50}	M_3: pA_2	M_1: pA_2	M_2: pA_2
Atropine	8.42	8.99	9.46	9.05
4-DAMP	8.30	8.83	9.12	8.16
(R)-HHD	7.51	8.35	8.71	6.83
HHSiD	7.26	7.76	7.92	6.54
UH-AH 37	6.96	8.07	8.64	7.15
Telenzepine	6.90	7.87	8.86	7.51
Mequitamium	6.72	8.14	8.68	8.36
Pirenzepine	6.19	6.87	8.08	6.23
Nuvenzepine	6.12	7.03	7.74	6.63
p-F-HHSiD	6.00	7.49	6.77	6.09
Himbacine	5.89	7.10	8.05	8.06
AF-DX 384	5.72	7.41	8.61	8.58
AQ-RA 741	5.40	6.97	8.44	8.62
(S)-HHD	4.64	6.07	5.97	5.25

These agonist and antagonist activities suggest that vasodilation elicited by muscarinic stimuli in endothelium-intact vasculature of rat kidney is mediated by muscarinic M_3-receptors.

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CHARACTERIZATION OF MUSCARINIC RECEPTORS IN GUINEA-PIG GALLBLADDER

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Acetylcholine contracts gallbladder smooth muscle via muscarinic receptors. We have recently reported that the functional muscarinic receptors of guinea-pig (g-p) gallbladder are not of M_1, M_2 and M_3 subtypes regarding the affinity profile of pirenzepine, AF-DX 116 and 4-DAMP (1). We present here the antagonistic affinities of various selective muscarinic antagonists obtained at both functional (pA_2 values) and radioligand binding (pK_i values) experiments (Table).

	pA_2	Slope	pK_i	n_H
Pirenzepine	7.9±0.1	0.8±0.1	7.7±0.2; 6.4±0.2	
4 DAMP	8.3±0.2	0.8±0.1	8.3±0.5	0.9±0.04
HHSİD	7.3±0.1	1.1±0.1	6.6±0.4	1.3±0.04
pfHHSID	7.6±0.1	1.0±0.1	6.1	0.9
Methoctramine	7.7±0.2	0.9±0.2	8.3	0.9
AF-DX 116	6.7±0.1	1.1±0.2	6.7	1.0
DABDMA	6.5±0.2	1.1±0.3		

Acetylcholine or carbachol was used as agonist in functional experiments. A comparison of affinity values indicates that the functional muscarinic receptors in g-p gallbladder display a novel pharmacological profile, which is not consistent with either M_1, M_2 or M_3 receptors. ^{3}H-QNB binds to g-p gallbladder smooth muscle homogenates with a K_D of 0.367±0.06 nM and a Bmax of 224±44 fmol/mg protein at 37 °C after 90 min of incubation. The analysis of the binding displacement curves with pirenzepine reveals the presence of two binding sites at this tissue one of which is consistent with M_2 and the other is in agreement with the functional receptor subtype. In conclusion, it is likely that g-p gallbladder smooth muscle cells have both M_2 and M_4 muscarinic receptor subtypes according to the binding data presented here. However, functional experiments do not support the contribution of the M_2 - subtype to the contractile response.

(1) Kurtel et al. Arch int Pharmacodyn Ther 1990; 308; 39-46.

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DFFERENTIATION BETWEEN MUSCARINIC RECEPTOR SUBTYPES COUPLED TO ADENYLATE CYCLASE AND PHOSPHOINOSITIDE HYDROLYSIS IN CANINE TRACHEAL SMOOTH MUSCLE CELLS

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To determine the muscarinic receptor (mAChR) subtypes involved in the contraction of tracheal smooth muscle, the mAChRs were characterized by a binding assay using [^{3}H]NMS and by functional approaches. Freshly isolated tracheal smooth muscle cells (TSMCs) are shown to possess high density (5.12 fmol/10^5cells) of mAChRs with K_D of 280 pM. Displacement of [^{3}H]NMS binding demonstrated mAChRs with low affinity for pirenzepine (K_i=220 nM), indicating that no M_1 receptor existed in TSMCs. Furthermore, investigation using AF-DX 116 and 4-DAMP revealed that M_2 and M_3 receptors existed in TSMCs. Activation of mAChRs led to decrease in cAMP, increase in IPs accumulation and contraction in tracheal smooth muscle. Pirenzepine (M_1 antagonist) displayed low affinity for antagonizing cAMP inhibition, IPs formation and contraction induced by carbachol (K_i=237, 80, and 35 nM). AF-DX 116 (M_2 antagonist) blocked cAMP inhibition with high affinity (K_i=33 nM) while it antagonized IPs formation and contraction with low affinity (K_i=190 and 90 nM). 4-DAMP (M_3 antagonist) inhibited IPs formation and contraction with high affinity (K_i=0.73 and 1.1 nM) and cAMP inhibition with low affinity (K_i=9.2 nM). There was a good agreement between the estimates for the inhibition constants of muscarinic antagonists for the competitive inhibition of [^{3}H]NMS binding and those measured by antagonism of the functional responses. These results conclude that: 1) both M_2 and M_3 receptors exist in TSMCs; 2) inhibition of cAMP formation may be coupled to the M_2 receptors; and 3) M_3 receptors appear to be predominant in mediating the carbachol-induced IPs formation and contractile response of tracheal smooth muscle. (supported by NSC81-0412-B182-4, CMRP-340 and 273).

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CHARACTERIZATION OF PREJUNCTIONAL MUSCARINIC AUTORECEPTORS IN THE RAT URINARY BLADDER

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Acetylcholine (ACh) release from cholinergic nerves of the rat urinary bladder is controlled by a prejunctional muscarinic negative feed-back mechanism. To characterize the type(s) of muscarinic receptor (MR) inhibiting the neurotransmitter release, we investigated in the rat urinary bladder strip the overflows of ^{3}H-ACh after pulse-labelling ACh stores with ^{3}H-choline. The increase by MR antagonists of ^{3}H-overflow evoked by field stimulation (3Hz,1ms,8V,540 pulses) was taken as a parameter for blockade of prejunctional MR. Simultaneously, the decrease of smooth muscle contraction was considered a measure for blockade of postjunctional MR. From concentration-response curves for pre- and postjunctional effects the concentrations which produced half-maximal response (-log EC_{50}) were determined and compared to the postjunctional affinities.

	Atropine	4-DAMP	LG50643	Pirenzepine	AF-DX116	Methocramine
pA_2(Schild plot)	9.40	9.21	9.00	6.77	6.19	5.82
$-logEC_{50post}$	8.47	8.13	7.60	5.97	5.60	5.10
$-logEC_{50pre}$	8.50	8.17	7.67	6.57	5.80	6.60

The comparison of the rank orders of affinity and potency values confirmed postjunctional MR as an M_3 receptor. The selectivity of methoctramine (30 fold) and the high potency of 4-DAMP for prejunctional MR might indicate that muscarinic autoreceptors in the rat urinary bladder are not either single M_1, M_2 or M_3 subtypes or a heterogeneous mixture of these subtypes.

