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GRANT NUMBER: DAMD17-94-J-4011

TITLE: Ethanol Disruption of Synaptic Neurotransmission

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REPORT DATE: October 1995

TYPE OF REPORT: Midterm

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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19960206 005

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1995	Midterm 22 Sep 94 - 21 Sep 95

4. TITLE AND SUBTITLE
Ethanol Disruption of Synaptic Neurotransmission

5. FUNDING NUMBERS
DAMD17-94-J-4011

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Donald Guthrie Foundation for Medical Research
Sayre, Pennsylvania 18840

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The goal of this research is to understand how acute and chronic ethanol administration disrupts synaptic transmission in the central nervous system. The underlying hypothesis is that *ethanol depresses neurotransmission at neurotransmitter receptors by altering receptor-G protein interactions.*

The results provide partial support for our hypothesis. However, ethanol's actions were not universal, and receptor subtypes were not equally affected. The significance of ethanol's actions is indicated by the disruption of signaling processes (adenylate cyclase, intracellular Ca^{2+}, cell adhesion).

Ethanol disrupted the following processes: 1) receptor-G protein interactions as seen in ligand binding studies in receptors expressed by stably transfected CHO cells; 2) muscarinic receptor control of G protein GTPase activity and guanine nucleotide binding in brain tissues; 3) the balance between inhibitory and stimulatory G protein influences on adenylate cyclase, 4) Ca^{2+} responses of m1 and m5 receptors; 5) muscarinic mediated cell adhesion response in a non-neuronal cell line. The quantity of experimental material has been a limiting factor in studies with cultured cells. We initiated studies on the influence of chronic ethanol treatment on neurotransmitter receptors, including the adoption of Western blots (G proteins) and Northern blots for receptor mRNA. To analyze control of receptor expression, the promoter regions of m1-m5 were cloned, sequenced and characterized.

14. SUBJECT TERMS
ethanol; alcohol abuse; synaptic transmission; acetylcholine; cholinergic receptors; muscarinic; G proteins; signal transduction; calcium; adenylate cyclase; cAMP; GTPase; phospholipase C; inositol triphosphate; calcium

15. NUMBER OF PAGES
112

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Roberts Arnstan 10/18/95

PI - Signature Date

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5. Introduction

The overall goal of this research is to increase our understanding of the mechanisms by which acute and chronic ethanol administration disrupts synaptic transmission in the central nervous system.

Ethanol and other short chain alcohols depress neuronal excitability in the central nervous system, although the molecular mechanisms underlying ethanol intoxication, tolerance, and dependence are poorly understood. The overall hypothesis driving this research is that *ethanol depresses neurotransmission at neurotransmitter receptors by altering receptor-G protein interactions.* Sub-hypotheses include the following: 1) Ethanol interferes with the coupling of neurotransmitter receptors with transducer G proteins as evidenced by altered guanine nucleotide sensitivity of receptor binding properties and altered receptor control of the G protein cycle; 2) Ethanol interferes with transmission mediated by only certain subtypes of muscarinic receptor; 3) Animals which show tolerance and physical dependence to ethanol display altered receptor and/or G protein expression or receptor-G protein interactions. This research is expected to increase our understanding of acute and chronic ethanol action on signal transduction processes in the brain, and evaluate a novel mechanism that may contribute to altered synaptic transmission during ethanol intoxication and tolerance/dependence.

The specific objectives of the research were to determine the effects of acute and chronic ethanol administration on the following processes:

1. Guanine nucleotide regulation of agonist binding to a) muscarinic receptors in cell cultures stably transfected with a gene encoding a single subtype of muscarinic receptor (m1, m2, m3, m4 or m5), b) cultured cell lines expressing defined receptor subtypes, c) other neurotransmitter receptors expressed in brain tissue.
2. Receptor control of G protein cycle function (i.e., receptor-stimulated GTPase activity, GDP release and GTP binding). These experiments were performed in neuronal membranes isolated from different brain regions or cell cultures expressing different profiles of neurotransmitter receptors.
3. Receptor signal transduction processes as evidenced by agonist stimulation of second messenger production. The purpose of these experiments was to establish the functional significance of ethanol-induced alterations of receptor-ligand and receptor-G protein interactions.

During the period covered by this mid-term report we characterized the acute effects of ethanol on muscarinic acetylcholine receptors in cultured cell lines expressing defined receptor subtypes and in certain intact tissues. Moreover, substantial progress has been made in defining the effects of ethanol on G protein cycle activity (both basal and muscarinic receptor-mediated). A study on the effects of ethanol on muscarinic stimulation of G protein GTPase activity (an indication of turnover in the G protein cycle) has been completed. Studies on other neurotransmitter systems have begun. A study on the acute influence of ethanol on muscarinic receptor-mediated adenylate cyclase activity has been completed. The latter study revealed a confounding and powerful effect of ethanol on G protein-mediated stimulation of the enzyme's catalytic activity in which the stimulatory actions of guanine nucleotides and sodium fluoride were both enhanced. We have characterized muscarinic control of intracellular calcium concentrations in CHO cells stably transfected with specific muscarinic receptor subtypes as well as in SK-N-SH cells expressing m3 receptors, and have demonstrated ethanol inhibition of these processes in m1- and m5-expressing cells. We have demonstrated a powerful effect of ethanol on a novel muscarinic receptor-mediated signal transduction process in a non-neuronal cell line, cultured small cell lung cancer cells. The

first cohorts of rats chronically-exposed to ethanol are being processed. Protocols to measure transcriptional control of receptor expression, mRNA production, and G protein expression were developed to complement ligand binding measures of receptor function in the chronically-treated animals. To permit these chronic treatment studies, the promoters of m1–m5 muscarinic receptors were cloned, sequenced, and partially analyzed (homology and footprinting), and reporter gene constructs designed to evaluate ethanol induced changes of functional activity were produced.

In all, approximately 40% of the proposed research has been completed. However, the entire team of researchers has been recruited and trained , and we anticipate that all work can be completed within the specified grant period. A number of publications have come from the supported work, although the majority of work has yet to be prepared for publication.

Specific Accomplishments during the present reporting period include the following determinations:

1. effects of ethanol on the guanine nucleotide sensitivity of agonist binding to muscarinic receptors in a cell line (SK-N-SH) expressing m3 muscarinic receptors
2. characterization of CHO cells stably transfected with specific subtypes of muscarinic receptor (ethanol studies partially completed), including the influences of heat and alkylation on receptor binding and coupling to G proteins
3. effects of ethanol on ligand (agonist and antagonist) binding to m2 and m4 receptor expressed by cultured CHO cells
4. effects of ethanol on the guanine nucleotide sensitivity of agonist binding to muscarinic receptors in different regions of rat brain (including cerebral cortex, hippocampus and striatum), as well as the atrium which expresses only m2 receptors
5. acute effects of ethanol on muscarinic receptor-stimulated guanine nucleotide binding to G proteins in different regions of rat brain
6. acute effects of ethanol on muscarinic receptor-stimulated G protein GTPase activity in different regions of rat brain
7. acute effects of ethanol on adenylate cyclase control by muscarinic receptors, guanine nucleotides, sodium fluoride, forskolin and other agents in brain tissue
8. modified our vivarium facility for performance of chronic ethanol treatment studies; initiated chronic treatment schedules
9. developed a spectrophotometric assay for the physiological activation of muscarinic receptor based on muscarinic receptor-induced increases in intracellular calcium concentration using a calcium-sensitive fluorescent dye
10. utilized a calcium-sensitive fluorescent dye to characterize muscarinic control of intracellular calcium content in single cells and small groups of cultured cells (SK-N-SH and CHO cells); established techniques to look at phasic vs tonic calcium responses and receptor desensitization; observed differences in the actions of agonists on different muscarinic receptor subtypes
11. utilized a calcium-sensitive fluorescent dye to establish an inhibitory action of ethanol on muscarinic receptors in CHO cells expressing m1 and m5 muscarinic receptors, using novel protocols in which each cell served as its own control
12. cloned and sequenced the promoter regions of m1–m5 receptors; designed and produced reporter constructs incorporating luciferase enzyme to evaluate ethanol-induced changes in transcriptional control mechanisms

13. utilized a novel cell adhesion assay to determine the effects of ethanol on signal transduction in a non-neuronal cell line; demonstrated ethanol inhibition of m3 receptor mediated-cell adhesion in SCC-9 cells
14. demonstrated a lack of acetylcholinesterase activity in cultured CHO cells
15. adopted Western blot procedures to quantify G protein expression using specific antibodies

These results are discussed in the narrative section immediately following. This narrative is divided into 3 sections: 1) Ligand Binding Studies, 2) G Protein Studies, and 3) Signal Transduction Studies.

Goals for the second half of the project period include the determination of the following:

1. effects of chronic ethanol treatment on the guanine nucleotide sensitivity of agonist binding to muscarinic receptors in different regions of rat brain
2. effects of chronic ethanol treatment on muscarinic receptor-stimulated G protein cycle activity (e.g., guanine nucleotide binding to G proteins and receptor-stimulated G protein GTPase activity) in different regions of rat brain.
3. effects of chronic ethanol treatment on the expression of muscarinic receptor subtypes and G proteins, by examination of nuclear extracts and expressed levels of proteins and mRNA
4. effects of acute and chronic ethanol treatment on receptor-ligand interactions and receptor control of G protein cycle to other neurotransmitter systems.
5. effects of chronic ethanol treatment on muscarinic receptor signal transduction processes, especially receptor-mediated changes in intracellular Ca^{2+} concentrations and production of second messengers (e.g., cAMP, IP3).
6. complete characterization of acute ethanol effects on muscarinic receptor subtypes expressed in transfected CHO cells
7. influence of ethanol on the modulation of melatonin release.
8. publish work on ethanol effects already obtained, as outlined in the following sections

6. Body

Background: Acute ethanol administration produces a wide range of depressant effects on neuronal excitability (Roth, 1979; Deitrich et al., 1989), although the precise mechanisms underlying ethanol intoxication, tolerance, and dependence have not been clearly defined. However, it is noteworthy that ethanol inhibits synaptic transmission at lower concentrations than it disrupts axonal conductance or the electrical properties of nerve cells (e.g., Deitrich et al., 1989; Faber and Klee, 1977).

The cholinergic system is affected by ethanol in several ways (Faber and Klee, 1977). Perhaps the most consistent neurochemical effect of acute ethanol administration is a decrease in acetylcholine release from brain tissue *in vivo* and *in vitro* (Erickson and Burnam, 1971; Phillis and Jhamandas, 1971; Erickson and Graham, 1973; Morgan and Phillis, 1975. Moreover, acetylcholine release is more sensitive to ethanol than is the release of other brain monoamines (Clark et al., 1977; Kalant and Grose, 1967). Variable effects of ethanol on brain acetylcholine levels, turnover, and cholinergic enzyme activities have also been reported (Moss et al., 1967; Rawat, 1974; Okonmah et al., 1989; Hunt and Dalton, 1976). Cholinergic involvement in ethanol intoxication is also suggested by physostigmine antagonism of ethanol-induced sleep time in mice, as well as choline potentiation of ethanol-induced hypomotility in rats (Pohorecky et al, 1979).

Several acute effects of ethanol on postsynaptic muscarinic processes have been described (Deitrich et al., 1989). Systemic administration of ethanol selectively enhances the sensitivity of hippocampal pyramidal cells to acetylcholine (as well as somatostatin) (Mancillas et al., 1986). Ethanol inhibits antagonist binding to muscarinic receptors (but only at very high concentrations, IC50's >1 M), and the potency of short chain alcohols in inhibiting antagonist binding is directly related to the length of the carbon chain (Fairhurst and Liston, 1979). Ethanol, at physiologically tolerable concentrations, selectively decreases agonist binding affinity to muscarinic receptors by decreasing hydrophobic binding effects (Waelbroeck et al., 1984; 1985). Ethanol has been reported to inhibit muscarinic receptor-mediated stimulation of [^{32}P] incorporation into synaptosomal phosphatidic acid (Smith et al., 1986), but not muscarinic receptor-mediated inhibition of adenylate cyclase activity in the striatum (Rabin, 1985).

Recent evidence has implicated guanine nucleotide-dependent transducer proteins (G proteins) in the actions of ethanol. Ethanol increases the activation of adenylate cyclase by G_S (e.g., Luthin and Tabakoff, 1984; Rabin and Molinoff, 1983). Hoffman and Tabakoff (1990) have proposed that ethanol selectively enhances the rate of activation of G_S (an action that is normally catalyzed by an interaction with receptors) as well as the interaction of G_{Sa} with guanine nucleotides. On the other hand, Bauché et al. (1987) have presented evidence that ethanol also disrupts G_i-mediated control of adenylate cyclase in rat brain. Charness et al. (1988) have demonstrated differential regulation by ethanol of the expression of both $G_{i\alpha}$ and $G_{s\alpha}$ in several neuronal cell lines.

We have demonstrated that compounds in a second class of global central nervous system depressants, the volatile general anesthetics, inhibit muscarinic neurotransmission by disrupting receptor coupling to transducer G proteins (Aronstam and Dennison, 1989; Aronstam et al., 1986; Anthony et al., 1988; 1989; Narayanan et al., 1988) . This disruption was initially detected as a depression in the guanine nucleotide sensitivity of agonist binding to the receptors (Aronstam et al., 1986; Dennison et al., 1987). Similar effects were observed with a second G_i-coupled receptor, the a_2-adrenergic receptor of rat cerebral cortex (Baumgartner et al., 1990). In the present study, we explored the possibility that ethanol has similar effects on muscarinic receptor-G

protein interactions in rat brain by examining the effect of ethanol on muscarinic regulation of two aspects of G protein function, GTPase activity and guanine nucleotide binding.

B. Ligand Binding Studies

Influence of acute ethanol administration on the guanine nucleotide sensitivity of ligand binding to neuronal receptors.

a. Antagonist binding. Receptor binding was measured using appropriate radiolabelled probes by filtration procedures (Aronstam and Carrier, 1982). For example, for muscarinic receptors an aliquot of tissue (30-50 μg protein brain tissue) was incubated with [^{3}H]*N*-methyl-scopolamine ([^{3}H]MS) for 90 min at room temperature in a medium containing 50 mM Tris-HCl, pH 7.4, and 2 mM $MgCl_2$. The suspensions were then filtered through glass fiber filters (Schleicher and Schuell, #32) using a Brandel filtration manifold. The incubation tubes and filters were washed twice, and the radioactivity content of the filters determined by liquid scintillation counting. Non-specific binding was determined in the presence of 10 μM unlabelled antagonist. Binding parameters were determined by nonlinear regression analysis using a mass action expression for ligand binding to a single population of non-interacting sites: $B = Bmax \cdot C/(C + K_D)$, where B is binding, Bmax is the binding site concentration, C is the ligand concentration, and K_D is the dissociation constant. [^{3}H]Antagonist binding to rat brain is, in general, well-described by this model. Analyses were performed using a nonlinear curve fitting program (Delta-Graph Pro) .

b. Agonist binding. The guanine nucleotide sensitivity of agonist binding to muscarinic acetylcholine receptors, an indication of receptor-G protein coupling, was determined in [^{3}H]antagonist/agonist competition experiments. For example, muscarinic agonist binding was inferred from the ability of carbamylcholine to inhibit [^{3}H]MS binding. Binding isotherms were resolved where possible into their constituent components using nonlinear curve fitting procedures and the following model for two classes of non-interacting binding sites, $B = (R_H)C/(C + K_H) + (1- R_H)C/(C + K_L)$, where K_H and K_L are the dissociation constants associated with receptor populations displaying high and low affinity agonist binding (R_H and $1- R_H$). Agonist binding is often well-described by this two site model.

The coupling of the receptors considered in the present research to effector mechanisms is mediated by G proteins (Gilman, 1987; Rodbell, 1980). Guanine nucleotides lower agonist affinity by converting receptors from a state of high affinity to a state of lower affinity (Berrie et al., 1979; Birdsall et al., 1984). This suggested that high affinity agonist binding involves receptors which are coupled to G proteins, while low affinity binding involves uncoupled receptors. This interpretation was supported by reconstitution experiments utilizing purified receptors and G proteins (Flori and Sternweis, 1985; Haga et al., 1986). We assessed the association of muscarinic receptors with G proteins by measuring agonist binding in the presence and absence of 10 μM 5'-guanylylimidodiphosphate (Gpp(NH)p), a stable analog of GTP. Non-linear regression analyses revealed changes in the proportions of high and low affinity receptors, as well as changes in the affinities of the receptor subpopulations. The magnitude of these changes (particularly the fractional conversion of high affinity receptors to a low affinity state) was used as an index of receptor-G protein coupling. Other indications of the state of receptor-G protein coupling were [^{3}H]MS binding (affinity and apparent number of sites; G protein-coupled receptors have enhanced [^{3}H]antagonist binding that parallels their depressed agonist binding. Other hallmarks of receptor-G protein interactions which we have begun to consider are heat and alkylation treatments which preferentially inactivate G proteins. Heating involved holding membranes at 50°C for 10 minutes. Alkylation involved treating membranes (1 mg protein/ml) with 1 mM N-ethylmaleimide for 15 minutes at 37°C.

Results:

The responses of muscarinic acetylcholine receptors to ethanol were studied in 1) a neuroblastoma cell line expressing m3 muscarinic receptors, 2) rat atrium which expresses exclusively m2 muscarinic receptors, 3) CHO (Chinese hamster ovary) cells stably transfected with the human genes for m1, m2, m3, m4 and m5 receptor subtypes. These results were compared to muscarinic receptors in membranes isolated from brain regions expressing different populations of muscarinic receptors. As noted, certain baseline measures, including studies dealing with brain regions (notably brainstem), were performed before the initiation of the present grant and formed part of the rationale for the design of the present study. They represent the baseline for analysis of the data obtained in other systems and are included to increase the clarity of the presentation.

Influence of ethanol on the guanine nucleotide sensitivity of agonist binding to muscarinic receptors from rat brain.

Agonist binding was determined in rat cerebral cortex (cortex), striatum, hippocampus and brainstem in carbamylcholine/[^{3}H]N-methylscopolamine ([^{3}H]MS) competition studies. The brainstem expresses predominantly m2 receptors (71–84%); the cerebral cortex expresses receptor subtypes is the following order of density, m1>m4>m2>m3; in hippocampus the order is m1>>m4=m2>m3; in striatum the order is m4>m1>m2>m3; in atrium virtually all muscarinic receptors are of the m2 subtype (Wall et al., 1992; Levey et al., 1991; 1992; Li et al., 1991; Yasuda et al., 1993).

In work which provided the rationale for the present study, we demonstrated that carbamylcholine binding to muscarinic receptors in rat brainstem (predominantly m2 receptors) was depressed by guanine nucleotides, and that this depression was modulated by ethanol (Aronstam et al., 1993). Ethanol did not significantly alter carbamylcholine binding, but it virtually eliminated the ability of Gpp(NH)p to lower carbamylcholine binding affinity. This "guanine nucleotide effect" on agonist binding is a widely observed phenomenon, and is believed to reflect uncoupling of the receptor-G protein complex. Typical curves for rat brainstem receptors are demonstrated in Figure 1. These binding curves were performed at 20°C, the optimal temperature for demonstrating receptor-G protein interactions in ligand binding studies (Aronstam and Narayanan, 1988).

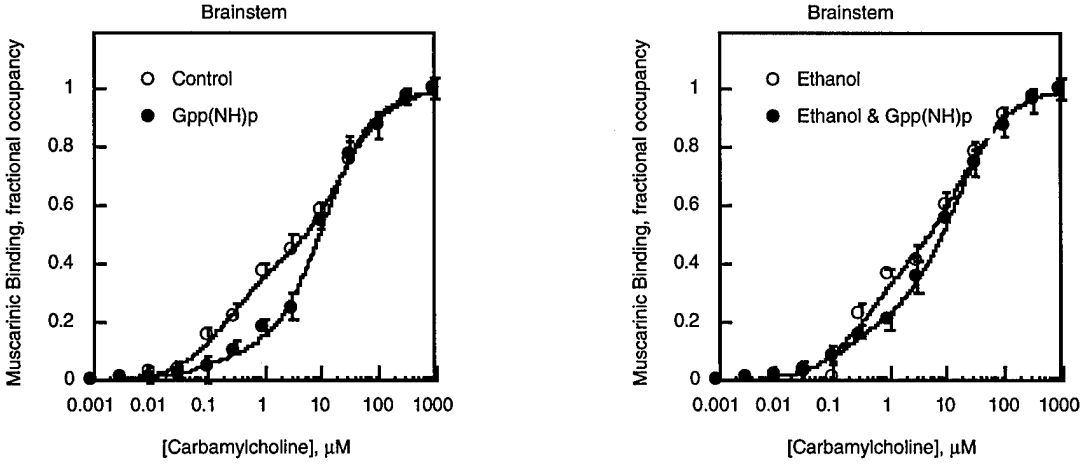


Figure 1. Influence of ethanol on carbamylcholine binding to muscarinic receptors in rat brainstem membranes. Agonist binding was determined in carbamylcholine/[^{3}H]MS competition studies. Binding was performed in the absence or presence of Gpp(NH)p (50 μM) and/or ethanol (100 mM), as indicated. Points and bars represent the means and standard deviations from 5 determinations. Lines are drawn according to nonlinear regression fits to a two receptor population model which revealed the parameters listed in Table 1. (Data from Aronstam et al., 1993).