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MUSCARINIC RECEPTORS IN THE RAT ISOLATED PORTAL VEIN.

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The rat portal vein displays spontaneous myogenic activity. When stimulated by carbachol (CCh) the amplitude of the rhythmic contractions increases and, at higher doses a tonic contracture develops. We investigated the characteristics of muscarinic stimulation in isolated portal veins of male Wistar rats by measuring the isometric contractile activity. After a 1 h period of equilibration cumulative concentration-response curves for the muscarinic agonists were constructed. The agonists used were (E_{max}-values in mN) of the increase of the amplitude and the contracture, respectively, are given in brackets; Mean±S.E.M., n=6; - indicates no response): arecoline (16.1±1.03, -), aceclidine (15.5±1.75, 11.7±1.33), bethanechol (15.4±1.75, 11.5±1.43), acetylcholine (15.3±2.75, 11.6±1.36), oxotremorine (15.2±1.22, -), muscarine (13.6±1.56, 16.4±1.67), CCh (12.5±0.-54, 14.3±0.53), metacholine (12.3±0.57, 11.0±1.75), McN 343 (7.1±0.93, -), and pilocarpine (7.1±1.00, -), respectively. In order to characterize the receptor subtypes involved pA_2-values for several muscarinic antagonists were determined for both the phasic and the tonic response. Atropine (unspecific) 9.68±0.07, 9.98±0.11; pirenzepine (M_1) 6.90±0.15, 7.25±0.25; AF-DX 116 (M_2) 6.54±0.06, 6.78±0.17; 4-DAMP (M_3) 9.55±0.14, 9.84±0.08; p-FHHSiD (M_3) 7.53±0.10, 7.87± 0.15. According to the pA_2-values of the antagonists found in other tissues (Eglen & Whiting 1990, *J.Auton.Pharmacol.* **19**, 233), we conclude that both types of response to muscarinic stimulation are mediated predominantly by the muscarinic M_3-receptor subtype.

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ESTROGEN MODULATION OF CHOLINERGIC RECEPTORS IN GUINEA-PIG MYOMETRIUM

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Ovarian steroid treatment modifies the contractile response to various neurotransmitter substances, including cholinergic drugs in different tissue from various animal species. It was therefore of interest to obtain information on estrogen induced changes in muscarinic receptor density and/or affinity in myometrial membranes from guinea-pigs. Prepubertal female guinea-pigs (Dunkin Hartley 200-220 g) were pretreated s.c. for 4 days with 200 µg/Kg 17ß-estradiol in a peanut oil vehicle (E-treated) and a second group of animals was injected in parallel with oil only (control). Uterine weights were controlled at the end of the treatment and are expressed as wet weight. Microsomal fraction was obtained from scraped myometrium as described in Maggi et al. (1988) and was stored frozen at -80°C until used for ligand binding studies. To study the muscarinic receptors in guinea-pig myometrium from control and E-treated uteri, the membrane fraction was incubated with ^{3}H(-)QNB. The E-treatment induces a significant increase in uterine weight ranging from 198.5±20.3 to 686.9±33.2 mg (N=12, P<0.001). Radioligand binding studies reveal a significant reduction in E-treated myometrium receptor density (B_{max}) as compared to that of the control group, while K_D values remain unchanged as shown in the following table:

Myometrium	B_{max} [fmol/mg]	K_D [nM]	N of exp.
Control	1194.5±184.4	0.19	4
E-treated	564.7±32.9 (P< 0.05)	0.18	3

This work was supported by grants from CNR (Aging project)

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[^{3}H]AFDX384: CHARACTERIZATION OF BINDING AND AUTORADIOGRAPHIC LOCALIZATION

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The Thomae Pharmaceutical Company introduced a compound, AFDX384, which appears to be highly selective for the M_2 receptor subtype. We have used the tritiated form ([^{3}H]AFDX384) (Dupont NEN) to characterize M_2 receptor binding in rat brain tissue homogenates and to localize these receptors via autoradiography. Our results indicate that the compound binds selectively and reversibly to a single class of receptors whose binding characteristics and/or receptor density differ from those labeled by [^{3}H]Pz (M_1), [^{3}H]QNB + 100nM Pz (non M_1), or [^{3}H]AFDX116 (the predecessor of AFDX384). Nonlinear regression analyses of kinetic binding experiments revealed a k_{+1} = .0078 min^{-1} nM^{-1}, a k_{-1} = .053 min^{-1}, and calculated k_d = 6.79nM. Nonlinear regression analyses of saturation experiments suggest a k_d = 7.8nM and B_{max} = 37fmol/mg tissue. Because [^{3}H]AFDX384 appears to be somewhat unstable, these experiments were conducted at 4°C and the results closely approximate values derived from room temperature experiments. Autoradiographic localization revealed high levels of specific binding in regions previously shown to be replete with M_2 receptor subtypes. The highest levels of binding were found in the caudate putamen, olfactory tubercle, thalamic nuclei, nucleus accumbens, superior colliculus and layer IV of the cortex.

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THE KINETICS OF DISAPPEARANCE AND THE ROLE OF GLYCOSYLATION IN THE POLAR EXPRESSION OF NATIVE AND EXPRESSED MUSCARINIC RECEPTORS IN *XENOPUS* LAEVIS OOCYTES

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Two native (**M**-Rs) and a cloned (**m1**-R) muscarinic receptors exhibit different polarity of hemispheric expression in ***Xenopus*** oocytes. The two native receptor subtypes are characterized by qualitatively different responses. They were identified by pharmacological techniques and by specific anti-sensense oligonucleotides as **M1**- and **M3**-like. While the density of the **M3**-Rs is comparable on both hemispheres, **M1**-Rs and **m1**-Rs exhibit marked preference for the animal hemisphere of the oocyte. Inhibition of total protein synthesis with cycloheximide or specific inhibition of **M1**-R or **M3**-R synthesis with complementary antisense oligonucleotides yielded muscarinic receptors' half-lives of 2.5-5 hrs. Inhibition of glycosylation with tunicamycin caused a rapid decrease of **M1**-R functional expression, though it did not affect the functional expression of either **M3**-Rs or **m1**-Rs. This effect of tunicamycin was not caused by decreased **M1**-Rs stability, since subsequent kinetics of functional expression decrease with cycloheximide did not change. We conclude that although functional receptor expression requires proper glycosylation for **M1**-Rs, it is not a general pre-requisite for the functional expression of muscarinic receptors.

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REGULATION OF m4 MUSCARINIC RECEPTOR GENE EXPRESSION.

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Each of the five muscarinic receptors has been shown to have a characteristic pharmacological profile and the mRNA coding for each receptor subtype has a unique expression pattern within the CNS. It is obvious from these studies that the expression of the muscarinic receptor subtypes is tightly regulated. This regulation is most likely to occur at the level of transcription and would be conferred by cis acting elements in the genome in the vicinity of the gene itself.

In order to investigate the transcriptional control of expression of these genes, genomic clones containing the coding region for the m4 gene have been isolated from a rat cosmid library. One of the clones isolated, R3-6, contains approximately 25kb of upstream sequence from the coding region and approximately 10kb of downstream sequence. Since a full length cDNA clone for the m4 gene is unavailable to aid in the location of the transcription initiation site within the genomic clone, primer extension, nuclease protection assays and PCR (polymerase chain reaction) techniques have been used in an attempt to identify this sequence within the clone.

A preliminary study was also performed to determine if the genomic clone contains sequences that will drive expression of the m4 gene and if it contains any tissue specific elements. Stably transfected cell lines were made using the cosmid clone and these were then assayed for m4 expression. The result obtained show that the cosmid clone is able to drive expression of the rat m4 gene when transfected into the human cell line IMR32, which endogenously expresses m4. No expression of m4 was seen when the cosmid clone was transfected into CHO cells which do not endogenously express the m4 gene. These data suggest that at least some of the tissue specific elements are present within this clone. Work is now proceeding to identify those sequences within the clone that are responsible for m4 specific expression.

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DISTINCT KINETIC BINDING PROPERTIES OF N-[^{3}H]-METHYL-SCOPOLAMINE AFFORD DIFFERENTIAL LABELING AND LOCALIZATION OF M_1, M_2 AND M_3 MUSCARINIC RECEPTOR SUBTYPES.

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Three classes of muscarinic receptors in mammalian brain have been postulated on the basis of equilibrium and kinetic binding data. However, equilibrium binding assays have not permitted the selective labeling and localization of the M_1, M_2 and M_3 receptor subtypes due to the overlapping affinities of muscarinic antagonists. We have developed a differential labeling strategy, based on the distinct binding kinetics of N-[^{3}H]-methylscopolamine (NMS) for the m1-m4 muscarinic receptor subtypes in A9L transfected cell lines, for the quantitative autoradiographic localization of the M_1, M_2 and M_3 muscarinic receptor subtypes in the primate brain. The M_1 (m1) receptor was directly labeled with 3 nM [^{3}H]-pirenzepine. The M_2 (m2) subtype was labeled with short (2 min) pulses of [^{3}H]-NMS following a preincubation with 0.3 μM pirenzepine to occlude the m1, m3 and m4 sites. Selective labeling of the M_3 (m3) receptor was obtained by preincubating with 0.5 nM unlabeled NMS for 5 min, to partially occlude the m1, m2 and m4 sites, prior to a 60 min incubation with 0.5 nM [^{3}H]-NMS, followed by a 60 min dissociation in the presence of 1 μM atropine. Autoradiographic analysis demonstrated that the distribution of M_1 and M_2 subtypes corresponded well to previous studies. The kinetic labeling strategy described here also permitted the visualization of the M_3 receptor subtype. The M_3 receptor distribution was largely coincident with the pattern of M_1 sites. In the primate brain, M_3 receptors were most prevalent throughout the superior and inferior temporal gyri, the hippocampus, the anterior and dorsal sectors of the caudate nucleus, and certain thalamic nuclei. This approach for localizing M_1, M_2 and M_3 receptor subtypes may help clarify the observed heterogeneity of muscarinic receptor mRNAs with the mapping of the distribution of the putative receptor proteins. (Supported by NS19065 and NS25785).

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MUSCARINIC TOXIN 1 (MT-1) RECEPTORS IN THE RAT BRAIN: QUANTITATIVE AUTORADIOGRAPHIC LOCALIZATION

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Muscarinic toxin 1 (MT-1), a protein toxin, has been isolated from the green mamba (Dendroaspis angusticeps) venom by gel filtration on sephadex G-50, chromatography on the ion-exchangers Bio-Rex 70 and Sulphopropyl-Sephadex C-25 and reversed-phase HPLC. MT-1 has 64 amino acids and four disulfides and a formula weight of 7200. Ultracentrifugation gave a molecular weight of 6900. MT-1 was labelled by ^{125}I and used for autoradiographic studies.
The distribution of MT-1 receptors in rat brain sections (10μm) was studied by quantitative autoradiography. High densities of MT-1 receptors were found in the striatum, amygdaloid nucleus, nucleus accumbens, hippocampus and cortex. Low densities of MT-1 were found in the hypothalamus, brain stem, cerebellum and white matter. The localization of MT-1 receptors was similar to those of the muscarinic M1 receptors (as labelled by ^{3}H-pirenzepine). Moreover, pirenzepine displaced ^{125}I-MT-1 from its receptors with high affinity. From our results it is possible to suggest that MT-1 is a novel ligand that binds specifically to the M1 muscarinic receptors.

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DEVELOPMENT AND UTILIZATION OF A PANEL OF ANTISERA SELECTIVE FOR EACH OF THE SUBTYPES OF MUSCARINIC CHOLINERGIC RECEPTOR.

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Using fusion proteins we have produced a set of polyclonal antisera that have been characterized using an immunoprecipitation protocol. Cells transfected with the cDNA encoding a single subtype of muscarinic receptor were used to demonstrate that each antiserum precipitated >90% of the appropriate subtype with no (<2%) precipitation of any other subtype. These selective antisera were then used to quantitatively assay subtypes of muscarinic receptors in a variety of tissues and experimental paradigms.

The determination of the distribution of these receptors in rat brain demonstrates that m1, m3, and m4 receptors are found in highest concentrations in the rostral portions of the brain (cortex, hippocampus, striatum, olfactory tubercule) with lower levels found in more caudal areas (thalamus/hypothalamus, pons/medulla, and cerebellum). m2 receptors are more evenly distributed while m5 receptors have been reliably found only in midbrain, striatum, and hippocampus. In peripheral tissues, m3 receptors were highest in the parotid while m2 receptors predominated in the lung, ileum, and bladder of the rat.

Several clonal cell lines were examined with surprising results. For example, SK-N-SH cells express three subtypes (m1, 32%; m2, 22%; m3, 42%) at high density.

Chronic administration of atropine to rats results in strong upregulation of m3 (70%), mild upregulation of m1 and m4 (10-20%), and no change in m2 receptors. Fimbria-fornix lesion results in a very similar pattern with strong upregulation of m3 (80%), mild upregulation of m1 and m4 (10-25%), and a decrease (25%) in m2 receptors. These results suggest that m3 and probably m1 and m4 receptors are, in general, localized postsynaptically to ACh while at least a fraction of the m2 receptors are localized on presynaptic neurons originating in the septum.

The ontogeny of each of the subtypes was determined in rat brain. The density of each receptor increased dramatically during the second week following birth and no major differences between subtypes were observed.