These experiments were extended to agonist binding in rat brain cerebral cortex, striatum, and hippocampus, as well as rat atrium. These are presented in the next series of figures and the binding parameters are summarized in Tables 1 and 2.

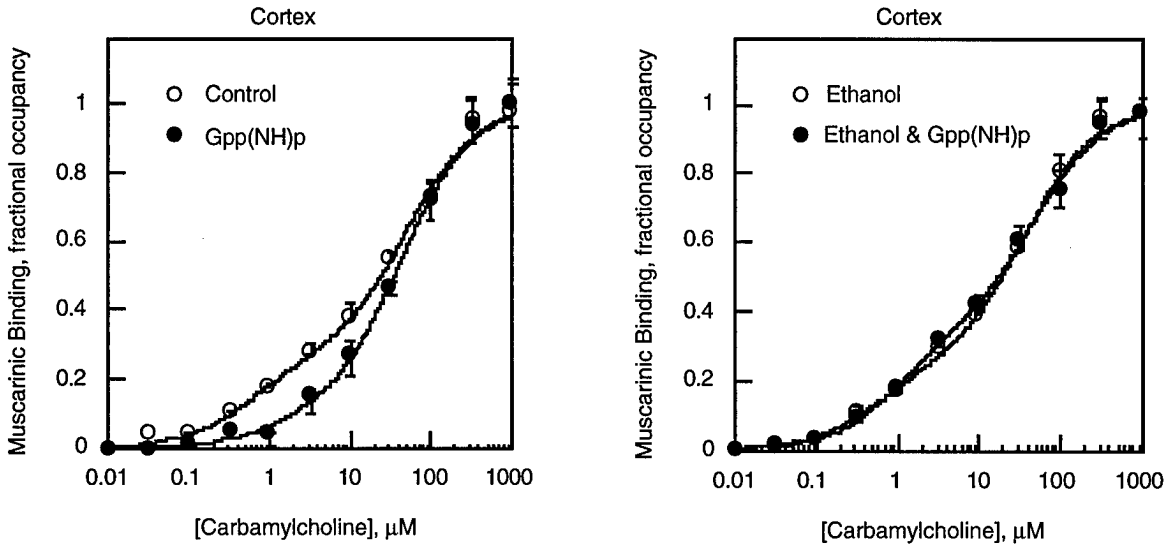


Figure 2. Influence of ethanol on carbamylcholine binding to muscarinic receptors in rat cerebral cortex membranes. Agonist binding was determined in carbamylcholine/[^{3}H]MS competition studies. Binding was performed in the absence or presence of Gpp(NH)p (50 μM) and/or ethanol (100 mM), as indicated. Points and bars represent the means and standard deviations from 3 determinations. Lines are drawn according to nonlinear regression fits to a two receptor population model which revealed the parameters listed in Table 1.

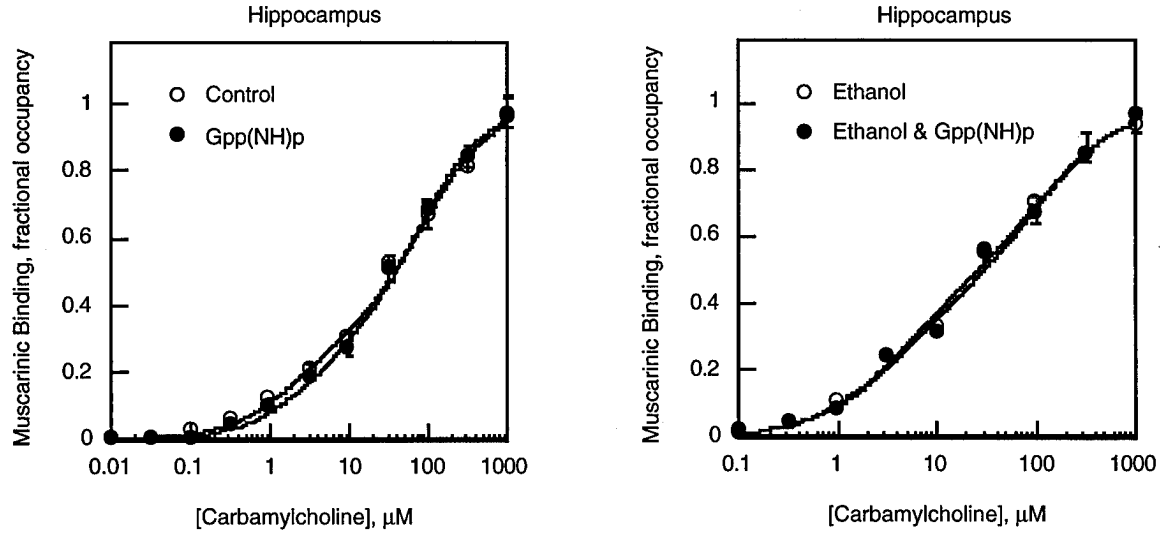


Figure 3. Influence of ethanol on carbamylcholine binding to muscarinic receptors in rat hippocampal membranes. Agonist binding was determined in carbamylcholine/[^{3}H]MS competition studies. Binding was performed in the absence or presence of Gpp(NH)p (50 μM) and/or ethanol (100 mM), as indicated. Points and bars represent the means and standard deviations from 3 determinations. Lines are drawn according to nonlinear regression fits to a two receptor population model which revealed the parameters listed in Table 1.

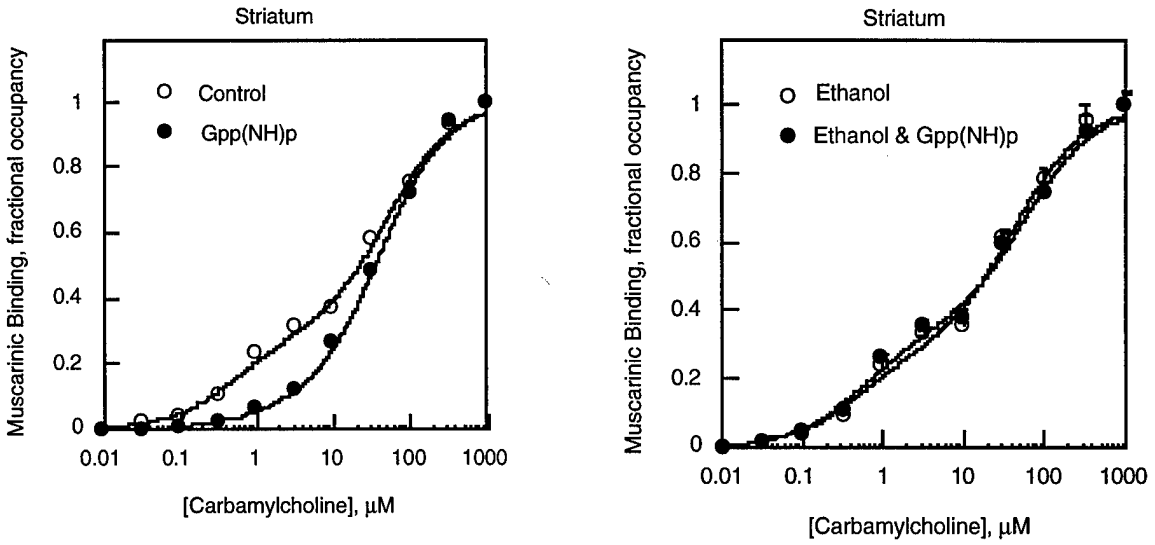


Figure 4. Influence of ethanol on carbamylcholine binding to muscarinic receptors in rat striatal membranes. Agonist binding was determined in carbamylcholine/[^{3}H]MS competition studies. Binding was performed in the absence or presence of Gpp(NH)p (50 μM) and/or ethanol (100 mM), as indicated. Points and bars represent the means and standard deviations from 3 determinations. Lines are drawn according to nonlinear regression fits to a two receptor population model which revealed the parameters listed in Table 1.

Influence of ethanol on the guanine nucleotide sensitivity of agonist binding to m2 muscarinic receptors expressed in rat atrium.

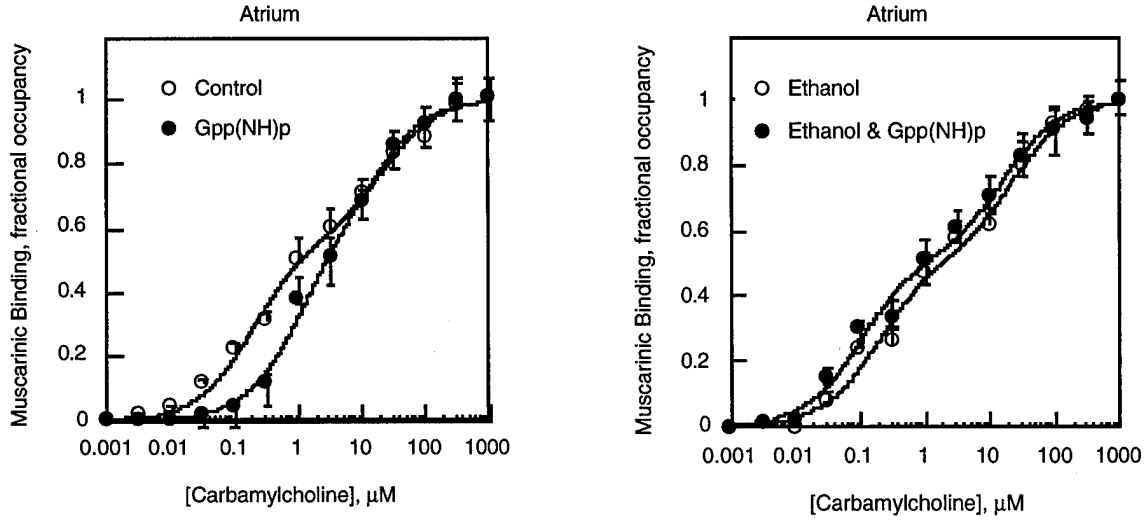


Figure 5. Influence of ethanol on carbamylcholine binding to muscarinic receptors in rat atrial membranes. Agonist binding was determined in carbamylcholine/[^{3}H]MS competition studies. Binding was performed in the absence or presence of Gpp(NH)p (50 μM) and/or ethanol (100 mM), as indicated. Points and bars represent the means and standard deviations from 3 determinations. Lines are drawn according to nonlinear regression fits to a two receptor population model which revealed the parameters listed in Table 1.

Discussion: Agonist binding to muscarinic receptors involved multiple components in each tissue examined. The binding parameters associated with the nonlinear fit of the binding curves to a two receptor population model are summarized in Table 1. The guanine nucleotide, Gpp(NH)p decreased agonist binding affinity in each tissue (except the hippocampus) in a manner which usually suggested a decrease in the proportion of high affinity binding sites, although changes were also noted in the dissociation constants associated with the high and low affinity binding sites. In the atrium, which had the one of the greatest guanine nucleotide effects on agonist binding, the proportion of high affinity binding sites paradoxically increased, although the apparent affinity associated with the high affinity binding decreased by a factor of 5. It is clear from these studies that the "guanine nucleotide effect" on agonist binding is most pronounced in those tissues which express the greatest proportion of m2 receptors (brainstem and atrium). It is also evident that m2 receptors display the greatest proportion of high affinity receptors (and, thus, the highest agonist affinity) of any receptor subtype. An intermediate effect is seen in the cerebral cortex and striatum, and no effect is seen in the hippocampus. This raises the possibility that the guanine nucleotide effect is a property to receptors coupled to the Gi transducer protein (as are the m2 and m4 receptors) and not a property of muscarinic receptors coupled to the $G_{q/11}$ transducer protein (i.e., m1, m3, m5).

Ethanol (100 mM) alone had little effect agonist binding affinity. However, ethanol largely suppressed the ability of 10 μM Gpp(NH)p to influence agonist binding, This was seen in the cortex, striatum and atria. These results are consistent with results obtained in brainstem membranes (Aronstam et al., 1993), and are consistent with our hypothesis that ethanol disrupts receptor-G protein interactions.

Table 1. Influence of ethanol and guanine nucleotide on carbamylcholine binding parameters in a variety of tissues.

Tissue	Condition	Bmax, high	Kd1	Kd2
Brainstem	Control	0.403	0.224	21.5
	Gpp(NH)p	0.086	0.092	10.92
	Ethanol	0.41	0.374	18.9
	Ethanol + Gpp(NH)p	0.213	0.196	13.8
Cortex	Control	0.269	0.58	49.0
	Gpp(NH)p	0.097	1.13	42.1
	Ethanol	0.246	0.53	35.5
	Ethanol + Gpp(NH)p	0.350	1.00	47.6
Hippocampus	Control	0.311	2.00	87.3
	Gpp(NH)p	0.270	2.70	71.5
	Ethanol	0.465	4.06	115.3
	Ethanol + Gpp(NH)p	0.427	3.89	108.7
Striatum	Control	0.292	0.511	48.6
	Gpp(NH)p	0.065	0.950	38.3
	Ethanol	0.288	0.518	41.6
	Ethanol + Gpp(NH)p	0.345	0.579	56.7
Atrium	Control	0.565	0.190	22.2
	Gpp(NH)p	0.610	0.980	18.2
	Ethanol	0.512	0.187	21.8
	Ethanol + Gpp(NH)p	0.518	0.098	15.9

Binding parameters were determined by nonlinear regression analysis using a model incorporating two independent binding sites characterized by the dissociation constants Kd1 and Kd2. Bmax, high is the fraction of receptors displaying high affinity agonist binding (i.e., Kd1). These analyses were performed using the DeltaGraph Pro 3.5 computer program on a Macintosh microcomputer; the small differences in parameters for brainstem compared to those reported in Aronstam et al. (1993) probably reflect the use of this different analysis program.

In an alternate analysis, we made the assumption that Gpp(NH)p and ethanol did not affect the individual dissociation constants associated with the high and low affinity binding components, only the distribution of the receptors between high and low affinity states (i.e., G protein-coupled and -uncoupled states). Using the average dissociation constants for the high and low affinity binding components of 0.39 ± 0.17 and 32 ± 13 μM, respectively, we obtained the distributions of high and low affinity binding in the various tissues and treatment conditions listed in Table 2.

Table 2. Influence of ethanol and guanine nucleotide on the fraction of high affinity (i.e., G protein-coupled) muscarinic receptors different tissues.

Tissue	Control	Gpp(NH)p	Ethanol	Ethanol & Gpp(NH)p
Brainstem	0.49	0.31	0.47	0.37
Cortex	0.20	0.03	0.22	0.24
Striatum	0.23	0.03	0.24	0.26
Atrium	0.67	0.54	0.61	0.69

These parameters were obtained by nonlinear regression analyses performed using a model incorporating high and low affinity dissociation constants of 0.39 and 32 μM, respectively.

This analysis suggested 1) that Gpp(NH)p decreased the fraction of high affinity binding sites in each tissue, 2) that ethanol had little effect on the proportion of receptors displaying high affinity binding, and 3) that ethanol decreased the ability of Gpp(NH)p to decrease the proportion of receptors displaying high affinity binding. Again, this is consistent with our hypothesis that receptor-G protein interactions are altered by ethanol.

SK-N-SH Cells. SK-N-SH are a neuroblastoma cell line which express predominantly the m3 form of muscarinic receptors, as revealed by Northern blot analysis, pharmacological analysis, and immunoprecipitation, even though mRNA transcripts for all 5 receptor subtypes are expressed (Baumgartner et al., 1992). Saturation [^{3}H]MS binding to SK-N-SH cell membranes is shown in Figure 6. The affinity of [^{3}H]MS was ≈0.64 nM.

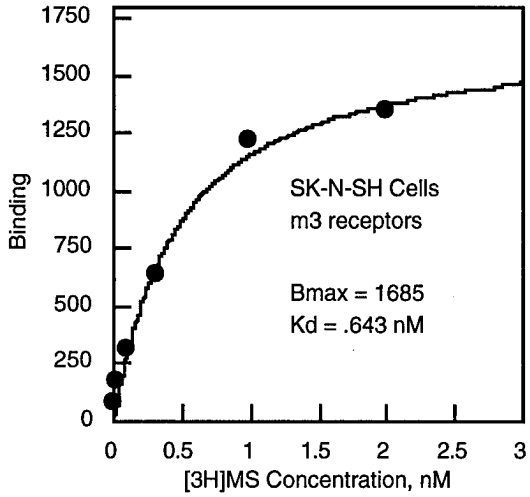


Figure 6. Binding of [^{3}H]MS to m3 muscarinic receptors expressed by SK-N-SH neuroblastoma cells. Results are from a typical experiment. The Bmax (illustrated in cpm) correspond to a receptor density of . The dissociation constant was 0.64 nM. The line is drawn according to a nonlinear fit to a single binding site model. These binding levels corresponds to a receptor concentration of 1.26 pmol/mg membrane protein.

The influence of ethanol on receptor-G protein interactions was investigated by studying carbamylcholine inhibition of 1 nM [^{3}H]MS binding. A major difficult in all studies with cultured cells (both SK-N-SH and CHO cells) was the paucity of available tissue. Thus, the tissue content was kept at a minimum and the concentration of [^{3}H]MS was raised to increase detectibility. This would have the effect of shifting competition curves to the right and possibly obscuring effects on high affinity binding. A typical experiment is presented in Figure 7. Gpp(NH)p was found to decrease the ability of carbamylcholine to inhibit [^{3}H]MS binding. This effect was mitigated by 100 mM ethanol, in agreement with finding in brain tissues.

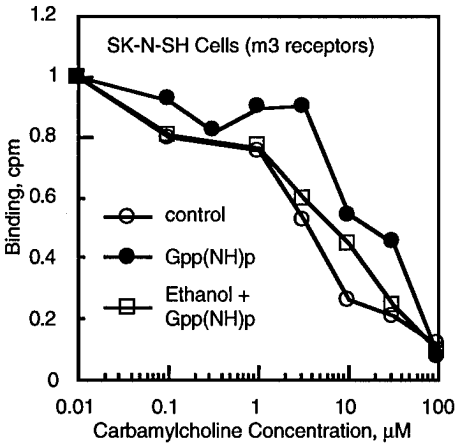


Figure 7. The influence of ethanol on agonist binding to m3 muscarinic receptors expressed by SK-N-SH cells. The binding of 1 nM [^{3}H]MS was measured in the presence of the indicated concentrations of carbamylcholine in the absence or presence of 10 µM Gpp(NH)p and 100 mM ethanol. as indicated in the Legend. Each point is the average of three determinations from a single experiment, which varied by less than 10%.

CHO cells transfected with specific muscarinic receptor subtypes.

In order to ascertain the receptor subtype specificity of the guanine nucleotide and ethanol effects on agonist binding, we have examined muscarinic binding to specific receptors expressed in stably transfected CHO cells. In addition to the paucity of tissue encountered with SK-N-SH cells (e.g., only a few mg tissue total wet weight per flask–enough for perhaps 15 assay tubes), the concentration of muscarinic receptor fluctuated greatly in the different cultures and was always much less than the receptor density of brain tissue or SK-N-SH cells. Thus, tissue availability was a limiting factor. While a substantial number of experiments have been performed, these experiments are not yet complete. These studies

are being pursued on a continuing basis as tissue is produced. We have not been able to define the conditions which provide for maximum receptor expression. We have never observed muscarinic receptor expression to be totally lost. As a consequence of this situation was have tended to work at high levels of receptor saturation. This distorts binding parameter values, especially in competition studies, although the same qualitative effects should be observed.

m2 and m4 muscarinic receptors expressed in CHO cells.

The even numbered muscarinic receptors couple efficiently to the inhibition of adenylate cyclase through the G1 transducer G proteins. Insofar as the "guanine nucleotide effect" on agonist binding is seen most clearly in tissues expressing large quantities of m2 receptor, we examined these receptor subtypes first to see if the ethanol effect most vigorously involves this receptor subtype.

We have previously noted another effect of guanine nucleotides on muscarinic binding in tissues expressing large quantities of m2 receptor, namely an increase in the binding affinity of muscarinic antagonists. For example, we found the Gpp(NH)p increased the apparent level of [^{3}H]MS binding in rat atria by almost 25%. This increase is probably related (and may contribute to) the more popularly studied decrease in agonist binding affinity. Whatever, the source of this consistent effect, it appears to be a useful empirical indication of receptor-G protein interactions: The conformational change associated with receptor-G protein coupling engenders an increase in antagonist binding as well as a concomitant decrease in agonist binding.

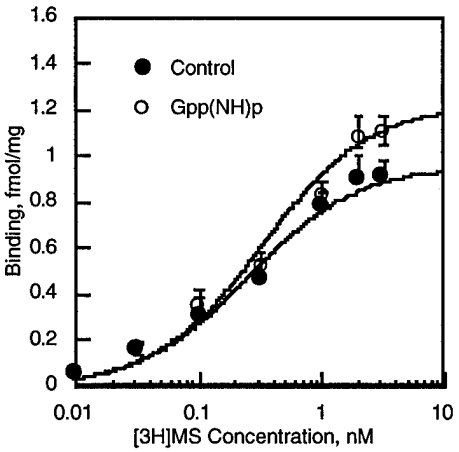


Figure 8. Influence of Gpp(NH)p on [^{3}H]MS binding to m2 muscarinic receptors in membranes isolated from rat atrium. The specific binding of [^{3}H]MS was measured in the absence and presence of 10 μM Gpp(NH)p. In the presence of the guanine nucleotide the level of binding increased from 0.96 to 1.23 pmol/mg protein. The apparent dissociation constant was not significantly affected (0.25 vs 0.32 nM, in the absence and presence of Gpp(NH)p, respectively). Data is from Aronstam et al., 1995.

A typical binding curve to m2 muscarinic receptors expressed in CHO cells is depicted in Figure 9. The binding was well-described by a model incorporating a single population of binding sites. In this example, the receptor concentration was 1.7 pmol/mg membrane protein and the apparent Kd was 0.43 nM. Receptor densities ranged from 0.3 to 1.8 pmol/mg membrane protein in the different culture preparations. The reason for this wide variation was not clear. The expression of muscarinic receptors was never entirely lost and new cultures from frozen stocks were used frequently.

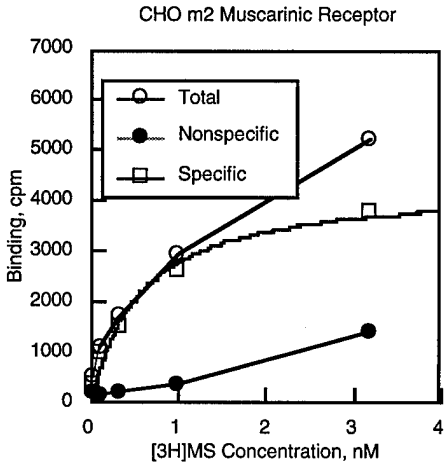


Figure 9. [^{3}H]MS binding to m2 muscarinic receptors expressed in stably transfected CHO cells. Total, background and specific (i.e., total minus background) binding is shown. Each point is the average from 3 determinations which varied by less than 10%. The line through the specific binding is based on parameters derived from non-linear regression analysis.

The influence of Gpp(NH)p and ethanol on [^{3}H]MS saturation curves to m2 receptors expressed in CHO cells is shown in Figure 10. As with atrial m2 receptors, the guanine nucleotide increased binding, increasing the apparent number of binding sites and affinity. Ethanol decreased [^{3}H]MS binding, and totally blocked the ability of the guanine nucleotide to increase antagonist binding. This is consistent with ethanol interference with receptor-G protein interactions.

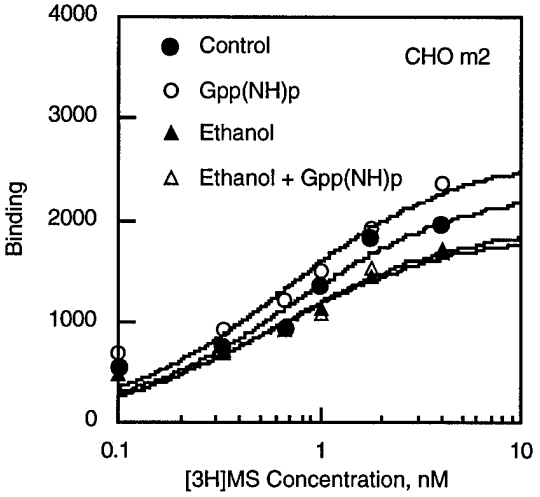


Figure 10. Typical [^{3}H]MS saturation curves for CHO cells transfected with m1 muscarinic receptors. Lines are drawn according to nonlinear regression analyses which revealed the binding parameters listed in Table 3. Binding was measured in the absence or presence of 100 mM ethanol and/or 10 μM Gpp(NH)p, as indicated. Gpp(NH)p increased [^{3}H]MS binding, in agreement with similar results obtained with receptors in brainstem membranes and atria (tissues rich in m2 receptors [e.g., Aronstam et al., 1995]), and this increase was blocked by ethanol.

Table 3. Binding parameters of [^{3}H]MS binding to m2 muscarinic receptors expressed in stably transfected CHO cells.

Condition	**Bmax, fmol/mg protein**	**Kd, nM**
Control	865 ± 35	0.71 ± .15
Gpp(NH)p	983 ± 27	0.66 ± .10
Ethanol	696 ± 41	0.59 ± .13
Gpp(NH)p + Ethanol	723 ± 84	0.63 ± .09

The influences of Gpp(NH)p and ethanol on [^{3}H]MS saturation curves to m4 receptors expressed in CHO cells are illustrated in Figure 11. As with m2 receptors expressed by

CHO cells and rat atrium, the guanine nucleotide increased antagonist binding, increasing the apparent number of binding sites without affecting binding affinity. Ethanol decreased [^{3}H]MS binding (again, affecting the apparent number of binding sites), and totally blocked the ability of the guanine nucleotide to increase antagonist binding. These results are also consistent with ethanol interference with receptor-G protein interactions.

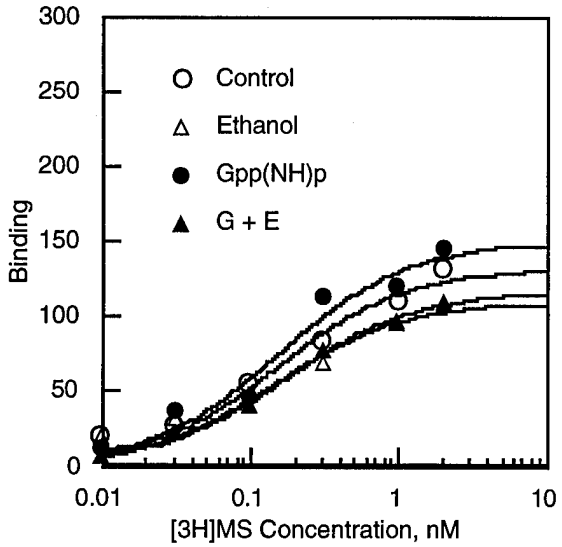


Figure 11. Influence of ethanol on the binding of [^{3}H]MS to m4 muscarinic receptors expressed in transfected CHO cells. Lines are drawn according to nonlinear regression fit to a single binding site model which revealed the parameters listed in the accompanying Table. Points are the mean from 3 determinations.

Table 4. Binding parameters of [^{3}H]MS binding to m4 muscarinic receptors expressed CHO cells measured in the presence of 100 mM ethanol and/or 10 μM Gpp(NH)p. Parameters are from the experimental data presented in Figure 11.

Condition	**Bmax, fmol/mg protein**	**Kd, nM**
Control	49.7	0.148
Gpp(NH)p	56.1	0.151
Ethanol	41.5	0.143
Gpp(NH)p + Ethanol	43.0	0.168

The influence of ethanol on m4 receptors and their coupling to G proteins was also studied in agonist/[^{3}H]antagonist competition studies. The guanine nucleotide Gpp(NH)p lowered agonist affinity (and probably increased [^{3}H]MS affinity), thereby shifting the competition curves to the right. This is usually interpreted as reflecting a binding conformation associated with an uncoupled receptor. In contrast, ethanol (100 mM) had no effect on the position of the agonist/[^{3}H]antagonist competition curve. Unexpectedly, ethanol did not affect the ability of Gpp(NH) to shift the competition curve to the right. These experiments are being repeated to be certain of the accuracy of this observation. The reason for this apparent contradiction of results illustrated in Figure 11 is not yet clear. This experiment suggests that ethanol does not, in fact, affect receptor-G protein coupling with m4 receptors. Further work is required to understand this discrepancy. It is possible that m2 and m4, although both coupled to Gi proteins) are differently affected by ethanol.

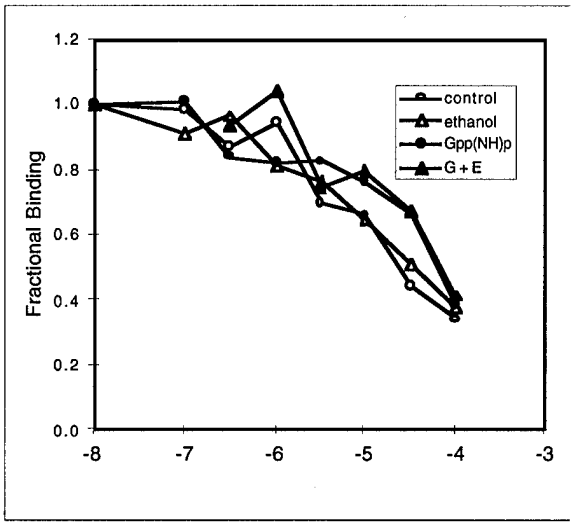


Figure 12. Influences of Gpp(NH)p and ethanol on carbamylcholine inhibition of [^{3}H]MS binding to m4 receptors expressed in CHO cells. The binding of 1 nM [^{3}H]MS was measured in the absence or presence of 100 mM ethanol and/or 10 μM Gpp(NH)p. Data is from a representative experiment performed in duplicate.

CHO cells expressing m1, m3 and m5 muscarinic receptors

Fewer experiments have been performed with CHO cells expressing the odd-numbered muscarinic receptors, since it is generally more difficult to observe the "guanine nucleotide effect" with these receptors. An example of [^{3}H]MS binding to m1 receptors is shown in Figure 13. Unexpectedly, both Gpp(NH)p and ethanol depressed [^{3}H]MS binding. We are repeating these experiments to be certain of the results.

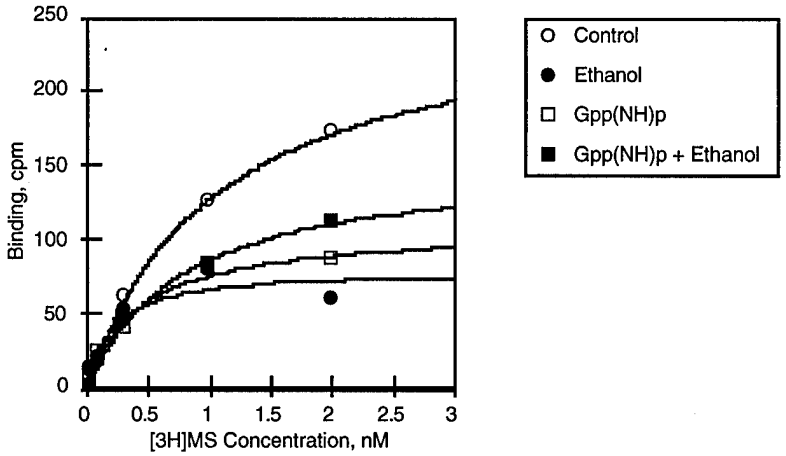


Figure 13. Typical [^{3}H]MS saturation curves for CHO cells transfected with m1 muscarinic receptors. Lines are drawn according to nonlinear regression analyses which revealed that ethanol and Gpp(NH)p decreased the apparent number of binding sites. The two effects were not additive. The binding levels corresponded to 55 fmol of receptor/mg membrane protein in control cells, compared to 28, 32 and 37 fmol/mg in the presence of ethanol (100 mM), Gpp(NH)p (10 μM) and ethanol + Gpp(NH)p, respectively (parameters from non-linear regression analyses).

On the other hand, [^{3}H]MS binding to m3 receptors expressed in CHO cells was strongly stimulated by Gpp(NH)p. We have not yet determined the influence of ethanol on the guanine nucleotide's action.

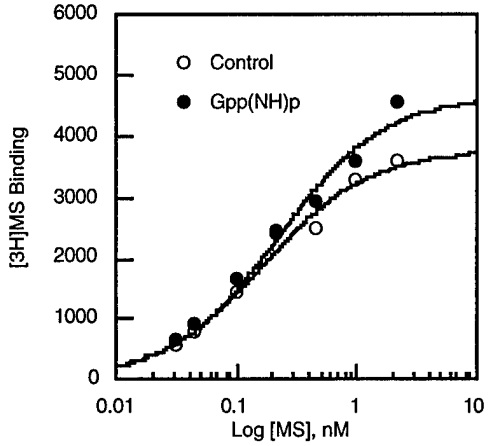


Figure 14. Saturation curves for [^{3}H]MS binding to m3 muscarinic receptors expressed in CHO cells. In the absence of Gpp(NH)p the receptor density was 1.6 pmol/mg membrane protein; in the presence of 10 μM Gpp(NH)p receptor density was 2.0 pmol/mg. The apparent affinity was not significantly different in the presence of guanine nucleotide (0.16 vs 0.21 nM).

Alkylation of muscarinic receptors with N-ethylmaleimide has long been known to increase the binding affinity of muscarinic receptors (Aronstam et al., 1978). This effect is though to reflect alterations in receptor-G protein coupling. It is interesting that alkylation of membranes from CHO cells expressing the m3 receptor shifted carbamylcholine/[^{3}H]MS competition curves to the left. We have initiated experiments to determine the influence of ethanol on this shift. These approaches are being exploited to glean the maximum amount of information on receptor-G protein interactions from ligand binding studies.

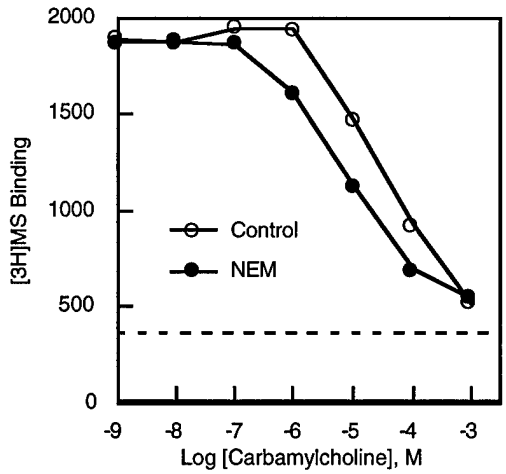


Figure 15. Influence of alkylation with N-ethylmaleimide (NEM) on carbamylcholine inhibition of [^{3}H]MS binding to m3 receptors expressed by CHO cells. The binding of 0.32 nM [^{3}H]MS was measured in the presence of the indicated concentrations of carbamylcholine. Binding was measured to m3 receptors in untreated membranes and in membranes that had been treated with 1 mM NEM for 15 min at 37°C. The dashed line indicates the level of nonspecific binding.

Another method for disrupting receptor-G protein interactions involves heating the membranes at 50°C for 10 min. This rapidly inactivates G proteins, as evidenced by decreases in low Km GTPase activity and receptor-stimulated guanine nucleotide binding; the receptor per se is not altered insofar as receptor density and antagonist binding is not affected. We found that this mild heating of membranes from CHO cells expressing both m2 and m3 altered carbamylcholine inhibition of [^{3}H]MS binding (Figure 16). Carbamylcholine inhibition curves were shifted to the right (i.e., to lower agonist affinity) by destroying the G proteins.

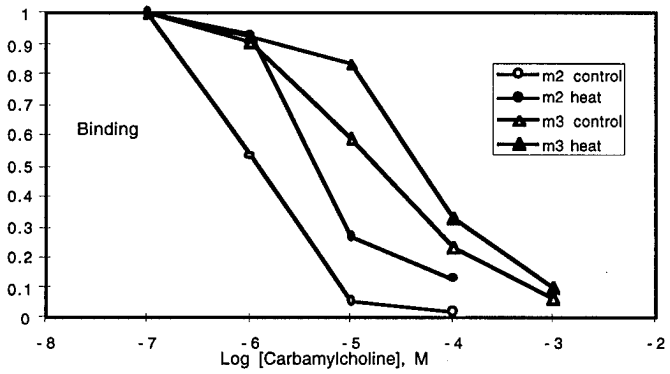


Figure 16. Influence of heat (50°C 10 min) on carbamylcholine inhibition of specific [^{3}H]MS binding to m2 and m3 receptors expressed by CHO cells. The binding of 1 nM [^{3}H]MS was measured in the presence of the indicated concentrations of carbamylcholine.

Saturation binding of [^{3}H]MS to m5 receptors expressed In CHO cells is demonstrated in Figure 17. The level of specific binding (32 fmol/mg membrane protein) was consistently the lowest of any of the receptor subtypes expressed in CHO cells. Consequently, we are attempting to alter culture conditions to enhance expression and to harvest a larger quantity of membranes.

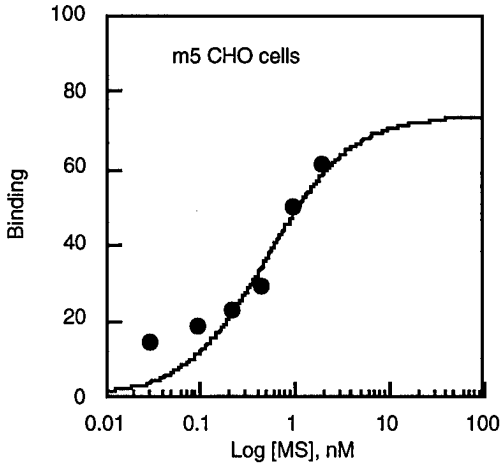


Figure 17. Saturation binding of [^{3}H]MS to m5 muscarinic receptors expressed by CHO cells. Receptor density was 32 fmol/mg membrane protein and the dissociation constant was 0.50 nM.

Other neurotransmitter receptors.

An important aspect of this study is the generality of ethanol's actions with respect to other neurotransmitter systems. To this end we have initiated studies with α1 and α2-adrenergic receptors (using [^{3}H]prazosin and [^{3}H]aminoclonidine as the probes) and D1 and D2 dopaminergic receptors (using [^{3}H]SCH23390 and [^{3}H]methylspiperone as the probes) and cerebral cortex as the tissue. The conditions for visualizing receptor-G protein interactions vary with each receptor subtype, and this has slowed our progress. We have demonstrated guanine nucleotide sensitivity of agonist binding to serotonin 5-HT_{1A}, a2-adrenergic, and adenosine A_1 receptors in rat brain using the same 50 mM Tris-HCl, pH 7.4 buffer containing 2 mM $MgCl_2$ which was successfully used with muscarinic receptors. In these studies the ability of a guanine nucleotide (Gpp(NH)p) to inhibit the binding of a tritiated agonist was used as an index of receptor-G protein coupling. As an example of this analysis, the influence of ethanol on the ability of Gpp(NH)p to inhibit

[^{3}H]oxotremorine binding to m2 muscarinic receptors expressed in rat brainstem is shown in Figure 18.

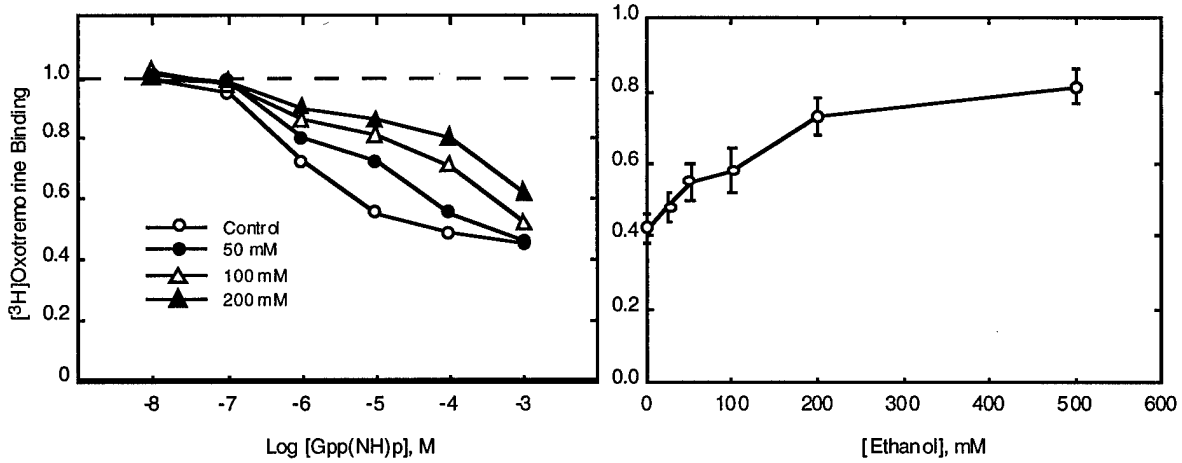


Figure 18. Influence of ethanol on the guanine nucleotide sensitivity of high affinity [^{3}H]oxotremorine-M binding to muscarinic acetylcholine receptor from rat brainstem. Fig. 18A: The binding of [^{3}H]oxotremorine-M was measured in the presence of the indicated concentrations of Gpp(NH)p in the presence of 0 (○), 50 (●), 100 (△) and 200 (▲) mM ethanol. Binding is expressed as fraction of specific binding measured in the absence of Gpp(NH)p. Each point represents the mean from 3 experiments which varied by less than 10%. Fig. 18B: [^{3}H]Oxotremorine-M binding was measured in the presence of 10 μM Gpp(NH)p and the concentration of ethanol indicated on the abscissa. Means and standard deviations from 4 experiments are indicated. *Significantly different from the level of binding in the absence of ethanol ($p < 0.01$; ANOVA and Dunnett's test). Figure from Aronstam et al., 1993.