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m3-MUSCARINIC RECEPTOR SPECIFIC ANTISERA FOR USE IN STUDIES OF MUSCARINIC RECEPTOR DESENSITISATION

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Stimulation of CHO cells transfected with human m3-muscarinic receptor cDNA results in a biphasic production on Ins(1,4,5)P_3, the early phase of which is susceptible to rapid agonist induced desensitisation. In order to dissect the molecular mechanism of this desensitisation event we set out to produce an m3-receptor specific antisera. A region of the third intracellular loop of human m3-receptor cDNA (nucleotides 1132-1489) was amplified by PCR and subcloned into a bacterial expression plasmid to produce a fusion protein with glutathione-S-transferase (Pharmacia). The resulting 43Kd fusion protein was purified and used to immunise two rabbits. Antisera was screened by Western blots of bacterial extracts. The final bleed (following seven immunisations) produced high titer antisera against the m3-receptor portion of the fusion protein that was readily purified from the glutathione-S-transferase specific antisera. When used in Western blots of membranes from CHO cells transfected with m1-m5 human muscarinic receptor cDNA, only the lane from the m3-receptor transfects showed positive immunoreactivity. In this case a diffuse 97Kd band comprising of 2-4 closely running bands was observed. Immunoprecipitation of solubilisation receptors from CHO/m3 transfects labelled with ^{35}S-methionine also isolated a diffuse band running at approx 97Kd that was superimposable with that identified by Western blotting. This band was not present in non-transfected CHO cells. This antisera is now being used in studies of post-translational modification of m3-receptors following agonist stimulation.

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MUSCARINIC RECEPTOR-MEDIATED CONTROL OF HIPPOCAMPAL BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) mRNA EXPRESSION

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Quantitative in situ hybridization and northern blot analysis techniques were used to determine the effects of removal of the cholinergic input on levels and topographical distribution of BDNF mRNA in the hippocampus of adult rats. First, the effects of partial and full fimbrial transections, which result in, respectively, partial and near-total cholinergic deafferentation were compared. Twenty one days following partial unilateral fimbrial transections there were signficant decreases of BDNF mRNA expression throughout the hippocampal formation. The largest decreases were noted in pyramidal CA1 and CA3 layers and in the dentate gyrus. The decreases amounted to 22-36% reductions compared to unlesioned control animals. BDNF mRNA levels were decreased to a greater extent (50-69%) following full unilateral fimbrial transections. Quantitative northern blot analysis indicated that hippocampal BDNF mRNA (2.4 and 4.4 kb species) was decreased by 29% and 68%, 3 weeks after partial or full unilateral fimbrial transections, respectively. The extent of the reductions in BDNF mRNA levels correlated with reductions in acetylcholinesterase staining density and cholinergic terminal density determined by quantitative autoradiographic analysis of [^{3}H]vesamicol binding sites. Second, we found that chronic treatment with atropine (20 mg/kg for 14 days) decreased BDNF mRNA levels in the pyramidal CA1, CA2 and CA3 layers and in the dentate gyrus by 54%. In contrast, chronic treatment with nicotine (1.18 mg/kg for 14 days), a treatment known to desensitize nicotinic receptors, did not affect BDNF mRNA expression in the hippocampal formation. The findings provide evidence for cholinergic muscarinic regulation of BDNF mRNA expression in the adult rat hippocampal formation and they suggest the existence of a tonic stimulation of hippocampal BDNF mRNA synthesis by cholinergic afferents originating in the septum. (Supported by HFSPO, Japan and French Foundation For Alzheimer Research, Los Angeles, CA).

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ONTOGENY OF MUSCARINIC ACETYLCHOLINE RECEPTOR EXPRESSION AND IN VIVO MUSCARINIC RECEPTOR MEDIATED IMMEDIATE EARLY GENE INDUCTION IN THE RAT CNS.

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Postnatal ontogeny of muscarine receptor (mAChR) subtypes in CNS sections at days 1,7,14,21,28,35 and adult (3mo) was studied by quantitative ligand autoradiography, Northern analyses and in situ hybridization histochemistry (ISHH). Li was used in cerebral cortical slices (350x350um) to study inositol phospholipid (PI) hydrolysis by prelabeling with myo[-2-3H]inositol (0.5uM) in Krebs buffer ($95\%O_2$,$5\%CO_2$). [^{3}H](-)quinuclidinylbenzilate, a nonsubtype selective specific antagonist, [^{3}H]cismethyldioxolane, a label of super high affinity agonist states, [^{3}H]pirenzepine, an M1 antagonist, [^{3}H]AF-DX 384, an M2 antagonist and [^{3}H]hemicholinium-3, a Na+-dependent high affinity choline uptake inhibitor were each used as previously described. Quantitative ISHH was done via ^{35}S-labeled oligonucleotide probes for m1-m5 mRNA or cRNA for proto-oncogenes. Previous data show immediate early genes (IEG) respond rapidly and transiently to CNS mAChR stimulation in vitro and in vivo. In vivo basal expression and maximal induction of c-fos, c-jun, egr-1, jun-B and jun-D by oxotremorine (OXO;3mg/kg;ip) treatment was determined during ontogeny. Northern blot hybridization studies were done via ^{32}P-labeled cRNA probes with RNA extracted from rat CNS. Data show basal levels of some IEG transcripts are barely detectable, while many show significant induction and differential changes in levels of proto-oncogene expression at various postnatal times. Differential progressive changes in m1-m5 mRNA and mAChR levels occur. Increased mAChR density, in concert with varied mAChR-mediated IEG production is consistent with the concept that differential IEG induction may account for long term changes in cellular phenotype and transcription factors play a role in regulation of mAChR gene expression. (MH-43024).

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DIFFERENTIAL IN VIVO INDUCTION OF IMMEDIATE EARLY GENES IN THE CNS OF LONG- AND SHORT-SLEEP MICE VIA MUSCARINIC RECEPTOR ACTIVATION.

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Long- and short-sleep (LS/SS) mice show a differential sensitivity to ethanol (ET). Data show different responses to muscarine acetylcholine receptor (mAChR) agonist or antagonist treatment (Rx), CNS regional distribution of mAChR mRNA, mAChR subtypes and inositol lipid hydrolysis (Watson et al., EJP 183(5):1620, 1990). Pharmacogenetic approaches can be used to probe drug mechanisms. Immediate early gene (IEG) expression shows differential genomic responsivity. Data show significant differences in mAChR-mediated activation-transcription coupling in LS vs SS determined by Northern analyses and in situ hybridization histochemistry via cRNA probes. In vivo basal expression and maximal induction of c-fos, c-jun, egr-1, jun-B and jun-D by oxotremorine (OXO 0-5mg/kg;ip) Rx was assessed. RNA extracted from brains excised at t=0,15,30,45,60,90,120 and 180m was assayed by Northern analyses. Peak induction of c-fos mRNA (2.2kb) is 67x and 136x at 30 and 90m in SS and LS respectively. Significant basal c-jun RNA (2 transcripts of 2.9 and 3.6kb) is seen. SS c-jun mRNA peak induction is 2x at 60m. Basal egr-1 expression is seen, with peak induction (3x,120m) vs (7x,90m) in SS and LS, respectively. jun-D is only expressed constitutively but in high abundance. Significant basal jun-B expression is seen only in SS, with 3x peak induction at 90m that stays >180m. LS jun-B induction is significantly greater, with 40x peak induction at 45m and levels at basal by 180m. Induction kinetics for c-fos, egr-1 and jun-B are notably greater in LS. OXO induced expression of c-fos, c-jun, jun-B and egr-1 genes show characteristic dose-response relations to 0-5mg/kg Rx, which is blocked by scopolamine (10mg/kg;ip) Rx 15m before OXO Rx. Rapid and transient genomic responses may be mediated via mAChRs. Differential in vivo IEG induction may account for long term changes in cellular phenotypes. (MH-43024).

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1,2,3,4-TETRAHYDRO-9-AMINOACRIDINE (THA) MAY INTERACT WITH CHOLINERGIC PRESYNAPTIC RECEPTORS TO REGULATE *IN VIVO* ACETYLCHOLINE RELEASE IN THE STRIATUM OF ANESTHETIZED RATS

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1,2,3,4-Tetrahydro-9-aminoacridine (THA, tacrine) is a potent cholinesterase inhibitor which is under consideration for the treatment of Alzheimer's disease. This study examines the effect of microdialysis of THA on the extracellular concentrations of acetylcholine (ACh) in the striatum of the anesthetized rat using a *in vivo* brain microdialysis method. THA increased the extracellular concentration of ACh in a concentration dependent manner (1 μM - 1 mM). At concentrations producing equivalent effects on ChE activity, THA (100 μM) produced a smaller increase in the concentration of ACh than physostigmine (10 μM) both in the presence of atropine (1 μM). It has been well established that muscarinic presynaptic receptors participate in the regulation of the release of ACh, thus atropine increases ACh release by blocking the presynaptic receptors, i.e., blocking the feed back machanism. Since THA bound significantly to both muscarinic and nicotinic receptors in rat striatum while physostigmine did not show significant binding, the smaller increase in the concentration of ACh induced by THA in the presence of atropine is probably due to the direct interaction of THA with presynaptic cholinergic receptors.

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KINETICS OF SOLVOLYSIS AND *IN VIVO* MUSCARINIC RECEPTOR BINDING OF N-(2-BROMOETHYL)-4-PIPERIDINYL DIPHENYLACETATE: AN ANALOGUE OF 4-DAMP MUSTARD.

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N-(2-chloroethyl)-4-piperidinyl diphenylacetate (4-DAMP mustard) is known to irreversibly block muscarinic receptors through the formation of an aziridinium ion which selectively alkylates muscarinic receptor subtypes other than M_2. Recently, *in vivo* treatment with 4-DAMP mustard has been shown to irreversibly alkylate muscarinic receptors in both central and peripheral tissues. It is possible that the *in vivo* distribution of the alkylating agent may be influenced by the rate at which the aziridinium ion is formed. A rapid generation of the positively charged aziridinium ion may prevent the drug from reaching the brain, thus allowing for a selective antagonism of M_3 receptors in peripheral tissues only. In an attempt to increase the kinetics of aziridinium ion formation, we have prepared the bromine analogue of 4-DAMP mustard. N-(2-bromoethyl)-4-piperidinyl diphenylacetate (4-DAMP bromo mustard) was synthesized and the molecular formula confirmed by mass analysis. The 4-DAMP bromo mustard was shown to cyclize in phosphate buffer to the corresponding aziridinium ion with a first-order rate constant (k_1) of 0.07 min^{-1} at 0°C (pH 7.4). At 25°C and 37°C, the formation of the aziridinium ion was nearly instantaneous (100% cyclized within 20 seconds) at neutral pH. The rate constants (k_2) for the hydrolysis of the aziridinium ion at 25°C and 37°C (pH 7.4) were 0.0027 and 0.010 min^{-1}, respectively, in excellent agreement with the published rate constants for the hydrolysis of the aziridinium ion formed from 4-DAMP chloro mustard. *In vivo* treatment with 4-DAMP bromo mustard in rats resulted in an inhibition of muscarinic receptor binding in peripheral, but not central, tissues suggesting that the quickly formed aziridinium ion does not penetrate the blood brain barrier.

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PHARMACOLOGY OF MUSCARINIC RECEPTOR SUBTYPES MEDIATING SPINAL ANALGESIA IN THE RAT

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At present, three subtypes of muscarinic receptors have been defined pharmacologically (M_1, M_2 and non-M_1,non-M_2), although at least 5 distinct receptor genes have been identified by molecular biological techniques (m1-m5). Muscarinic receptors in rat spinal cord were assessed by *in vitro* binding techniques using the tertiary ligand ^{3}H-quinuclidinyl benzilate (^{3}H-QNB) and the quaternary ligand ^{3}H-*N*-methylscopolamine (^{3}H-NMS). As expected, ^{3}H-QNB labeled significantly more sites than ^{3}H-NMS (Bmax's = 578 ± 59 and 304 ± 35 fmol/mg protein, respectively), possibly reflecting access of the former ligand to a sequestered population of muscarinic receptors. Lumbar-sacral cord contained a higher density of ^{3}H-QNB binding sites than thoracic or cervical cord. Selective muscarinic antagonists inhibited binding in whole cord with the same rank order of potency for both ligands, i.e. 4-DAMP > methoctramine > AF-DX 116 > pirenzepine. Nonlinear regression analysis of the pirenzepine and methoctramine inhibition curves indicated 5-10% were M_1- and 90-95% were M_2-receptor subtypes. Muscarinic systems in lumbar spinal cord mediate the analgesic effects of cholinergic agonists administered intrathecally (i.t.). For example, (+)-*cis*-methyldioxolane produced analgesia in the tail-flick and hot-plate assays, with an ED50 of about 10 nmol. These analgesic effects were antagonized by low doses of the M_1 selective antagonist pirenzepine (ID50 ≈ 1.0 nmol, i.t.) and by methoctramine, an M_2 selective antagonist (ID50 ≈ 7.0 nmol, i.t.). Thus, both M_1 and M_2 subtypes are involved in the spinal analgesia produced by cholinergic drugs. (Supported in part by NS 28847 to ETI).

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CHRONIC NGF TREATMENT ALTERS HIPPOCAMPAL MUSCARINIC RECEPTOR FUNCTION IN RATS WITH FIMBRIAL TRANSECTIONS.