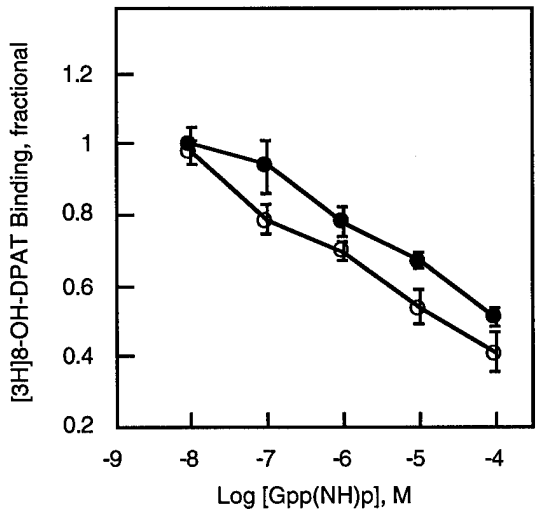


Figure 19. Guanine nucleotide sensitivity of the binding of 1 nM [^{3}H]8-hydroxy-dipropylaminotetralin ([^{3}H]8-OH-DPAT) to 5-HT_{1A} receptors expressed in membranes from rat brain hippocampal membranes. Binding was measured in the presence of the indicated concentrations of Gpp(NH)p. Results from two experiments are shown, the error bars refer to the variability associated with the binding measurements from two series of experiments (N=4 in each case). Non-specific binding was determined in the presence of 100 μM 5-HT, and represented less than 20% of the total binding. The binding medium contained 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, and 1 mM diothiothreitol. The binding incubation was carried out for 10 min at 37°C.

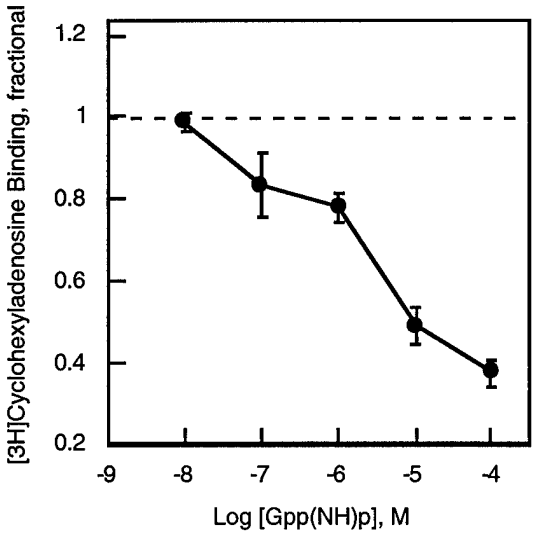


Figure 20. Guanine nucleotide sensitivity of the binding of 1 nM [^{3}H]cyclohexyladenosine to adenosine A1 receptors expressed in membranes from rat forebrain (telencephalon and diencephalon). Binding was measured in the presence of the indicated concentrations of Gpp(NH)p. Non-specific binding was determined in the presence of 1 mM theophylline and represented less than 20% of the total binding. The binding medium contained 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, and 1 mM diothiothreitol. The binding incubation was carried out for 60 min at 37°C. The membranes were incubated with adenosine deaminase (0.2 unit/ml) for 30 min at room temperature to remove endogenous adenosine.

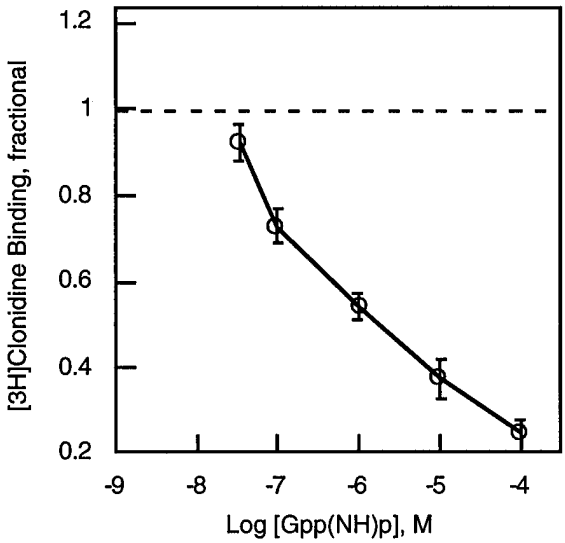


Figure 21. Guanine nucleotide sensitivity of the binding of 1 nM [^{3}H]clonidine to α_2-adrenergic receptors expressed in membranes from rat cerebral cortex. Binding was measured in the presence of the indicated concentrations of Gpp(NH)p. Non-specific binding was determined in the presence of 100 µM unlabelled clonidine. The binding medium contained 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, and 1 mM diothiothreitol. The binding incubation was carried out for 45 min at room temperature. Each point and bar represents the mean and standard deviation from 4 experiments.

In summary, a great deal of information has been obtained in ligand binding experiments which supports our hypothesis that receptor-G protein interactions are disrupted by ethanol. Receptor-G protein interactions were most readily visualized in m2 and m4, compared to m1, m3 and m5, muscarinic receptors. Thus, in intact tissues, these interactions were most readily visualized in brainstem and atrial tissue, which express mostly m2 receptors. In these studies ethanol decreased the sensitivity of agonist binding to inhibition by guanine nucleotide in a manner which is consistent with our hypothesis that ethanol disrupts receptor-G protein interactions (Aronstam et al., 1993).

A number of challenges present themselves when dealing with muscarinic receptors expressed by cultured cell lines, especially a relative paucity of experimental material. Moreover, a number of observations inconsistent with our hypothesis, and with our results in intact tissues, have been made on muscarinic receptors expressed in CHO cells. These

points are the focus of continued study. Novel physical and chemical mechanisms for disrupting receptor-G protein interactions (i.e., heat and alkylation) are being used to gain further insight into ethanol's actions and the nature of muscarinic receptors expressed in foreign systems. The optimal assay conditions for visualization of receptor-G protein interactions with the various neural receptors differ, although conditions have been identified for use with a number of receptor systems. Ethanol effects on these interactions are in progress.

C. G Protein Studies

1. Influence of acute ethanol administration on muscarinic receptor-stimulated G protein GTPase activity.

The overall hypothesis guiding this research is that ethanol disrupts synaptic signaling by altering receptor-G protein interactions. In these experiments we characterized the effect of ethanol on receptor control of two aspects of the G protein cycle, guanine nucleotide binding and hydrolysis. Guanine nucleotide binding is increased as a consequence of G protein interaction with stimulated receptors, while GTPase activity is a convenient measure of turnover in the G protein cycle. We further proposed to characterize a third reaction of the G protein cycle, namely the release of GDP from the inactive $G_{\alpha\beta\gamma}$ complex (an action catalyzed by interaction with the receptor). Below we present studies on ethanol's effects on muscarinic receptor-stimulated guanine nucleotide binding and hydrolysis. Measurements of GDP release have so far proven too unreliable to provide useful information. This probably reflects the compounding of the variablities inherent in G protein loading efficiency, receptor action, and release quantification.

Methods

Materials: [γ-^{32}P]Guanosine triphosphate ([^{32}P]GTP; ≈6000 Ci/mmole) and [^{35}S]guanosine 5'-(γ-thio)triphosphate ([^{35}S]GTP; ≈1000 Ci/mmole) were purchased from DuPont-NEN (Boston, MA).

Tissue Preparations: Adult, male Wistar rats (Harlan Sprague-Dawley, Indianapolis, IN) were decapitated and brain areas (especially the striatum) dissected and homogenized in 20 volumes of TED buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol) containing 10% sucrose using a Teflon-glass tissue grinder. The homogenate was spun at 1,000 x *g* for 10 min. The pellet was resuspended in the same buffer and spun under the same conditions. The supernatants were combined and centrifuged at 17,000 x *g* for 20 min. The pellet was resuspended in TED buffer (without sucrose) and washed twice by centrifugation. The resulting pellet was suspended in TED buffer adjusted to pH 8.0 and incubated on ice for 30 min. This suspension was then centrifuged at 17,000 x *g* for 20 min, and the pellet resuspended in TED buffer, pH 7.4, and used without further treatment. Low Km GTPase activity was quite sensitive to elevated temperatures, so tissue preparation was carried out at 0-4°.

G Protein Activity Assay: GTPase activity was determined by the method of Cassel and Selinger (1976). The reaction mixture (100 µl) contained 75 mM Tris-HCl, pH 7.4, [γ-^{32}P]GTP (70-100,000 cpm), 1 µM unlabeled GTP, 2 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM 5'-adenylylimidodiphosphate, 5 mM phosphocreatine, 50 units/ml creatine phosphokinase, 50 µg bovine serum albumin, 0.1 mM EDTA, 0.2 mM EGTA, 1 mM cAMP, 100 mM NaCl, and 8-20 µg membrane protein. The reaction was initiated by the addition of protein, and was continued for 5 min at 37°. The reaction was quenched by the addition of 5% activated charcoal in 20 mM phosphoric acid (pH 2.5). The samples were kept on ice for 10 min, and then centrifuged at 7,000 x *g* for 10 min. The radioactivity content of the supernatant was determined by liquid scintillation counting. Low K_m GTPase activity was calculated by subtracting [^{32}P] released in the presence of 100 µM unlabeled GTP. Receptor-stimulated activity was measured by including acetylcholine and 10 mM physostigmine in certain assays. The muscarinic nature of this stimulation was established by including 10 µM atropine in a parallel series of incubations. Measurements in each experiment were performed in quadruplicate.

Results: A time course of the low K_m GTPase activity of striatal membranes is shown in Figure 1. Activity was linear over at least 20 min under these conditions. Basal activity in the presence of 1 μM GTP was 60 ± 12 pmol/mg/min (n=12) over a standard incubation period of 5 min at 37°. This stimulation was greatest in the striatum (m1, m3 and m4 receptors) but could also be detected in brainstem and atrium (two tissues which express predominantly m2 receptors). Measurements of low K_m GTPase activity is a particularly sensitive and specific measurement of activation of the G protein cycle. These studies are currently being extended to muscarinic receptor subtypes expressed by transfected CHO cells.

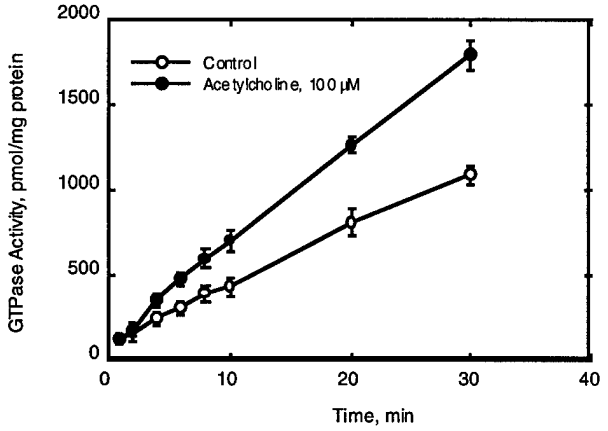


Figure 1. Time course for low K_m GTPase activity in rat striatal membranes: stimulation by acetylcholine. GTPase activity was measured with 1 μM GTP in the absence (O) and presence (●) of 100 μM acetylcholine. Each point and bar represents the mean and standard deviation from 3 experiments.

A concentration-response curve revealed a maximal stimulation of about 75% with an EC50 of about 1 μM (Figure 2). The stimulation by acetylcholine was completely prevented by preincubating the protein with 10 μM atropine (Figure 2).

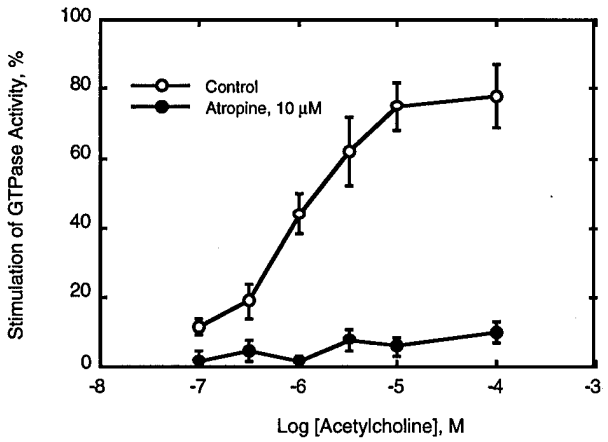


Figure 2. Acetylcholine stimulation of low K_m GTPase activity in rat striatal membranes. The percent stimulation of basal GTPase activity (i.e., activity measured in the absence of acetylcholine) was determined in the presence of acetylcholine at the concentrations indicated on the abscissa (O). In a parallel set of experiments the membranes were pretreated with 10 μM atropine for 30 min at 4° before GTPase activity was measured (●). Each point and bar represents the mean and standard deviation from 3 experiments.

A concentration response curve for atropine inhibition of acetylcholine-stimulated GTPase activity is presented in Figure 3. The relatively high concentrations of atropine required to inhibit acetylcholine stimulation (IC50 ≈ 0.8 μM) probably reflects the slow onset of atropine's action (the tissue was not preincubated with atropine in this experiment).

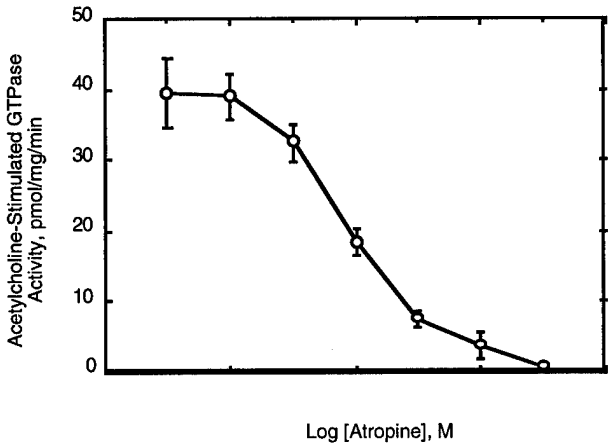


Figure 3. Atropine inhibition of acetylcholine-stimulated GTPase activity in rat striatal membranes. Atropine was included in the GTPase assay medium (i.e., the membranes were not preincubated with the atropine). Each point and bar represents the mean and standard deviation from 3 experiments.

Ethanol specifically inhibited acetylcholine-stimulated GTPase activity by up to 80% with an IC50 of ≈ 40 mM. Ethanol slightly increased basal activity measured in the absence of acetylcholine (10-15%). Selective inhibition of muscarinic receptor-stimulated GTPase activity was observed at several GTP concentrations from 0.5 – 2.0 μM (Fig. 4A). Double reciprocal plots (Fig. 4B) indicate that ACh increases the maximum enzymatic rate; this increase was largely reversed by ethanol.

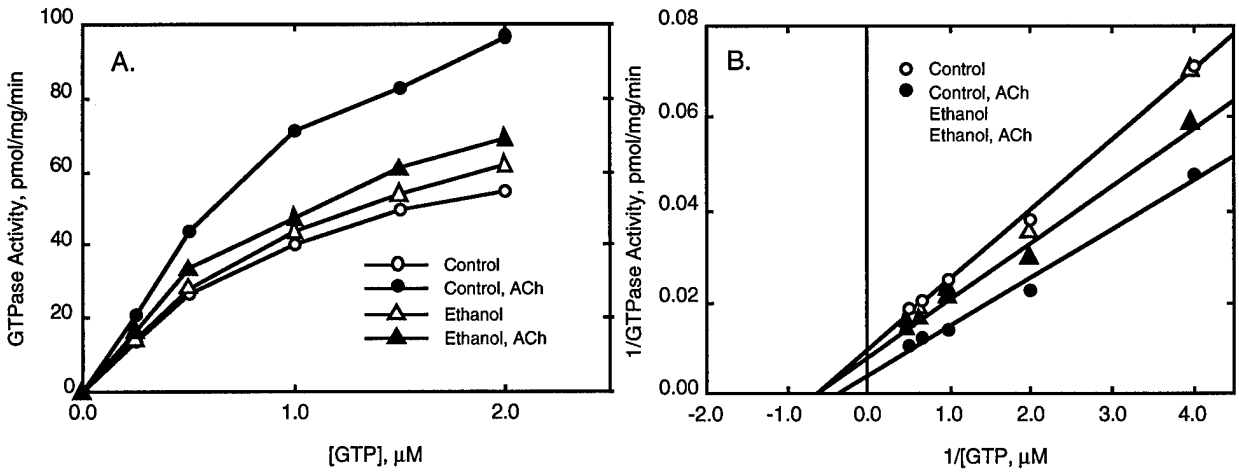


Figure 4. Influence of ethanol on basal and acetylcholine-stimulated GTPase activity determined at several concentrations of GTP. **A.** GTPase activity was measured in the absence (circles) and presence (triangles) of 200 mM ethanol in the absence (open symbols) and presence (closed symbols) of 100 μM acetylcholine. The GTP concentration of the assay medium is indicated on the abscissa. Each point represents the mean from 3 experiments which varied by up to 15%. **B.** Double reciprocal plots of the data presented in **A**. Lines are drawn according to linear regression analysis. (The control/ACh (△) line was essentially similar to the control/no ACh (▲) line, and is not shown for reasons of clarity.)

2. Influence of acute ethanol administration on neurotransmitter receptor-stimulated G protein GTPase activity.

In order to test the generality of the effect of ethanol on receptor-G protein coupling, the influence of ethanol on stimulation of a variety of neurotransmitter systems other than muscarinic was initiated. These characterizations are still in progress. To date, we have been able to reliably detect receptor-stimulated G protein GTPase activity in beta-adrenergic, α_2-adrenergic, and histaminergic

receptors. As an example, isoproterenol stimulation of G protein GTPase activity in neural membranes is shown in Figure 5 below. This stimulation was completely blocked by 100 μM propranolol. The influence of ethanol on this response has not yet been determined.

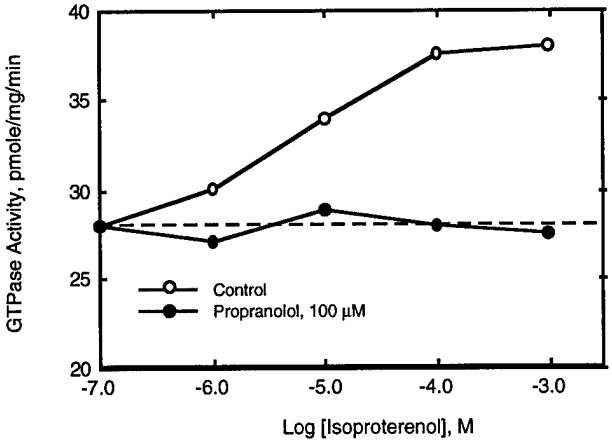


Figure 5. Isoproterenol stimulation of low K_m GTPase activity in rat neural membranes. GTPase activity was determined in the presence of isoproterenol at the concentrations indicated on the abscissa (O). In a parallel set of experiments the membranes were pretreated with 100 μM propranolol for 30 min at 4° before GTPase activity was measured (●). Each point represents the mean from 3 experiments.

3. Influence of acute ethanol administration on muscarinic receptor-stimulated G protein guanine nucleotide binding.

Methods: [^{35}S]Guanosine 5'-(γ-thio)triphosphate ([^{35}S]GTP; ≈1000 Ci/mmole) was purchased from DuPont-NEN (Boston, MA). Adult, male Wistar rats (Harlan Sprague-Dawley, Indianapolis, IN) were decapitated and brain regions dissected and homogenized in 20 volumes of TED buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA,.1 mM dithiothreitol) containing 10% sucrose using a Teflon-glass tissue grinder. The homogenate was spun at 1,000 x *g* for 10 min. The pellet was resuspended in the same buffer and spun under the same conditions. The supernatants were combined and centrifuged at 17,000 x *g* for 20 min. The pellet was resuspended in TED buffer (without sucrose) and washed twice by centrifugation. The resulting pellet was suspended in TED buffer adjusted to pH 8.0 and incubated on ice for 30 min. This suspension was then centrifuged at 17,000 x *g* for 20 min, and the pellet resuspended in TED buffer, pH 7.4, and used without further treatment. Tissue preparation was carried out at 0-4°C.