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The rationale for proposing that nerve growth factor (NGF) be used in the treatment of Alzheimer's disease is based upon pharmacological studies which show that chronic administration of rhNGF increases presynaptic cholinergic functions in the hippocampus of rats following fimbrial transections (Lapchak and Hefti, 1991). However, potential effects of chronic rhNGF on postsynaptic cholinergic markers have not been investigated previously. The present work assessed whether chronic rhNGF treatment (21 days) alters postsynaptic muscarinic receptors in the hippocampus of rats with unilateral fimbrial lesions, by determining whether muscarinic site densities and receptor-linked second messenger systems are affected. In cytochrome c (cc)-treated control rats, total muscarinic site densities ipsilateral to the lesion, determined using [^{3}H]QNB as ligand, were not changed compared to the contralateral unlesioned side. In addition, alterations in muscarinic-M_1 or M_2 sites, assessed using [^{3}H]pirenzepine and [^{3}H]AF-DX 384, respectively, were not observed. Similarly, in rats treated with rhNGF, muscarinic receptor site densities were not different in the lesioned compared to the unlesioned hippocampus, nor were they different from those quantitated in the cc-treated rats. In contrast, following full fimbrial transections, there was a discernable upregulation of muscarinic sites in the CA1 region (range: 30-35%) ipsilateral to the lesion in the rhNGF-treated, but not the control rats. Following partial fimbrial transections, the muscarinic-M1 receptor-mediated response, characterized by inositol triphosphate production by hippocampal slices, was increased by 61% on the lesioned side of cc-treated rats; this was not evident in the rhNGF-treated animals. rhNGF treatment did not alter oxotremorine-mediated increase in cyclic GMP levels in cc- or rhNGF-treated lesioned rats. In summary, the present findings suggest that the rhNGF-induced increases of presynaptic cholinergic functions in fimbriectomized rats may translate into functional changes at the level of postsynaptic muscarinic receptors in the hippocampus. (Supported by HFSPO and The French Foundation for Alzheimer's Research).

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ANTI-AMNESIC EFFECTS OF A NOVEL MUSCARINIC AGONIST, YM796.

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Accumulating evidence suggests that M_1 muscarinic agonists are possible agents for the symptomatic treatment of dementia of Alzheimer type. We herein report the further characterization of a novel muscarinic agonist, YM796 ((-)-S-2,8-dimethyl-3-methylene-1-oxa-8-azaspiro[4.5] decane L-tartrate monohydrate), compared with other cholinergic drugs. In vitro, YM796 exhibited preferential M_1 agonistic activity and had little or no affinity (Ki ≧85 μM) for other neurotransmitter receptors. In vivo, YM796 improved the learning deficit induced by scopolamine and the nucleus basalis magnocellularis-lesion at low doses (MED: 0.063 and 0.031 mg/kg po as salt, respectively), whereas induction of salivation, a peripheral cholinergic response, required much higher doses (ED_{30mg} = 22 mg/kg po). This selectivity of YM796 for anti-amnesic effects was much greater than that of other cholinergic agents, RS86, AF102B and tacrine. YM796 (MED: 0.2 mg/kg po) also improved the cognitive impairment induced by electroconvulsive shock in mice but tacrine did not up to 30 mg/kg po. In addition, anti-amnesic efficacy on the rat model of working memory was also observed. Thus, YM796 possesses M_1 agonistic activity and preferentially improves the cognitive impairment in various animal models presumably by the activation of M_1 receptors in the brain.

Note) MED; minimum effective dose, ED_{30mg}; the doses required to increase the salivary secretion by 30 mg.

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THE ROLE OF THE MUSCARINIC RECEPTORS SUBTYPES IN SLEEP REGULATION

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Our previous papers[1,2] suggested that specific muscarinic receptor subtypes (M_1, M_2 and M_3) are differentially involved in the cholinergic regulation of the sleep-wake cycle, particularly desynchronized sleep (DS) generation. Direct injection of highly selective muscarinic antagonists into the pontine reticular nucleus were performed in freely moving rats, in order to elucidate the anatomical localization of the muscarinic receptor subtypes involved in such control. Microinjections of carbachol (0.5 μg dose) induced a 52 % increase in DS in 6 hours' recordings of cerebral electric activity (by means of EEG), ocular movements (by EOG) and muscular electric activity (by EMG). Methoctramine, a highly selective M_2 muscarinic antagonist, induced a significant and dose-related decrease in the amount of DS (-44 %, -51 % and -68 %) and an increase in DS latency (+123 %, +150 % and +345 %) in comparison to control condition, following administration of 5, 10 and 15 μg doses, respectively, in 6 hours' recordings. The present results suggest that pontine muscarinic M_2 receptors are necessary for DS generation and maintenance.

References.
[1] L. Imeri et al., NeuroReport, 2:383-385, 1991.
[2] L. Imeri et al., NeuroReport, 3:276-278, 1992.

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INCREASED SUSCEPTIBILITY TO PYRETHROID (BIOALLETHRIN) EXPOSURE IN THE ADULT MOUSE NEONATALLY EXPOSED TO DDT - ALTERATIONS IN MUSCARINIC CHOLINERGIC RECEPTORS AND BEHAVIORAL VARIABLES

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DDT and bioallethrin have been shown to cause similar changes in muscarinic cholinergic receptors (MAChR) in neonatal mouse brain when given during the rapid brain growth. This neonatal exposure also led to similar permanent changes in MAChR and behavioral disturbances in the animals as adults. The purpose of the present study was to investigate whether neonates exposed to DDT would show an increased susceptibility to bioallethrin as adults and if the reaction is permanent. Ten day-old mice received an oral dose of DDT (0.5 mg/kg bw). At the age of 5 months the mice received bioallethrin 0.7 mg/kg bw/day per os for 7 days, controls received the vehicle. Behavioral testing, 24 hr after the last dose, showed significant differences both in mice receiving DDT neonatally and bioallethrin as adults, and in mice receiving the vehicle as neonates and bioallethrin as adults. The changes developed in mutually opposite directions and at tne age of 7 months the disturbances were even increased in the mice exposed neonatally to DDT, receiving bioallethrin as adults. In mice receiving the vehicle as neonates and bioallethrin as adults the disturbances were gone. Muscarinic receptor density, assayed in P2 fractions of cerebral cortex using ^{3}H-QNB, was significantly increased in mice receiving DDT as neonates and bioallethrin as adults. This increase persisted two months later. In conclusion this study shows that exposure to DDT during the peak of the brain growth spurt cause disturbances in brain function that increase the animals susceptibility to exposure of similar neurotoxic agents as adults.

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NEONATAL EXPOSURE TO A SINGLE SUB-SYMPTOMAL DOSE OF THE ORGANOPHOSPHATES DIISOPROPYL FLUOROPHOSPHATE OR PARAOXON INDUCES CHANGES IN BEHAVIOR AND MUSCARINIC DENSITY AT ADULT AGE

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Previous studies have shown an increased vulnerability of the cholinergic system to substances such as DDT, pyrethroids, and PCB's during rapid development of the neonatal mouse brain.

In this study an oral dose of either **diisopropyl fluorophosphate (DFP)** 0.075, 0.75, or 1.5 mg/kg body weight (bw), **paraoxon** 0.05, 0.5, or 1.0 mg/kg bw, or the vehicle, was given to the neonatal mouse. The degree of acetylcholinesterase (AChE) inhibition were approximately equal between the substances for the high, medium, and low dose respectively. Behavioral testing performed at the age of four months revealed that the two highest doses of DFP induced a significant ($p<0.05$) increase in spontaneous motor activity. The two higher doses of paraoxon induced a significant ($p<0.05$) decrease in spontaneous motor activity. Muscarinic receptor (MAChR) density, assayed in P2 fractions of the cerebral cortex of the adult mouse by using [^{3}H]QNB, showed dose-dependent alterations in receptor density between doses for both substances. DFP treated animals showed a dose dependent increase in density, whereas the paraoxon treated mice showed a dose dependent decrease i receptor density. The proportions of high-affinity or low-affinity binding sites, assayed in a displacement study using [^{3}H]QNB/carbachol, were not changed. **This study shows that two organophosphorus compounds causing equal AChE-inhibition induces different alterations in behavior and MAChR density in adult mice, when administered to the mice as neonates.**

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[^{3}H]AF-DX 384/MUSCARINIC-M_2 RECEPTOR BINDING SITES ARE INCREASED IN CERTAIN CORTICAL BRAIN REGIONS OF AGED MEMORY-IMPAIRED AS COMPARED TO MEMORY-UNIMPAIRED RATS

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Long-Evans rats (24 months) were classified into cognitively impaired or unimpaired subgroups based on their spatial learning ability in the Morris Swim Maze task. Using quantitative *in vitro* receptor autoradiography, the status of putative muscarinic M_1 ([^{3}H]pirenzepine, 15 nM), M_2 ([^{3}H]AF-DX 384, 2 nM; [^{3}H]acetylcholine, 15 nM) and high-affinity choline uptake ([^{3}H]hemicholinium-3, 10 nM) binding sites was investigated. No statistical differences were observed for the muscarinic M_1 receptor subtype in the aged impaired (n=10) as compared to the aged unimpaired (n=10) group in all cortical (frontal, parietal, occipital, temporal and entorhinal cortices), hippocampal layers of the CA1 region and dentate gyrus and subcortical (caudate-putamen and medial septum) regions analyzed. In contrast, a significant increase in putative muscarinic [^{3}H]AF-DX 384/M_2 binding sites was observed in frontal (n=8) and parietal (n=8) cortices, and in the dentate gyrus of the hippocampus (n=9) in the aged impaired group. Similar results were obtained for putative [^{3}H]acetylcholine/M_2 binding sites although they failed to reach significance (n=6). [^{3}H]hemicholinium-3 binding site densities were unchanged in all regions analyzed (n=3-6). In addition, choline acetyltransferase activity was unchanged in the cortex, hippocampus and caudate-putamen of aged impaired as compared to unimpaired rats (n=4). Since muscarinic M_2 receptors are considered to act as negative autoreceptors, an augmentation of their densities could cause a decrease in the release of acetylcholine; this possibly being associated with memory impairments.

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EFFECT OF SELECTIVE MUSCARINIC ANTAGONISTS ON PUPIL DIAMETER IN RABBITS.

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Muscarinic receptors play an important role in the control of pupil size. The presence of muscarinic receptors has been shown in both sphincter (M_3) and dilator muscles (M_2) (Honkanen et al., Invest. Ophthalmol. & Vis. Sci., 1990; Suzuki et al., Invest. Ophthalmol. & Vis. Sci., 1982). In this study, we examined the effect of several selective antagonists on pupil diameter in vivo to determine the receptor subtype that controls pupil size in rabbits. Selective antagonists, pirenzepine (PZP, 10-100 nM), AF-DX 116 (10 &100 nM), methoctramine (10 nM), himbacine (10 & 100 nM), 4- diphenyl-acetoxy-N-methyl-piperidine (4-DAMP, 3 & 10 nM), silahexacyclium (SHX, 50 nM) or p- fluorohexahydrosiladifenidol (p-F-HHSiD , 20 & 100 nM) were injected intracamerally into eyes of pigmented rabbits and pupil diameter was measured every hour from time 0 to 6 hours. There was no change in pupil diameter with PZP, AF-DX 116, methoctramine or himbacine. Para-F-HHSiD and SHX caused some increase in pupil diameter of about 5-12% respectively and 4-DAMP caused 10-24% increase in pupil diameter. This suggests that the antagonists that are selective for M_3 subtype were the most effective in inhibiting the receptors that control pupil size of rabbits. This is in agreement with previous reports which showed the presence of M_3 receptor subtypes in the iris sphincter (Honkanen et al., 1990). The lack of effect of M_2 antagonists suggests that the M_2 receptors in the dilator muscle may have minor role in determining pupil size in rabbits.

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BRONCHOSPASMOLYTIC ACTIVITY OF THE NOVEL MUSCARINIC ANTAGONIST DAC 5889

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DAC 5889 ((-)-3-(R)-1-Azabicyclo [2,2,2] oct-3-yl 2-oxo-5-phenylpiperidine-5-carboxylate) is a novel muscarinic ant-agonist with high affinity towards M_1 (pK_i = 8.3), intermediate for M3 (pK_i = 7.4) and low affinity for M_2-binding sites (pK_i = 6.4). It has been suggested that muscarinic antagonists with high affinity for M_1 and/or M_3-receptors might have a potential as bronchospasmolytics. Accordingly, the in vivo bronchospasmolytic activity and selectivity of DAC 5889 was investigated in guinea-pigs.
DAC 5889 (i.v.) inhibited the acetylcholine induced bronchoconstriction in 20-fold (ED_{50}=0.02 mg/kg) lower doses than the bradycardic effect (ED_{50}=0.40 mg/kg). Blockade of M_3-receptors may also result in an inhibition of salivation. Therefore the potency of DAC 5889 to antagonize the hypersalivation evoked by pilocarpine was examined. However, relative high doses were necessary (ED_{50}=0.70 mg/kg). The pronounced bronchoselectivity of DAC 5889 after i.v. administration was also observed in rats and rabbits and was less obvious for atropine or ipratropium bromide. Oral administration (0.3-3.0 mg/kg) of the active enantiomer of DAC 5889 (= DAC 6150) resulted in a significant and long lasting (1 mg/kg; > 4 h) inhibition of the acetylcholine induced bronchoconstriction without affecting the pilocarpine evoked hypersalivation. In conclusion: DAC 5889 is a novel muscarinic antagonist exhibiting bronchoselectivity after i.v. and p.o. administration in guinea-pigs.

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DENERVATION-INDUCED CHANGES IN HIPPOCAMPAL MUSCARINIC RECEPTOR SUBTYPES AND THEIR mRNAs

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Alzheimer's Disease is associated with cholinergic neuronal cell loss in the basal forebrain. Previous studies have shown that the major cholinergic projection to the hippocampus is derived from the medial septum and can be destroyed by lesions of the fimbria and fornix. The present study examined the expression of muscarinic receptor subtype mRNAs (m1, m3 and m4) by solution hybridization/RNase protection assay and the level of pharmacologically defined M_1 and non-M_1 muscarinic receptors by autoradiography in rat hippocampus following fimbria/fornix aspiration lesion. The receptor autoradiography data demonstrated that 3 weeks following unilateral lesion, there was an overall 20% increase in non-pirenzepine (non-M_1) displaceable [^{3}H](-)QNB binding in the denervated hippocampus (CA3: +35% and dentate gyrus: +16%). In bilaterally lesioned rats, the solution hybridization/RNase protection analyses indicated that hippocampal m3 and m4 receptor mRNAs were significantly increased (m3: +38.1% and m4: +40.79%) after 3 weeks lesion. However, the level of m1 receptor mRNA was not significantly affected by the lesion. These results suggest that: (a) the non-M_1 receptors are coded by the m3 and m4 genes; (b) the localization of these receptors is probably postsynaptic to the septohippocampal cholinergic terminals; and (c) they are upregulated following denervation.
Supported by a grant from the Stanley Foundation.