The binding of [^{35}S]GTP was measured using a filtration procedure. Unless otherwise indicated, neural membranes (15 μg protein) were incubated with 0.4 nM [^{35}S]GTP in TED buffer containing 2 $MgCl_2$ at pH 8.0 in a final volume of 1 ml. Nonspecific binding was measured in the presence of 10 μM GTP. After a 30 min incubation at 30°C, the suspension was filtered through a glass fiber filter (#32; Schleicher and Schuell; Keene, NH). The tubes and filters were washed twice with 5 ml TED buffer, and the radioactivity content of the filters was determined by liquid scintillation counting. Carbamylcholine and atropine were included in certain assays to identify muscarinic receptor-stimulated [^{35}S]GTP binding.

Results:

The level of high affinity [^{35}S]GTP binding was about the same in cortex, hippocampus and striatum (Figure 7). Binding was highly dependent on the presence of Mg^{2+}.

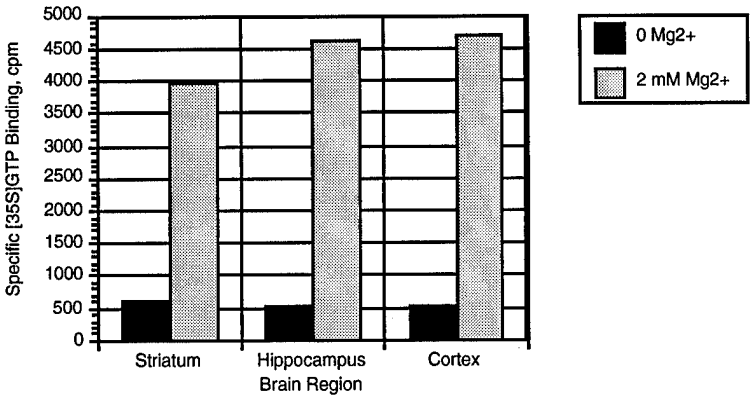


Figure 6. Regional distribution of specific [^{35}S]GTP binding in neural membranes from rat brain. Binding was measured in the absence and presence of 2 mM $MgCl_2$.

The concentration dependence of [^{35}S]GTP binding in cortical and striatal membranes is shown in Figure 7. The affinity of [^{35}S]GTP for its high affinity site was approximately 1 nM and did not differ in the different regions. Under mild heating conditions (50°C for 10 min), [^{35}S]GTP binding was severely depressed. This loss of binding activity closely paralleled the loss of low Km GTPase activity upon heating (Aronstam, unpublished results), and is characteristic of G proteins. This [^{35}S]GTP binding assay has also been used to follow G proteins during purification, suggesting that the high affinity [^{35}S]GTP binding measured in the present experiments reflects binding to heterotrimeric G proteins.

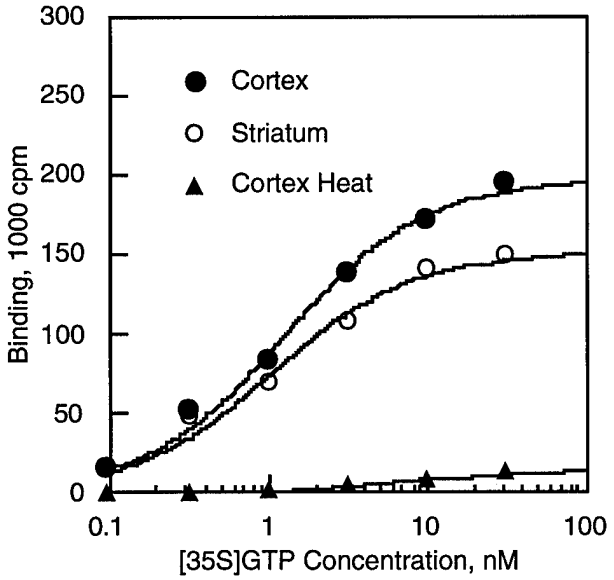


Figure 7. Concentration dependence for [^{35}S]GTP binding in two brain regions. Specific binding was measured in the cortex and striatum. Heating cortical membranes at 50°C for 10 min eliminated most binding, as well as low Km GTPase activity (not shown). Lines are drawn according to nonlinear regression to a single receptor population model which indicated maximum binding levels of 196940 in the cortex and 151649 in the striatum. The corresponding apparent affinity constants were 1.2 ± 0.2 and 1.0 ± 0.2 nM, respectively.

Muscarinic agonists consistently stimulated [^{35}S]GTP binding to neural membranes in striatum and cortex (e.g., Figure 8). This response is modest but reproducible. In the presence of 2 mM $MgCl_2$, carbamylcholine increased the binding of 0.4 nM [^{35}S]GTP by up to 20%. This increase was observed in all brain regions examined. We have not been successful at detecting cholinergic stimulation of [^{35}S]GTP binding to CHO or SK-N-SH membranes.

Ethanol significantly inhibited [^{35}S]GTP binding that was stimulated by acetylcholine (Figures 8 and 9), in agreement with our preliminary findings (Aronstam, 1993). This effect of ethanol was specific for the agonist-stimulated activity insofar as basal [^{35}S]GTP binding was not affected. There was an excellent correlation of between the ability of ethanol to inhibit carbamylcholine-stimulated [^{35}S]GTP binding and G protein GTPase activity (Figure 9).

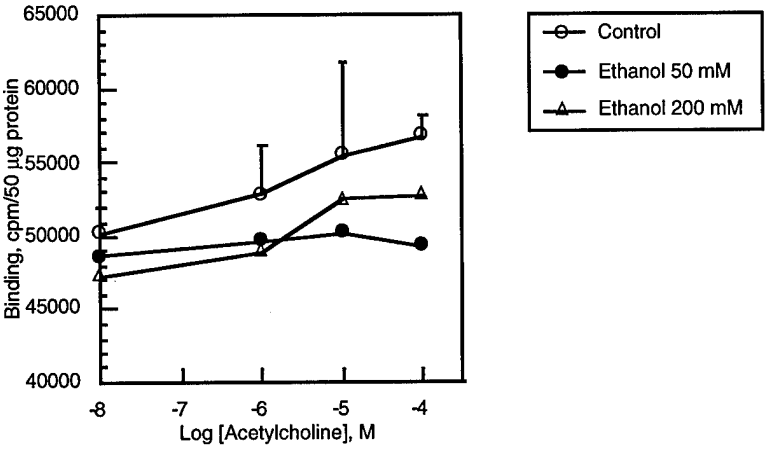


Figure 8. Stimulation of high affinity [^{35}S]GTP binding to cortical membranes by acetylcholine. Ethanol depressed this stimulation. Each point is the mean from 3 determinations; only standard deviations for the control curve are shown for clarity.

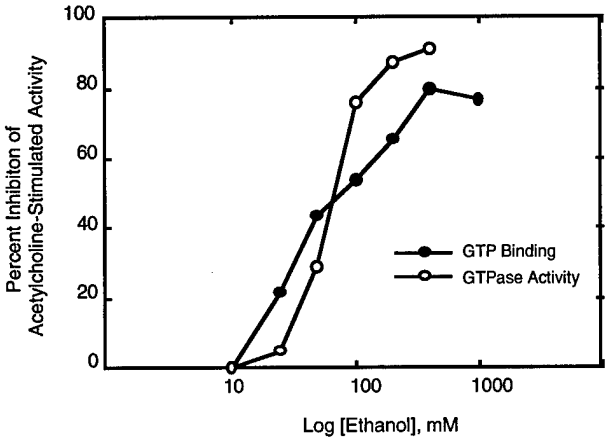
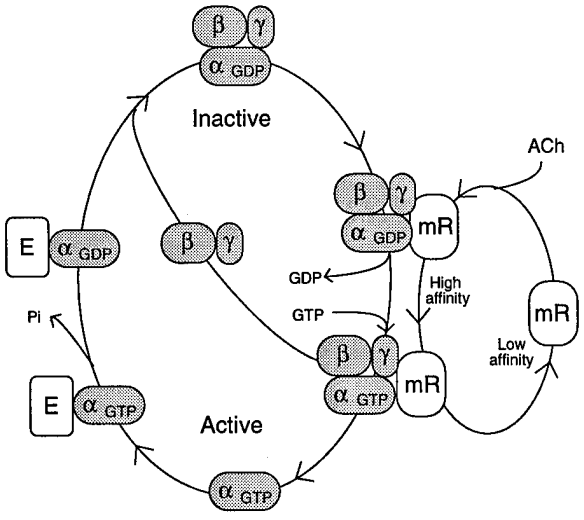


Figure 9. Comparison of the ability of ethanol to inhibit carbamylcholine-stimulated [^{35}S]GTP binding and G protein GTPase activity in rat striatal membranes. The inhibition is expressed as a fraction of maximum stimulated activity. Data is from a representative experiment.

Discussion. Results to date demonstrate that ethanol disrupts muscarinic regulation of G protein activity as evidenced by its inhibition of agonist-stimulated G protein GTPase activity and guanine nucleotide binding. The concentrations of ethanol required to produce this inhibition support the notion that these actions contribute to the intoxicating effects of ethanol.

Agonist binding to muscarinic (and many other hormonal and neurotransmitter) receptors is thought to engender an interaction of the receptor with the α subunit of transducer G proteins (Gilman, 1987; Iynegar et al., 1991). This interaction triggers a release of GDP from the α subunit. GTP then binds to the α subunit, initiating a dissociation of G protein subunits. The α_{GTP} subunit then interacts with an effector mechanism (e.g., enzyme or ion channel) altering its activity. This interaction is terminated by an intrinsic GTPase activity of the a subunit. The $G\alpha\beta\gamma$ complex then reassociates, completing the cycle. By catalyzing GDP release from the subunit, the receptor interaction accelerates turnover of the G protein cycle, seen in the present study as an increase in guanine nucleotide binding and



hydrolysis. Muscarinic receptor-mediated stimulation of both [^{35}S]GTP binding and [γ-^{32}P]GTP hydrolysis has been reported by others (Onali et al., 1983; Hoss et al., 1988; Hilf et al., 1989).

Concentrations of ethanol associated with impairment of reaction time in humans are about 4 - 6 mM. Ethanol concentrations associated with gross intoxication range from 30 - 50 mM, while lethal concentrations are generally above 80 mM (Ritchie, 1985). The IC50's associated with ethanol inhibition of muscarinic receptor-stimulated GTPase activity and GTP binding were approximately 40 to 80 mM, respectively. Thus, the present actions of ethanol might contribute to the physiological disturbances observed in ethanol intoxication.

In these actions, ethanol resembles a second class of CNS depressants, the volatile general anesthetics (Aronstam and Dennison, 1989). The general anesthetics interfere with receptor-G protein interactions in (at least) muscarinic and α_2-adrenergic systems, thereby depressing the guanine nucleotide sensitivity of agonist binding, receptor control of G protein function, and receptor regulation of adenylate cyclase activity (Aronstam and Dennison, 1989; Narayanan et al., 1988; Baumgartner et al., 1990. This similarity raises the possibility that disruption of synaptic transmission by a number of hydrophobic depressant compounds (i.e., compounds with access to interior membrane structures) involves an interference with receptor-G protein interactions. In this regard, it is noteworthy that the majority of neurotransmitter receptors couple to transducer G proteins, although we have found considerable variability in the susceptibility of different G protein-coupled receptors to the actions of volatile anesthetics. For example, adenosine A_1 and serotonin 5-HT-$_{1A}$ receptors were not affected by a volatile anesthetic (Martin et al., 1991).

In summary, we have presented further evidence that ethanol interferes with receptor control of the G protein cycle in muscarinic cholinergic systems. This is seen as an inhibition of muscarinic receptor control of G protein GTPase activity and guanine nucleotide binding. These effects are consistent with the effects of ethanol on the guanine nucleotide sensitivity of agonist binding, and are similar to the effects of volatile anesthetics on the same system. These findings further suggest that interference with receptor-G protein interactions is a common mechanism of action of compounds which nonspecifically depress synaptic transmission.

D. Synaptic Transmission Studies

Influence of ethanol on synaptic transmission processes.

Experiments have focused on 1) muscarinic receptor-mediated inhibition of adenylate cyclase, 2) the intracellular Ca^{2+} increase in response to muscarinic receptor activation, and 3) muscarinic receptor-mediated modulation of intercellular adhesion processes. The first two pathways were chosen because they represent the two major signal transduction pathways associated with muscarinic receptors: muscarinic receptors (especially m2 and m4) couple efficiently via Gi proteins to the inhibition of adenylate cyclase, while m1, m3 and m5 receptors couple efficiently via Gq/11 proteins to the stimulation of phospholipase C. Moreover, these pathways are associated with the activation of neural synaptic receptors regardless of the specific neurotransmitter. Phospholipase C catalyzes the breakdown of phosphatidylinositol in the membrane to two second messengers, inositol triphosphate and diacylglycerol. Inositol triphosphate releases Ca^{2+} from intracellular stores. This increase in Ca^{2+} in the cytoplasm in turn stimulates the influx of extracellular calcium. Thus, monitoring these two processes allows us to ascertain the functional significance of ethanol disruption of muscarinic systems. The third process (cell-cell adhesion) is a recently discovered muscarinic action that affords us the opportunity to consider the effects of ethanol on cholinergic signaling in non-neuronal cells. Completed studies characterizing ethanol's effect on muscarinic control of adenylate cyclase activity and cell-cell adhesion are presented below, along with summaries of on-going studies of Ca^{2+} responses to receptor activation in cultured cells.

1. Adenylate Cyclase Studies

The acute influence of ethanol on receptor control of cAMP concentration in brainstem membranes was determined to ascertain the functional significance of altered receptor-G protein interactions observed in ligand binding studies. These studies are presented below.

Methods. Adenylate cyclase activity was measured by the method of Salomon et al. (1974) with minor modifications. Each assay tube contained the following reagents in a final volume of 250 µl: 25 mM HEPES, pH 7.5; 1.33 mM EGTA; 1 mM dithiothreitol; 2 mM $MgSO_4$; 50 mM NaCl; 0.1 mM ATP; 1 mM cAMP; 1 µM GTP; 20 mM creatine phosphate; 10 units of creatine phosphokinase; 0.5 µCi [^{32}P] ATP; 50-100 µg of protein. Assays were carried out in quadruplicate; replicate values generally varied by less than 10%. The reaction was started by adding the membranes, and the incubation was continued for 10 min at 37°C. The reaction was stopped by immersing the assay tube in a boiling water bath for 1 min, followed by centrifugation at 4000 x g for 15 min. Purification of cAMP was carried out by the method of Mao and Guidotti (1974). An aliquot of the supernatant was loaded onto a neutral alumina column (0.7 x 4 cm) equilibrated with 0.06 M Tris-HCl, pH 7.5, and eluted with 5 ml of the equilibration buffer. The eluate was directly loaded onto a Bio-Rad AG 1x4 formate column (0.7 x 2 cm) equilibrated with water. The column was washed twice with 10 ml of water, and the bound cAMP was eluted with 5 ml of 1 N formic acid. An aliquot was examined to determine [^{32}P]cAMP content using a Beckman liquid scintillation counter. The recovery of cAMP curing the purification procedure was routinely monitored using [^{3}H]cAMP, and was always between 70 and 80%.

Results and Discussion. Basal adenylate cyclase activity ranged from 5 to 12 pmol/mg/min at 37°C in the various tissue preparations. Adenylate cyclase activity was routinely stimulated 2–3 fold by 5 µM forskolin. Acetylcholine had no significant on basal adenylate cyclase activity, but inhibited forskolin-stimulated activity by 20–40% in the various preparations (Figure 1). These findings are in agreement with results from many other laboratories.

Unexpectedly, the inhibitory effect of acetylcholine on forskolin-stimulated adenylate cyclase activity was markedly *increased* by ethanol (Figure 1A–C). Maximal inhibition was increased to 40–52%, and the effect was more marked at lower concentrations of acetylcholine. Thus, 100 mM ethanol increased the inhibition by 1 μM acetylcholine from 5 to 27% in one experiment (Figure 1A). The results were highly reproducible; results from three experiments are shown in Figure 1. The concentration dependence for this effect is summarized in Figure 2. At 50 mM ethanol, the effect was almost maximal, indicating that the effect occurs well within ethanol concentrations achieved following human ingestion.

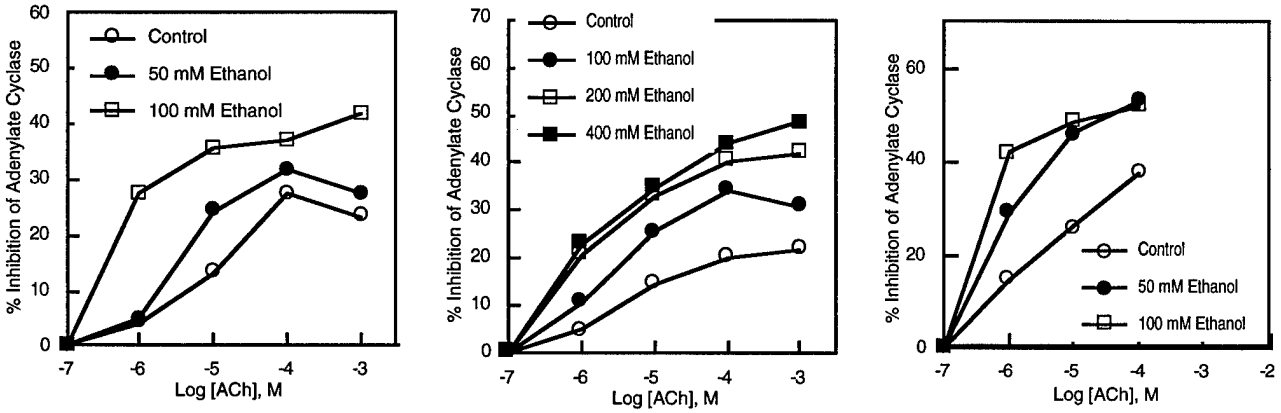


Figure 1. Influence of ethanol on acetylcholine inhibition of forskolin-stimulated adenylate cyclase activity in rat brainstem membranes. Results from three experiments are shown. Each point represents the average from 4 determinations which varied by less than 2 pmol/mg/min.

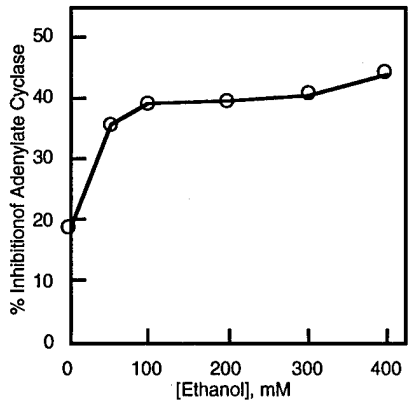


Figure 2. Concentration dependence of the influence of ethanol on adenylate cyclase measured in membranes isolated from rat brainstem. The fractional inhibition of adenylate cyclase activity by 1 μM acetylcholine was measured in a 10 min assay performed at 37°C. Each point represents the mean from four determinations that varied by less than 10%.

It was unclear how to reconcile this finding with earlier indications from 1) ligand binding, 2) GTPase modulation studies and 3) pharmacological studies, that suggested that cholinergic-G protein interactions are, in fact, disrupted by ethanol and that functional cholinergic synaptic transmission would be expected to be depressed. Adenylate cyclase in neural membranes is under reciprocal control by G protein-coupled rectors associated with both the inhibitory (with respect to adenylate cyclase) Gi protein and the stimulatory, Gs protein. Accordingly, we examined the influence of ethanol in our system on the stimulatory regulation of adenylate cyclase by one Gs-coupled receptor, the β-adrenergic receptor. We found that ethanol (200 mM) increased the ability of isoproterenol (a β-adrenergic receptor agonist) to stimulate adenylate cyclase activity in brainstem membranes (Figure 3). Thus, ethanol (200 mM) increased adenylate cyclase activity measured in the presence of 10 μM isoproterenol from 11 to 20 pmol/mg/min at 37°C.