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3-HEXYLOXY ANALOGS OF TZTP ARE POTENT AND SELECTIVE M_1 AGONISTS IN BRAIN OF RAT *IN VIVO*.

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Hexyloxy-TZTP (TZTP-A) and 5-hexenyloxy-TZTP (TZTP-B) analogs of 3-1,2,5-thiadiazol-4-yl-1,2,5,6-tetrahydro-1-methylpyridine (TZTP) are potent and selective M_1 agonists *in vitro*. *In vivo*, TZTP-A penetrated into the brain and decreased the *ex vivo* binding of the selective M_1 antagonist ^{3}H-pirenzepine (PZ) to cortex homogenates with an ED_{50} of 3 and 32 mg/kg, sc and po., but was a weaker inhibitor of binding to M_2 receptors in brain stem with an ED_{50} of 14 mg/kg sc. In a similar fashion, TZTP-B decreased *ex vivo* binding of the agonist ligand ^{3}H-oxotremorine-M and PZ with ED_{50} of 1 and 3 mg/kg sc. Turnover of dopamine may be modulated via M_1 heteroreceptors on dopamine terminals; TZTP-A and TZTP-B at doses as low as 1 mg/kg sc. increased levels of the dopamine metabolite dihydroxyphenylacetic acid (DOPAC) in striatum up to 50%. Furthermore, the increases in DOPAC levels produced by hexyloxy-TZTP were antagonized by the M_1-selective antagonist trihexyphenidyl. M_2 autoreceptors modulate acetylcholine (ACh) release and large increases in tissue levels of ACh induced by oxotremorine were not antagonized by TZTP-B. TZTP-A and TZTP-B alone transiently increased ACh levels up to only 60%. A lack of activity at M_3 receptors was indicated by no production of salivation by TZTP-A up to 10 mg/kg sc or 100 mg/kg po. TZTP-A inhibited PZ ex vivo binding with a t1/2 of 3 hours and, similarly, DOPAC levels were elevated up to 3 hours; however, no changes were evident at 6 hours. It is concluded that the hexyloxy-TZTP are potent and efficacious M_1 agonists *in vivo*, but have no activity at M_2 and M_3 receptors. Furthermore, they are orally bioavailable, of moderate duration *in rats* and suitable for development as M_1 agonists.

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REVERSAL OF PERFORMANCE DEFICITS INDUCED BY SCOPOLAMINE OR HEMICHOLINIUM-3 (HC3)

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The reversal of performance deficits induced by the muscarinic antagonist scopolamine has become a standard model in assessing the potential cognitive enhancing effect of cholinomimetics. However, there are several problems with this approach. In Alzheimer's disease the cholinergic receptors are not blocked by an antagonist, but receive a reduced input. The search for a cholinergic treatment for Alzheimer's has concentrated on the production of M_1 agonists. However, the reversal of scopolamine is best achieved by cholinergic agonists with little or no subtype specificity. This leads to a misleading view as to the mnemonic and/or potentially disruptive effects of the agonists chosen. Another approach is to induce mnemonic deficits by depleting central cholinergic stores using HC3. Both scopolamine and HC3 produce performance deficits in a variety of animal models of learning and memory. By comparing the effects of cholinomimetics on scopolamine and HC3-induced deficits, differences in the balance of positive and negative effects of general agonists and esterase inhibitors such as physostigmine can be readily compared. Male Long Evans rats were trained on a modified Dunnett delayed matching to position task. Following stabilisation rats were implanted with guide cannulae aimed at the lateral ventricle and allowed to recover. After restabilisation they were injected with physostigmine (0.025 - 0.4 mg/kg sc) or tacrine (0.2 - 5 mg/kg sc), alone, or following pretreatment with scopolamine (0.2 mg/kg) or HC3 (1 μg per rat icv). In general compounds can be tested at higher doses in the presence of scopolamine than following HC3. Moreover, the dose limiting effects on reversal are more readily apparent in HC3-treated than in scopolamine-treated animals. Similar dose-limiting effects are seen following agonists such as arecoline. These data suggest that HC3 may be a more valuable tool than scopolamine in assessing the potential usefulness of cholinomimetic treatments in mnemonic disorders.

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MUSCARINIC ANALGESIA IS MEDIATED BY NON-M1 MUSCARINIC RECEPTORS

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The analgesic potential of four 3-(3-alkylthio/oxy-1,2,5-thiadiazol-4-yl)-1,2,5,6 tetrahydro-1-methyl pyridines (TZTP's) was assessed in the mouse using the Grid Shock (MGS), Tail Flick, Hot Plate, and Writhing tests s.c. 30 min after administration. The occurrence of salivation and tremor was scored. The TZTP's were also assessed for displacement of ^{3}H-oxotremorine-M and ^{3}H-pirenzepine binding. Muscarinic subtype selectivity was determined in vitro, assessing inhibition of the electrically stimulated Rabbit Vas Deferens (RBVD; M1), reduction of contraction force in the Guinea Pig Atrium (GPA; M2), and production of contractions in the Guinea Pig Ileum (GPI; M3). Inositol phosphate hydrolysis (PI turnover) was assessed in BHK cells transfected with the human m1 receptor. The hexyloxy- and hexylthio-TZTP's displaced ^{3}H-oxotremorine and ^{3}H-pirenzepine with IC_{50}'s (nM) of 9.7, 6.5, and 7.0, 5.0, respectively, were active in the RBVD and PI turnover tests, inactive or moderately active in the GPA and GPI, and yielded analgesic (MGS) ED_{50}'s of 16.3 and 14.0 mg/kg, respectively. The propoxy- and 3-Cl-propylthio-TZTP's displaced ^{3}H-oxotremorine and ^{3}H-pirenzepine with IC_{50}'s (nM) of 1.6, 1.4, and 18.0, 1.9, respectively, were inactive in the RBVD and PI turnover tests and active in the GPA and GPI, and produced analgesic (MGS) ED_{50}'s of 0.7 and 0.9 mg/kg, respectively. The analgesic effects in the other tests used were in the same rank order of potency as seen in the MGS test. These results demonstrate that M1 activity is not a prerequisite for the analgesic effects of muscarinics.

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ABSTRACT INDEX

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1. A.B. NORMAN, G. BATTAGLIA, A.L. MORROW and I. CREESE, Eur. J. Pharmacol. 106 461-462 (1985).
2. IAN CREESE, *Neurotransmitter Receptor Binding*, Henry I. Yamamura, S.J. Enna, and Michael J. Kuhar (Eds), 189-233, Raven Press, New York (1985).

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