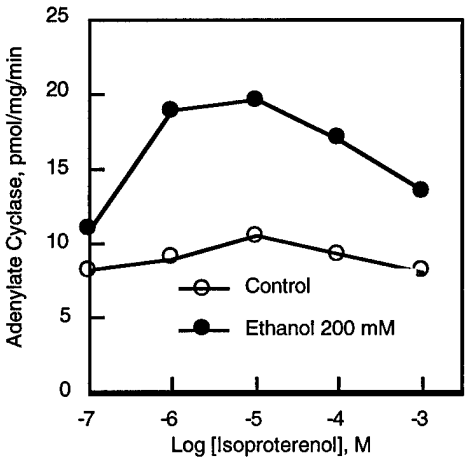
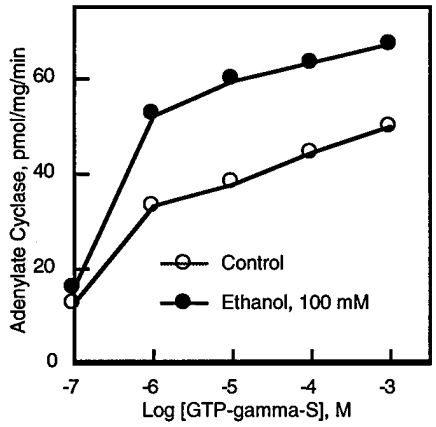
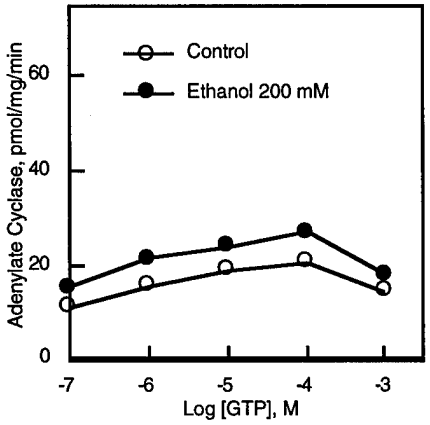


Figure 3. Influence of ethanol on isoproterenol stimulation of adenylate cyclase activity in membranes isolated from rat brainstem. Results are from a 10 min assay performed at 37°C in the presence of the indicated concentrations of isoproterenol. Each point represents the average from 4 determinations which varied by less than 2 pmol/mg/min.

In order to further define the nature of ethanol's action, we examined the influence of ethanol on the effects of guanine nucleotides on adenylate cyclase activity. Guanine nucleotides, such as GTP and its stable analogues, Gpp(NH)p and GTP-γ-S, interact directly with the G proteins, thereby affecting the catalytic activity of adenylate cyclase. GTP, Gpp(NH)p, and GTP-γ-S were found to markedly stimulate basal adenylate cyclase activity in brainstem membranes (Figure 4). The stable nucleotides, GTP-γ-S and Gpp(NH)p were more active in this regard. Ethanol was found to markedly enhance activation by each nucleotide (Figure 4A-C). At concentrations above 100 μM, there was a decrease in activity with both GTP and Gpp(NH)p.





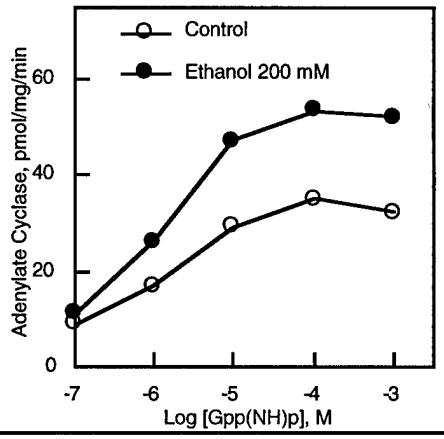


Figure 4. Influence of ethanol on guanine nucleotide stimulation of adenylate cyclase activity in membranes isolated from rat brain stem. Adenylate cyclase activity was measured in the presence of the indicated concentrations of GTP-g-S, Gpp(NH)p and GTP for 10 min at 37°C and the indicated concentrations of ethanol. Each point represents the mean from 4 determinations.

The influence of ethanol on NaF activation of adenylate cyclase activity is depicted in Figure 5. NaF activates G proteins by occupying a site on the G protein that is reserved for the gamma phosphate group of GTP. Ethanol was found to markedly enhance cyclase stimulation by NaF. This strengthens our suggestion that ethanol is having complex actions due to its interactions with (perhaps multiple classes of) G proteins.

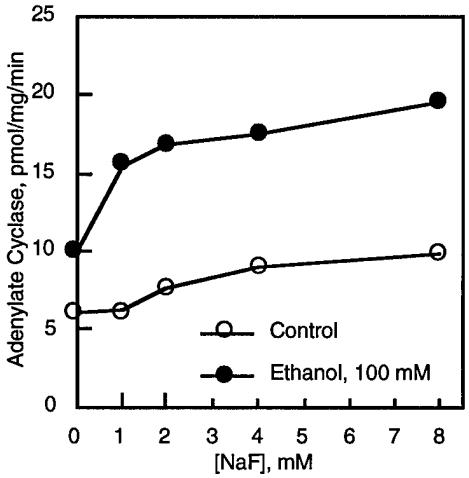


Figure 5. Influence of ethanol on NaF stimulation of adenylate cyclase in membranes isolated from rat brainstem. Adenylate cyclase activity was measured for 10 min at 37°C in the presence of the indicated concentrations of NaF and ethanol. Each point represents the mean from 4 determinations.

We have begun to extend these studies have been extended to a few other neurotransmitter systems. Preliminary results demonstrating the feasibility of measuring responses in β-adrenergic and α_2-adrenergic brain systems are depicted in Fig. 6. Activation and inhibition by forskolin and acetylcholine, respectively, are included for comparison purposes.

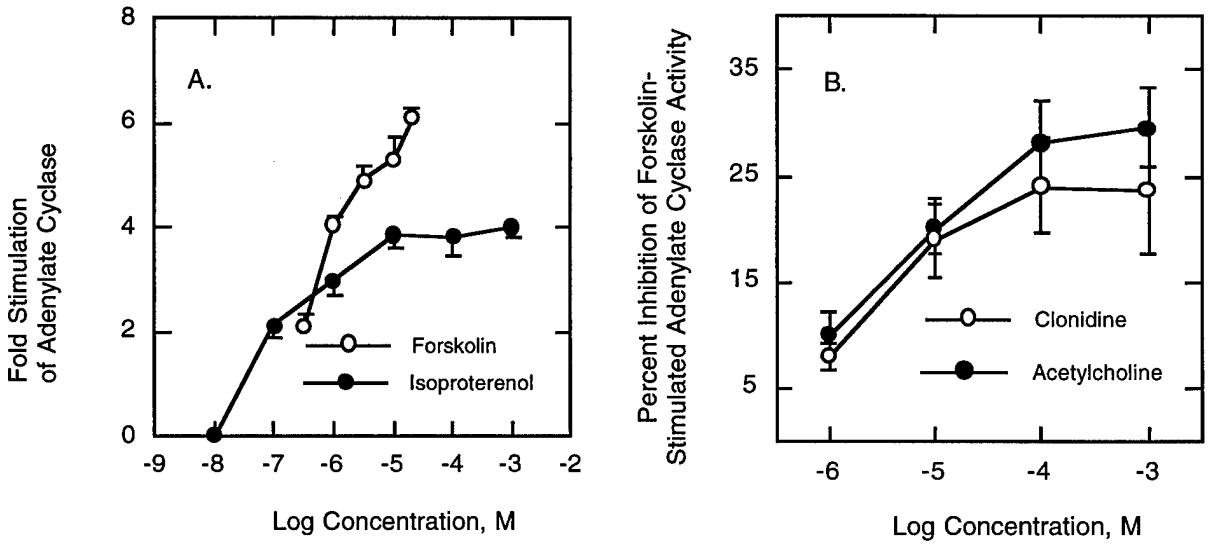


Figure 6. A. Stimulation of adenylate cyclase in whole brain homogenates by forskolin (acting directly on the catalytic subunit) and isoproterenol (acting via β-adrenergic receptors). B. Percent inhibition of forskolin (1 μM)-stimulated adenylate cyclase by clonidine and acetylcholine acting through α_2-adrenergic and muscarinic receptors, respectively. Each point represents the mean and standard deviation from 3 experiments.

These findings indicate multiple effects of ethanol on the control of adenylate cyclase. Basal activity was not consistently affected, suggesting that its effects reflect actions on the regulatory mechanisms (i.e., receptors and/or G proteins) rather than the catalytic subunit per se. In order to visualize an effect of acetylcholine, it is necessary to activate the enzyme. When this is done, the inhibition by ethanol is actually increased. These experiments raise the possibility that the effect of ethanol actually reflects an increase in the ability of guanine nucleotides (1 μM GTP is present in the assay medium) to stimulate adenylate cyclase activity, rather than an effect on cholinergic signal transduction mechanisms per se. It is likely that there is a balance between inhibitory (Gi-mediated) and stimulatory (Gs-mediated) influences on adenylate cyclase activity that is disrupted by ethanol. At low guanine nucleotide concentrations it seems likely that a stimulatory effect of ethanol on Gs predominates; there are some indications that this may change at higher nucleotide concentrations. Thus, it may not be possible to isolate ethanol's effects on cholinergic transmission using this approach. Accordingly, in subsequent experiments, we gave increased attention to other measures and aspects of cholinergic signaling, particularly measurements of intracellular calcium concentration regulation.

It is interesting that this effect of ethanol differs markedly from the effect of volatile general anesthetics, which also disrupt cholinergic (and other types) neurotransmission by disrupting receptor-G protein interactions. We have shown that the volatile anesthetics depress receptor-mediated inhibition of forskolin-stimulated adenylate cyclase in muscarinic and α-adrenergic systems (Anthony et al., 1990; Baumgartner et al., 1990).

2. Calcium responses to receptor stimulation.

This approach is particularly powerful because it permits the detection of receptor responses in both single cells and small groups of cells, and it has proven applicable to all of the muscarinic receptor subtypes, in addition to receptors for other neurotransmitters. While phospholipase C-coupled muscarinic receptors elicit the greatest changes in intracellular Ca^{2+} content (see below), there is sufficient cross-talk and indirect actions among the various signal transduction pathways to permit the detection of a Ca^{2+} response to all neurotransmitters we have examined to date. Thus, we can detect changes associated with stimulation of m1, m2, m3, m4 and m5 receptors (in SK-N-SH or CHO cells), although the largest responses are associated with the odd-numbered receptors.

Methods. SK-N-SH human neuroblastoma cells were grown to approximately 80% confluency in complete DMEM media (Dulbecco's Modification of Eagle's Medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and non-essential amino acids) in a 150 cm^2 culture flask. The cells were dislodged by treatment with Viokase, then washed and resuspended in Hank's salt solution. The cells were counted, pelleted and resuspended in DMEM media at a concentration of 1 x 10^5 cells/ml. Two ml of the cell suspension was plated on sterile glass coverslips (0.11 mm x 31 mm) in 35 mm x 10 mm Petri dishes. The cells were grown at 37° C in 5% CO_2 for approximately four days prior to measuring intracellular Ca^{2+}. CHO cells stably transfected with the genes for human muscarinic receptor subtypes were provided by Dr. Mark Brann (University of Vermont). CHO cells were grown in HAM'S F-12 (MOD) with L- glutamine (Mediatech, Fisher Scientific), 1X Penicillin/Streptomycin, and 5% fetal bovine serum. Intracellular Ca^{2+} measurements were made using the fluorescent dye Fura-2. The media was removed and the cells were incubated for 30 minutes in complete DMEM containing 2 μM Fura-2-AM, after which the media was replaced with fresh complete DMEM and the samples were incubated again for 30 minutes. The coverslips were then transferred to sample holders and 1.0 ml of complete DMEM media was added.

Intracellular Ca^{2+} measurements were performed using a SPEX Industries dual excitation wavelength fluorimeter. The samples were placed on the stage of the inverted microscope and either a single cell or a representative group of 3-4 cells was brought into focus. A scan (300 second, unless otherwise indicated) was then initiated using alternating 340 and 380 nm excitation wavelengths and an emission wavelength of 505 nm. The ratio of the fluorescent intensity at 505 nm after stimulation at 340 and 380 nm reflects the Ca^{2+} concentration; fluorescent emission after excitation at 340 nm increases when calcium is bound to Fura-2, while fluorescent emission after excitation at 380 nm decreases (e.g., see Figure 4 below). Since the ratio of intensities was measured, the signal is not affected by the absolute concentration of Fura-2 and the efficiency of Fura-2 loading is not a critical factor.

Carbamylcholine was used to stimulate the muscarinic receptors instead of the native transmitter acetylcholine because it is a close structural analogue of acetylcholine, but it is more stable because it is not degraded by esterase enzymes (but see Results below), and to facilitate comparison to binding experiments performed with carbamylcholine. The carbamylcholine was diluted in DMEM from a 0.1 M stock solution to a final concentration of 100 μM and was gently pipetted onto the coverslip at an appropriate time (generally at 60 seconds).

In some experiments EGTA was added to the medium just prior to the Ca^{2+} measurements to remove all of the extracellular calcium. In some experiments, fluorescent intensity was measured at 505 nm after excitation at 360 nm. This fluorescence is not sensitive to the Ca^{2+} concentration, and this procedure was utilized to determine divalent cation entry as signaled by Mn^{2+} quenching of the

fluorescent signal. When Mn^{2+} enters the cell it binds to the fluorescent dye, decreasing the emission of light at 505 nm.

Results and Discussion.

We characterized the Ca^{2+} responses to carbamylcholine stimulation of the m3 receptor expressed in SK-N-SH cells and all five muscarinic receptor subtypes expressed in CHO cells. This characterization included 1) time courses, 2) carbamylcholine concentration dependence, 3) initial phase (reflecting intracellular Ca^{2+} release) vs tonic response (reflecting extracellular entry) using EGTA, thapsigargin and Mn^{2+}, 4) pharmacology of the response (using specific receptor blockers), and 5) receptor desensitization. The influence of ethanol on these responses have been analyzed in CHO cell which express the m1 and m5 receptors; work on the receptors expressed in other CHO cells and in SK-N-SH cells is continuing. Ethanol was found to inhibit Ca^{2+} responses in most CHO m1 and m5 cells.

SK-N-SH cells (m3 Receptors): Typical responses of SK-N-SH cells to the muscarinic agonist carbamylcholine are presented in Figures 1-3. Every cell examined (several hundred) responded to carbamylcholine the absolute level of the response showed considerable variability, especially between sessions. We have been unable to ascertain the cause of this day -to-day and week-to-week variation. Within a session, the responses tended to be fairly constant (variability about 15%). The response of SK-N-SH cells to carbamylcholine could be divided into an initial phasic and a tonic plateau response (Figure 1). The plateau response was eliminated by including EGTA in the extracellular medium to chelate the calcium. The plateau response was also inhibited by Ni^{2+}, which blocks voltage-gated calcium channels.

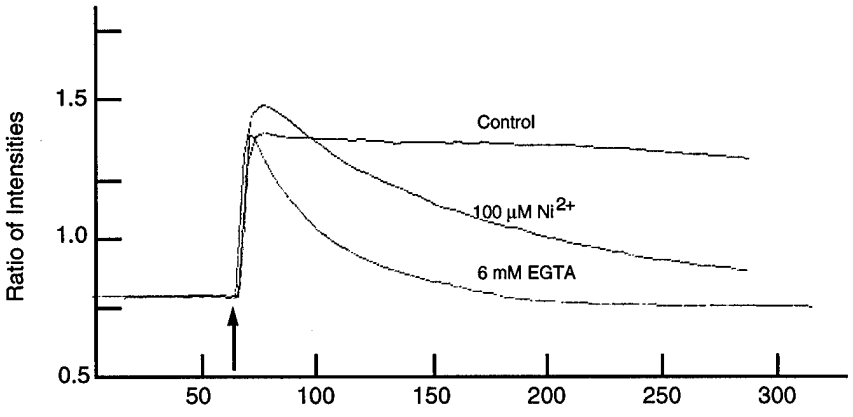


Figure 1. Carbamylcholine stimulation of Ca^{2+} influx in cultured SK-N-SH cells. Cells were loaded with the Fura-2 calcium sensitive dye and the ratio of fluorescent intensities measured after stimulation at 340 and 380 nm, which reflects the intracellular content of Ca^{2+}, was determined. The response could by broken into two components, an initial transient phase which reflected release of Ca^{2+} from intracellular sources and was not sensitive to extracellular Ca^{2+}, and a sustained plateau phase which reflected influx of extracellular Ca^{2+} and which was blocked by including EGTA or nickel in the extracellular medium..

The concentration dependence for carbamylcholine-elicitation of a calcium response is illustrated in Figure 2. Minimal responses were observed with 1 µM carbamylcholine, no response was obtained with 0.1 µM carbamylcholine, and maximal responses required 100 µM carbamylcholine. With an EC50 of ≈ 10 µM this would be appropriately classified as a low affinity response. It is

interesting that CHO cells expressing m3 receptors are considerably more responsive to carbamylcholine (see below); the reason for this discrepancy is not clear.

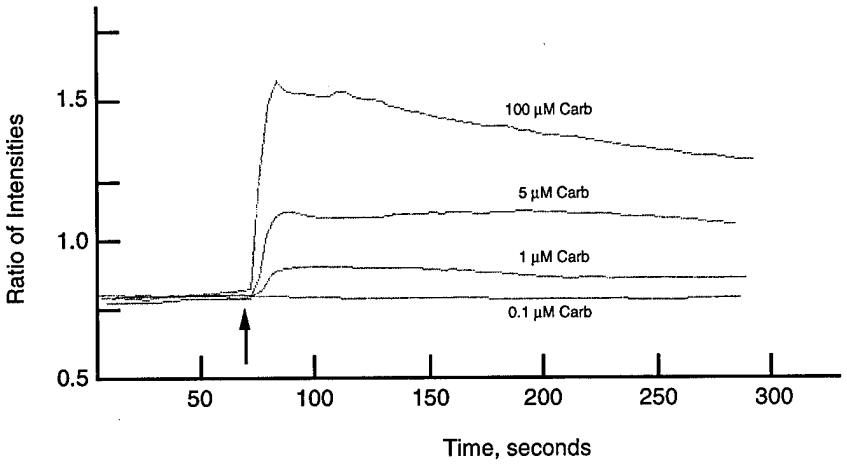


Figure 2. Concentration dependence for carbamylcholine stimulation of Ca^{2+} influx into cultured SK-N-SH cells. The agonist was added at the time indicated by the arrow. Results are from a representative experiment repeated five times.

The calcium response of SK-N-SH cells to carbamylcholine displayed a pharmacology that is appropriate for m3 muscarinic receptors. Thus, 4-DAMP, a selective m3 ligand, was particularly effective at inhibiting the response (Figure 3).

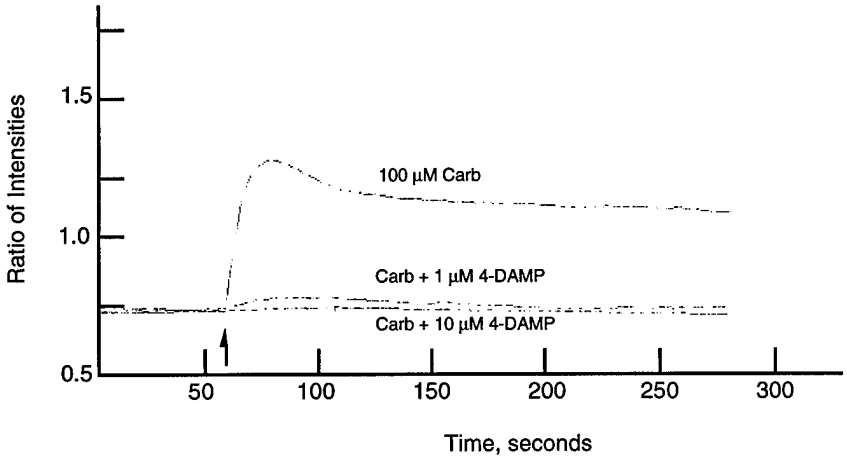


Figure 3. Inhibition of carbamylcholine stimulation of Ca^{2+} influx into cultured SK-N-SH cells by 4-DAMP. 4-DAMP is a m3-specific ligand. The 4-DAMP was added 5 minutes prior to recording the calcium responses. Results are from a representative experiment repeated at least 3 times.

CHO Cells (m1–m5 Receptors).

CHO cells expressing the m1, m3 and m5 muscarinic receptor.

The responses of CHO cells expressing these three receptor subtypes were essentially similar. These three receptors are coupled to phospholipase C activation and IP_3 generation via $G_q/11$ proteins. The magnitude of the calcium response was greater in these cells than in the SK-N-SH cells or in CHO cells expressing m2 or m4 receptors. The response of the Fura-2 to muscarinic stimulation is indicated in Figure 4, while typical responses to carbamylcholine are presented in Figure 5.

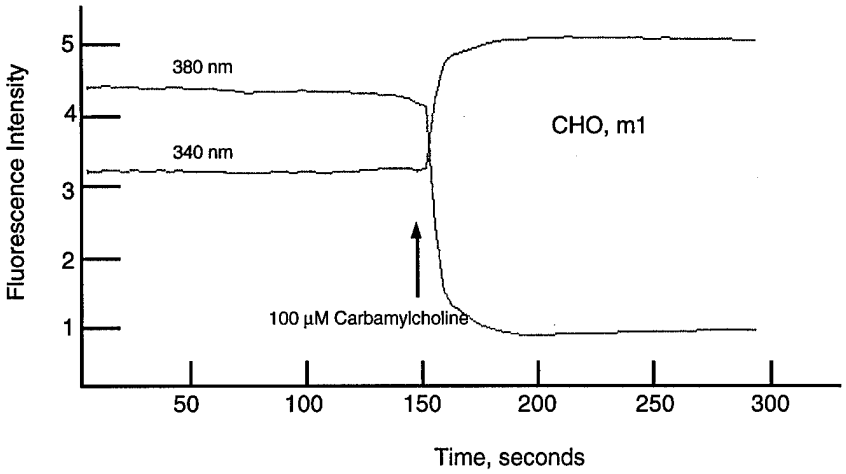


Figure 4. Response of cultured CHO cells expressing the m1 muscarinic receptor to carbamylcholine. Fluorescence emission intensity was monitored at 505 nm after excitation at 340 and 380 nm. Stimulation of cultured CHO cells expressing m1 muscarinic receptors with 100 µM carbamylcholine caused an increase in intracellular Ca2+.

Binding of Ca^{2+} to the fura-2 dye caused a decrease in fluorescence associated with excitation at 380 nm and an increase in the intensity associated with excitation at 340 nm. Fluorescence caused by excitation at 360 nm (not shown) was unaffected by carbamylcholine.

At 0.1 µM, carbamylcholine produced large responses in CHO cells expressing m1 and m3 receptors (e.g., Figure 5), although at this concentration carbamylcholine did not elicit a calcium response in SK-N-SH cells. It is also interesting that at 0.01 µM there was virtually no response to carbamylcholine. Thus, although their sensitivities to carbamylcholine differed, both SK-N-SH and CHO cells showed a steep rise in their dose response relationship with respect to muscarinic stimulation of intracellular calcium (i.e., at a concentration < one tenth the EC50 concentration, no response could be detected in either type of cell). This raises the likelihood of cooperative interactions in the generation of the calcium response at low agonist concentrations. The concentration dependencies for SK-N-SH cells and CHO m3 cells are summarized in Figure 6.

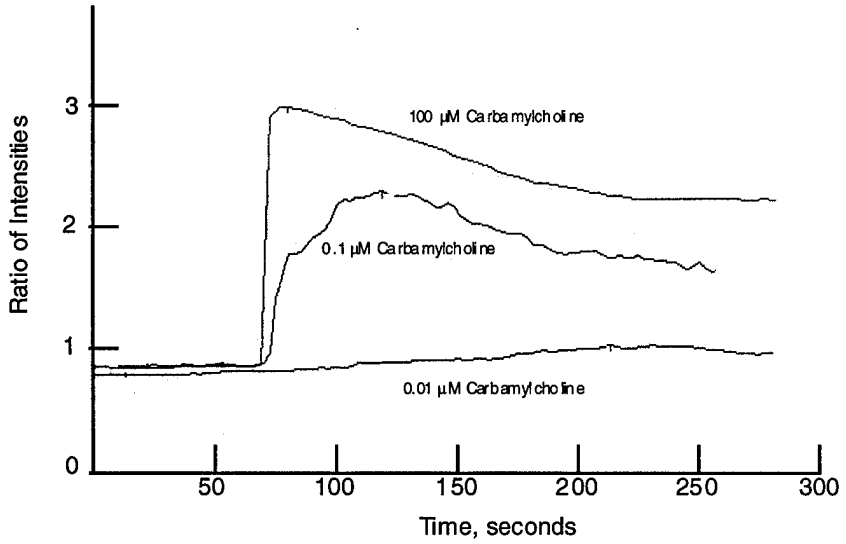


Figure 5. Typical responses of m1 receptor-expressing CHO cells to carbamylcholine. There was a vigorous response to 0.1 µM carbamylcholine, but almost no response to 0.01 µM carbamylcholine.

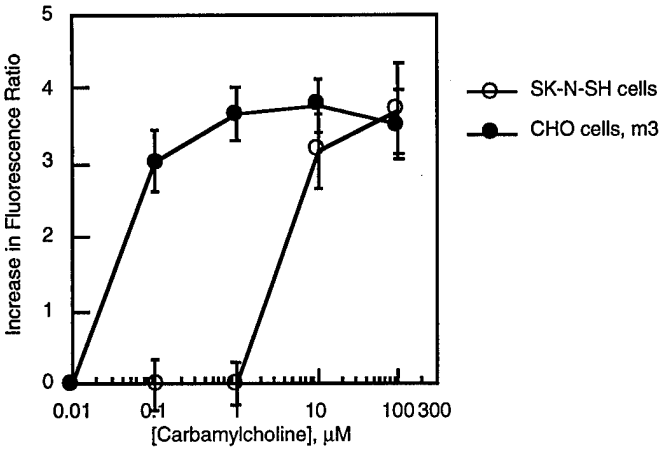


Figure 6. Concentration dependence for carbamylcholine stimulation of calcium responses mediated by m3 muscarinic receptors in SK-N-SH and CHO cells. The maximal change in fluorescent intensity ratios is plotted as a function of carbamylcholine concentration. The responses have been normalized to each other to be on the same scale. Each point and bar represents the mean and standard deviation from 3-5 determinations.

Thapsigargin inhibits the Ca^{2+}-dependent ATPase that is responsible for sequestering Ca^{2+} into the endoplasmic reticulum. Thus, in the presence of thapsigargin, the intracellular calcium concentration gradually rises. This effect is illustrated in m1-expressing CHO cells in Figure 7. Two aspects of this response are illustrated: 1) the onset of the thapsigargin response is variable. Thapsigargin is cell-permeable and its site of action is intracellular. Apparently it takes a variable amount of time for the drug to reach its site of action. No consistent pattern in the onset of this response was noted; 2) The rise in intracellular calcium caused by a functional depletion of intracellular stores causes an increase in the influx of extracellular calcium; thus, EGTA eliminated the plateau response to thapsigargin. Thapsigargin will be used to evaluate ethanol actions on intracellular calcium stores. Thapsigargin responses can be readily elicited in CHO cells that have not been transfected with a muscarinic receptor (Figure 8).

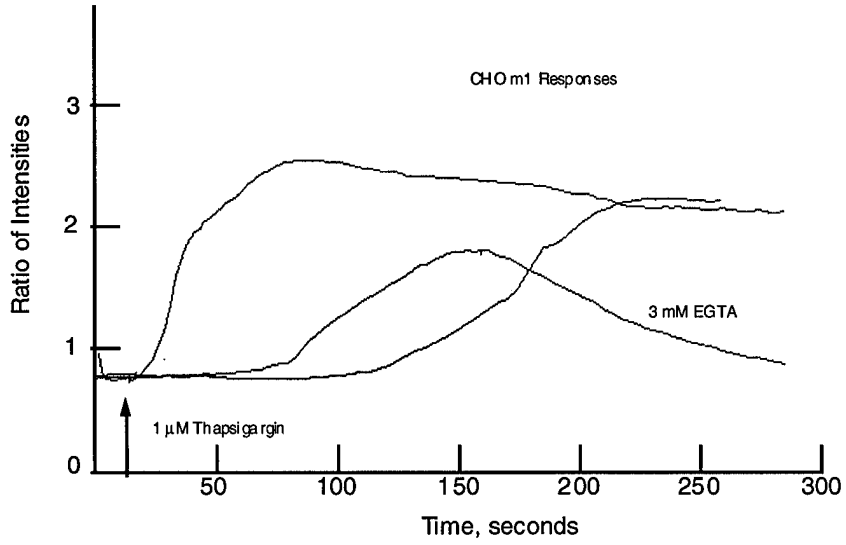


Figure 7. Responses of m1 receptor-expressing CHO cells to thapsigargin. Thapsigargin was added at the time indicated by the arrow. The response (increased intracellular calcium) occurred at very variable intervals, probably reflecting inconsistent uptake of the drug. In the presence of 3 mM EGTA, the response to thapsigargin was not sustained, indicating a later component dependent on the influx of extracellular calcium.

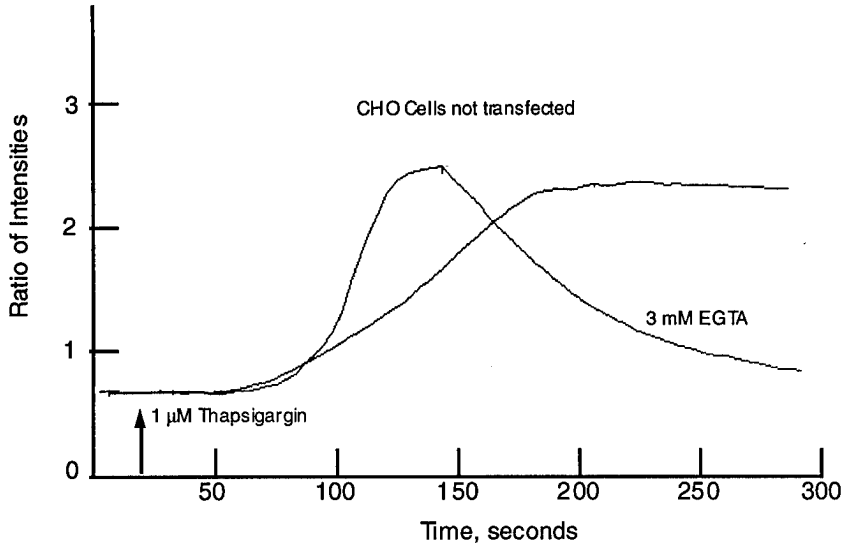


Figure 8. Responses of CHO cells not transfected with a muscarinic receptor gene to thapsigargin. Thapsigargin (1 μM) was added at the time indicated by the arrow. In the absence of EGTA the increase in intracellular calcium concentration was sustained; the fading of the response in the presence of EGTA indicated the stimulation of calcium entry pathways.

An example of the use of thapsigargin and Mn^{2+} to analyze calcium responses of CHO cells to muscarinic stimulation is presented in Figure 9. Fluorescent intensity was measured after excitation at 340 nm (this intensity increases in the presence of Ca^{2+}, and at 360 nm (a calcium-insensitive wavelength). Thapsigargin increased intracellular Ca^{2+}. Mn^{2+} had no influence on the apparent Ca^{2+} content, until carbamylcholine was added. Carbamylcholine induced an influx of extracellular divalent cations as reported by Mn^{2+}-induced quenching of the Fura-2 fluorescence. Similar protocols will be used to isolate ethanol effects on the entry of extracellular cations.

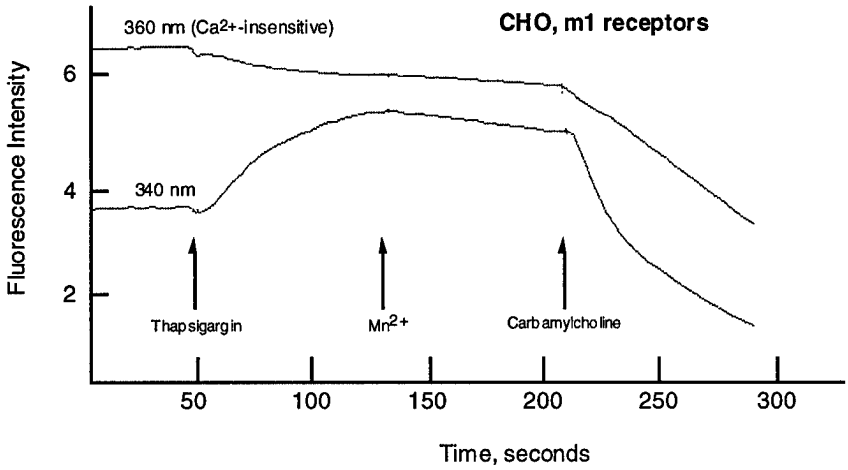


Figure 9. Influence of thapsigargin on the response of m1-expressing CHO cells to carbamylcholine. Fluorescence was measured after excitation at a calcium sensitive (340 nm) and an a calcium insensitive (360 nm) wavelength. Thapsigargin (1 μM), Mn^{2+} and carbamylcholine (100 μM) were added at the indicated times. Depletion of the intracellular calcium stores by thapsigargin prevented carbamylcholine from increasing calcium levels, but did not prevent carbamylcholine from increases divalent cation entry (as evidence by Mn quenching).

A consistent and unexpected difference between m1- and m5-expressing CHO cells was observed in the desensitization of their responses to muscarinic stimulation, as illustrated in Figures 10 and 11 of this section. Fura-2 fluorescence was monitored with excitation at 360 nm. As expected, addition of carbamylcholine elicited no change in the signal (fluorescence at this wavelength is insensitive to calcium concentration). When Mn^{2+} was administered at the same time, the

fluorescence signal diminished due to quenching upon Mn2+ entry. When Mn^{2+} addition was delayed for a few seconds (beyond carbamylcholine addition), Mn^{2+} entry (a surrogate for divalent cation entry) was markedly diminished. This represents a desensitization of the extracellular divalent cation entry response. If Mn^{2+} administration was delayed for 40 seconds, there was no apparent Mn^{2+} entry (Figure 10). In contrast, this desensitization took much longer to develop in the m5-expressing CHO cells. When Mn^{2+} + administration was delayed for a full two minutes, Mn^{2+} entry was still clear visible. With longer delays desensitization was observed. It is proposed to examine the influence of ethanol on the development of this desensitization.

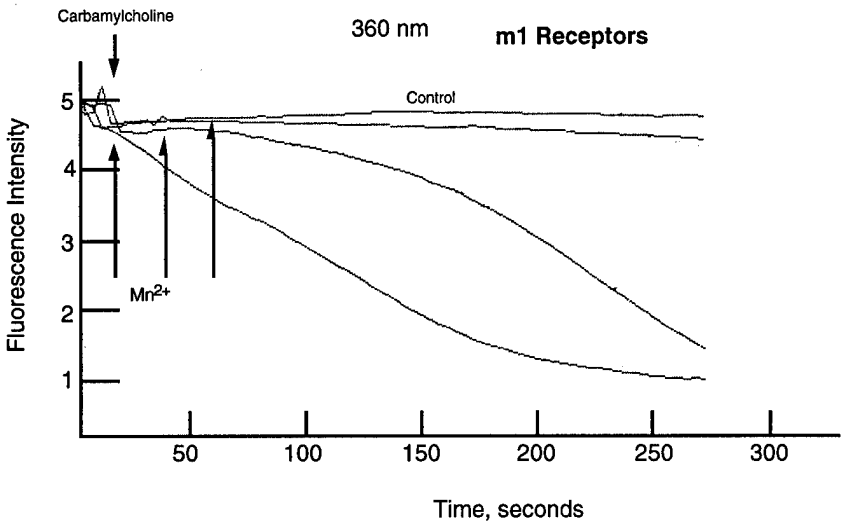


Figure 10. Exposure of m1-expressing CHO cells to carbamylcholine causes a time-dependent desensitization of the divalent cation permeability response. Fluorescence intensity at 505 nm was measured after excitation at the calcium-insensitive wavelength 360 nm. Mn^{2+} was added at the indicated times. The decrease in fluorescent intensity is due to quenching of Fura-2 fluorescence by Mn^{2+} and is a measure of divalent cation permeability.

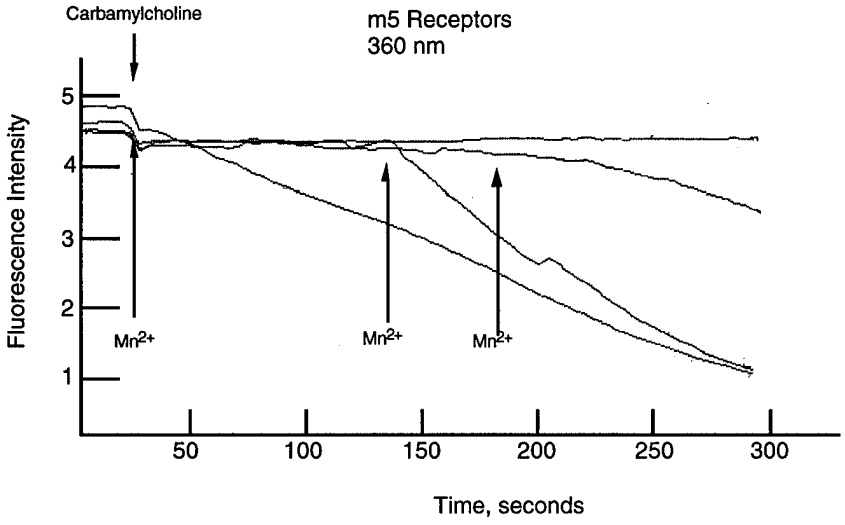


Figure 11. Exposure of m5-expressing CHO cells to carbamylcholine also caused a time-dependent desensitization of the divalent cation permeability response. However, this desensitization response took much longer to develop than with m1-expressing CHO cells (compare to Figure 9, above).

We are utilizing these approaches to demonstrate that ethanol acutely inhibits signal transduction through muscarinic receptors (Figures 12-15). These analyses are complicated by the fact that there are a number of distinct patterns of response at the single cell level (e.g., Kovacs et al., 1995; Reed, 1995). Pilot studies performed using CHO cells transfected with the m1 and m5 muscarinic

receptors are presented in Figures 12-15. In order to minimize the variability inherent in comparing responses from different cells and from experiments performed on different days with different culture stocks, we used each cell as its own control. The cells were stimulated with 1 μM carbamylcholine; when this response stabilized (≈ 3 minutes), the cells were washed with fresh media. After a few minutes, the calcium response was greatly diminished in virtually all cells, and the cell was again challenged with 1 μM carbamylcholine. Representative responses in m5-expressing cells are shown in Figures 12 and 13; responses in m1-expressing cells are shown in Figures 14 and 15. A number of difficulties in this analysis present: Importantly, the cells vary in the extent to which the first response could be eliminated by washing, and this seems to be related to the nature of the initial response. Thus, if there is a large plateau response (i.e., intracellular calcium levels remain elevated for an extended period of time), it is more difficult to reverse the response by washing. We are attempting to quantify this effect in order to understand relationship between the nature of the initial response, the efficacy of washing, and the magnitude of the secondary response. Meanwhile we are restricting our analysis to classes of cells that behave in a similar manner with respect to plateau and reversibility.

Studies to date indicate that ethanol (50 mM) markedly depresses calcium responses to 1 μM carbamylcholine in CHO cells expressing m1 and m5 muscarinic receptors. (Figures 13 and 15). These studies are being actively extended.

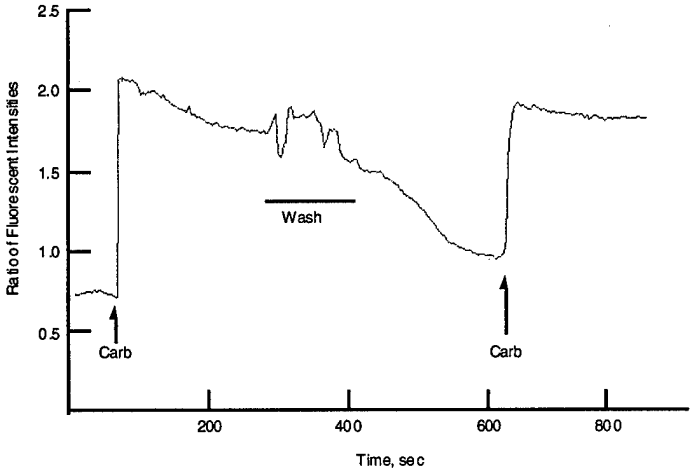


Fig. 12. Typical Ca^{2+} response of an m5-expressing CHO cell to 1 μM carbamylcholine (carb). After 3 min the carb was removed by rinsing with fresh buffer (bar); the cell was challenged with a second dose of carb 3 min later. Washing reversed the response by ≈ 75% and the second response was approximately equal to that of the first (note, however, the shift in baseline).

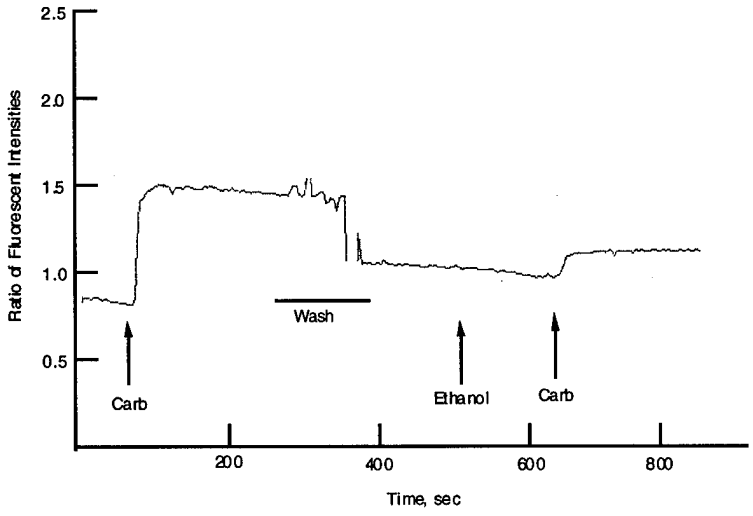


Fig. 13. Influence of ethanol (50 mM) on the Ca^{2+} response of CHO cells transfected with m5 muscarinic receptors. The second response to carbamylcholine was decreased by >70% (compare to Fig. 11).

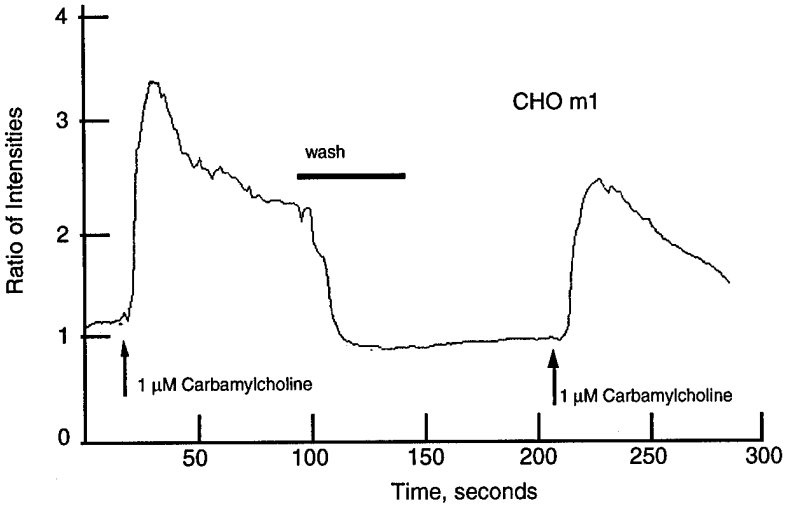


Fig. 14. Sequential responses to 1 µM carbamylcholine of CHO cells transfected with m1 muscarinic receptors. Carbamylcholine was added at the times indicated by the arrows. The bar indicates the period of washing with fresh media.

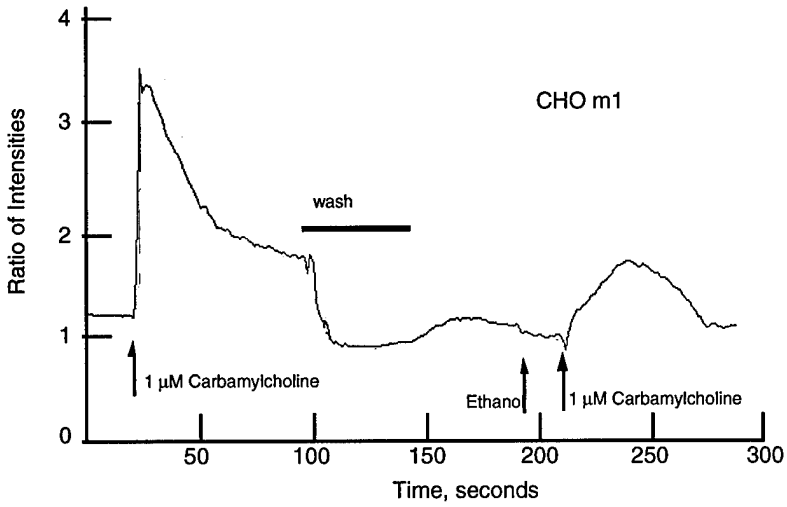


Fig. 15. Influence of ethanol (50 mM) on the Ca^{2+} response of CHO cells transfected with m1 muscarinic receptors. The second response to carbamylcholine was significantly decreased by compared to the response in Fig. 14.

CHO cells expressing the m2 and m4 muscarinic receptors.

The m2 and m4 muscarinic receptors are coupled via Gi proteins to inhibition of adenylate cyclase. Thus, their effects on the Ca2+ content of cells are considered to be indirect. Nevertheless, clear and consistent Ca2+ responses were characterized in CHO cells transfected with the genes for these receptor subtypes (Figures 14–16). These responses were consistently smaller than the responses in m1-, m3- ,and m5-expressing cells (i.e., the intracellular calcium concentration never rose as high). The onset of the responses also tended to be less abrupt. A typical response in an m4-expressing CHO cell is shown in Figure 14. The response is prolonged and involves an influx of extracellular calcium since the plateau response is diminished in the presence of EGTA (Figure 15). Also illustrated in Figure 14 is the lack of response of CHO cells to nicotine; CHO cells do not express nicotinic receptors. It is also shown in Figure 15, that the response of CHO m4 cells is not affected by pretreating the cells with 1 µg pertussis toxin (which ADP-ribosylates and inactivates the alpha subunits of Gi and Go); these G proteins not thought to be involved in m2 and m4-mediated responses.

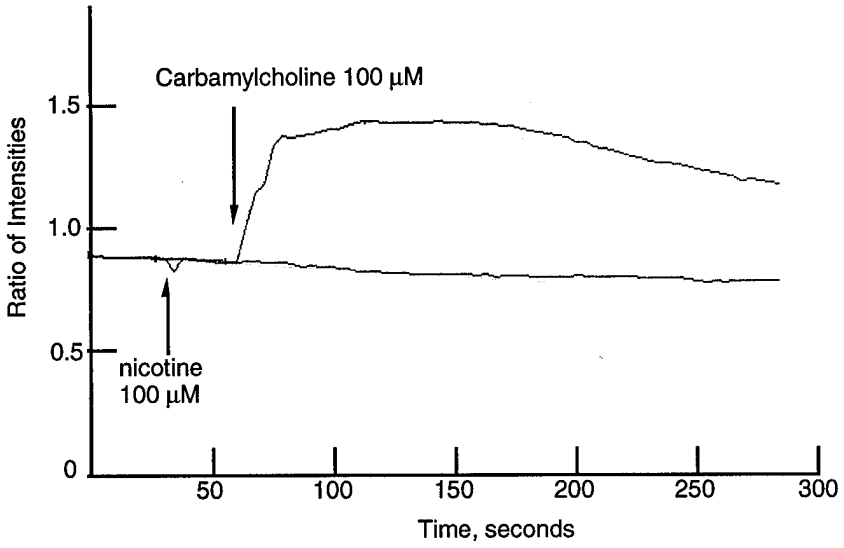


Figure 14. Responses of m4-expressing CHO cells to carbamylcholine. Note that the responses are considerably smaller (i.e., the increase in intracellular calcium concentration) is considerably smaller in m4 (and m2) expressing cells than in cells expressing m1, m3 or m5. Note that nicotine (100 μM) did not elicit a change in calcium content, indicating the absence of nicotinic receptors.

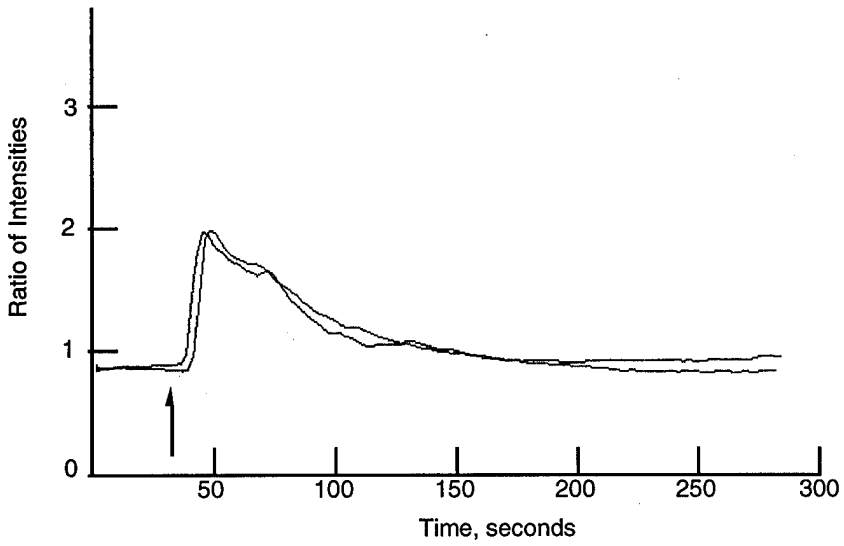


Figure 15. Responses of m4-expressing CHO cells to carbamylcholine are not affected by treatment with pertussis toxin. Note that these experiments were performed in the presence of 3 mM EGTA, hence the plateau response was not sustained. One cell was treated with 1 μg pertussis toxin for 30 min at 37°C prior to the experiment; no effect on the m4 muscarinic response was observed.

Carbamylcholine is frequently used as a substitute for acetylcholine because it is stable to the hydrolysis catalyzed by acetylcholinesterase (and other esterases) widely expressed in neural tissues. However, using the method of Ellman et al. (1961) we were unable to detect any acetylcholinesterase activity in plasma membrane fragments or in whole cell preparations of CHO cells. These studies used 1 μM acetyl-thio-cholinesterase as the substrate. Thus, acetylcholine could probably be safely used as the prototypical agonist in these studies.

CHO cells expressing m2 and m4 muscarinic receptors also respond to muscarinic stimulation by increasing the entry of extracellular divalent cations, as monitored by Mn2+ quenching of Fura-2 fluorescence (Figure 16).

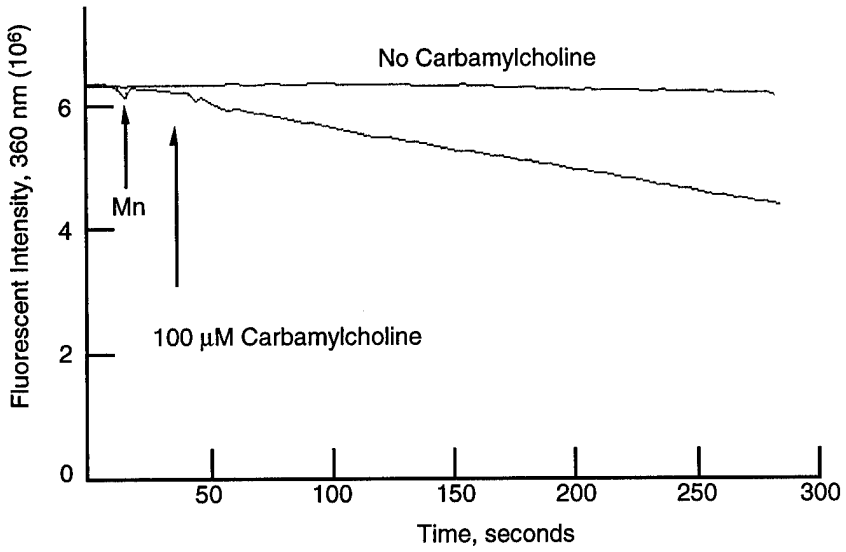


Figure 16. Response of m4-expressing CHO cells to carbamylcholine. Mn2+ and carbamylcholine (100 µM) were added at the times indicated by the arrows. In the absence of carbamylcholine, Mn^{2+} had no discernible effect (i.e., the cell did not admit divalent cations). Carbamylcholine was able to stimulate Mn2+ entry (and subsequent quenching of Fura-2 fluorescence).

In these studies we have utilized microfluorescence techniques to monitor the responses of cultured cells to muscarinic stimulation. Several parameters of this response were characterized, including concentration dependence, pharmacology, dependence on calcium from intracellular and extracellular sources, stimulation of extracellular divalent cation entry, and desensitization. Clear differences were observed in the responses of m2/m4 and m1/m3/m5/SK-N-SH cells. Strategies are being developed to deal with inter-experiment variability, including grouping cells according to nature of the response and using each cell as its own control. Preliminary results indicate that ethanol inhibits the response of m1 and m5-expressing receptors to muscarinic stimulation. Experiments currently underway will extend this analysis to each parameter in each cell type.

3. Cell Adhesion Studies

Effect of ethanol on signal transduction in small cell lung cancer cells expressing m3 muscarinic receptors.

Dr. Williams of the Guthrie Research Institute has recently discovered that activation of m3 muscarinic acetylcholine receptors increases E-cadherin binding activity in small cell lung carcinoma (SCLC) cells (Williams et al., 1993). We took advantage of this finding to examine the possibility that signal transduction at muscarinic receptors in non-neuronal cell types is also disrupted by ethanol. SCLC cells are thought to be derived from pluripotent endodermal cells and are known to possess some neuroendocrine properties. Dr. Williams has also shown that direct activation of protein kinase C (PKC) with phorbol 12-myristate 13-acetate (PMA) also increases E-cadherin activity in these cells.

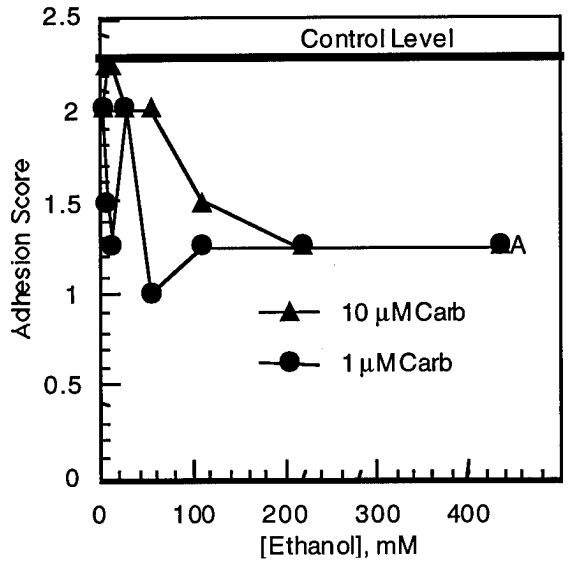
Methods: To examine the effects of ethanol on the muscarinic signal transduction, we measured E-cadherin-mediated adhesion induced by the mAChR agonist carbamylcholine in the presence and absence of ethanol. To ascertain the specificity of any ethanol effect with respect to muscarinic mechanisms, we also tested the effects of ethanol on E-cadherin-mediated adhesion induced by PMA.

The SCC-9 SCLC cell line was plated at a cell density of 4 x 10^5 cells/ml in 96 well microtiter plates. Cells were exposed to ethanol and incubated for 5 minutes at 37°C before the addition of carbamylcholine of PMA. After incubation for 30 minutes at 37°C, 2.5% glutaraldehyde was added to the microtiter wells to fix the cells. The cells were examined microscopically for evidence of E-cadherin-mediated cell-cell adhesion, and the amount of cell-cell adhesion was scored as follows: 0, no adherent cells; 1, few adherent cells; 2, most cells adherent; 3, all cells adherent.

Results and Discussion: The results from two studies are summarized in the following Tables and Figure:

[EtOH] mM	Control		10 μM Carb		1 μM Carb		1 μM PMA		0.1 μM PMA		Control	
435	0	0	0.5	1.0	1.0	1.0	0.5	1.5	1.5	2.0	0	0
217	0	0	1.0	2.0	0.5	1.5	2.0	2.0	2.0	2.5	0	0
109	0	0	1.0	2.0	0.5	1.5	2.0	2.5	2.0	2.5	0	0
54	0	0	2.0	2.5	0.5	2.0	2.0	2.0	2.0	2.5	0	0
27	0	0	2.0	2.0	0.5	2.0	2.0	2.5	2.0	2.0	0	0
13	0	0	2.0	2.5	0.5	1.5	2.5	2.5	2.5	2.5	0	0
7	0	0	2.0	2.5	0.5	1.5	2.5	2.5	2.5	2.5	0	0
3.5	0	0	1.5	2.0	2.0	2.0	2.5	2.5	2.5	2.5	0	0

[EtOH] mM	Control		10 μM Carb		1 μM Carb		1 μM PMA		0.1 μM PMA		Control	
435	0	0	2.0	2.0	1.5	2.5	2.5	1.5	1.5	1.5	0	0
217	0	0	1.5	2.5	2.0	2.5	2.0	2.0	2.0	2.0	0	0
109	0	0	2.0	2.5	2.0	2.5	2.0	2.5	2.5	2.5	0	0
54	0	0	2.0	2.5	1.5	2.5	2.5	2.5	2.5	2.5	0	0
27	0	0	2.0	2.5	2.5	2.5	2.5	2.5	2.5	2.0	0	0
13	0	0	2.5	2.5	2.0	2.5	2.5	2.5	2.5	2.5	0	0
7	0	0	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	0	0
3.5	0	0	2.5	2.5	2.5	2.5	2.0	2.5	2.5	2.5	0	0



Influence of ethanol on adhesion E-cadherin-mediated cell adhesion of SCLC cells. Control Level refers to the average adhesion score obtained in the absence of ethanol; this was the same in the presence of either 1 or 10 μM carbamylcholine. Each point is the average from 4 determinations from two experiments.

The results summarized in the accompanying Figure indicate that ethanol depresses cell adhesion stimulated by either 1 or 10 μM carbamylcholine. This depression was seen at ethanol concentrations as low as 54 mM in the presence of 10 μM carbamylcholine and 13 mM in the presence of 1 μM carbamylcholine. The concentration dependence of the inhibiting effect of ethanol suggests a competitive interaction with carbamylcholine, i.e., the inhibitory effect of ethanol can be overcome by increasing the concentration of muscarinic agonist. In contrast, ethanol did not affect cell adhesion stimulated by treatment with PMA. This indicates that ethanol can disrupt muscarinic signaling in a non-neuronal cell line, and that the disruption is "upstream" of (or perhaps unrelated to) the protein kinase step. It is also clear that ethanol can disrupt muscarinic receptor-mediated adhesion processes at concentrations well within concentrations achieved during intoxication.

Chronic Ethanol Treatment Studies.

We have initiated studies on the influence of chronic ethanol treatment on neurotransmitter receptors and their interactions with G proteins. These studies required 1) equipment modification of the cages to handle the liquid diet and 2) the establishment of baseline protocol measurements before utilizing this expensive material. The following processes are being considered: **1**) Muscarinic receptor expression and coupling to G proteins, as evidenced by ligand binding measurements and receptor control of G protein activity, **2**) levels of G protein expression (by Western blot analysis), and **3**) transcriptional and posttranscriptional control of receptor expression. We have established assays for G protein expression and transcriptional control. In particular, a plethora of new information regarding the control of receptor expression (especially the nature and activity of m1–m5 promoter regions) has been obtained. The latter processes have not been studied for muscarinic receptors in any tissues under any conditions, and this work represents a major contribution of this project.

1. Treatment

Methods. Four week old male Sprague-Dawley rats (Charles River) were stabilized at this facility for one week prior to introduction of the liquid diet. The animals were housed individually in shoebox cages with hardwood chip bedding (Northeast Products). The room environment is maintained at 70°F and 45% relative humidity with a 12 hour light/dark cycle and 12 air changes/hour. The cages are changed 2 times each week. Water is available ad lib in separate bottles which are changed weekly.

The diet mix was purchased from Purina Mills, Inc. The control diet (LD101) is mixed as: 230 g diet chow + 770 g deionized water. The alcohol diet (LD101A) is mixed as: 140 g diet chow + 50 g ethanol + 810 g deionized water. The diets are mixed in a mechanical blender for 30 seconds, and is prepared fresh daily.

Special feeder units were required for administration of the liquid diet because it is too thick to flow through conventional rat sipper tubes. The diet is weighed into the feeder units daily. Diet remaining from the previous 24 hour period is weighed upon changing to new feeders. The difference between the amounts weighed in and the amount weighed out is the amount given to the corresponding control animal. Initially 80 g of diet was made available to the experimental animals. The amount of pelleted feed (Rm #2000) was gradually reduced over the period of 1 week. Once the animals were on liquid diet exclusively, it was found that some were consuming the entire 80 g quantity, so the amount was increased to 90 g per day. In order to realize the maximum benefit from this study, we will divide the tissues and separately isolate 1) mRNA and nuclear extracts and 2) plasma membrane fractions.

Results:

The animals receiving alcohol exhibited increased urine output compared to control animals (observation based on soiling of bedding material). Almost no water consumption from the bottles has been noted in either the alcohol-fed or control animals. The alcohol groups are hyperactive, responding vigorously to cage handling. Otherwise, the animals appear normal and healthy. experiments with tissue from the chronic treatment studies are scheduled to begin in January. Treatment of a second cohort of subjects will be begin immediately thereafter.

2. Western Blot Analysis of Influence of Ethanol on G Protein Expression:

The levels of G proteins in brain membranes following chronic treatment with ethanol will be determined by Western blot analysis using specific antibodies. The levels of G proteins will be correlated with measures of receptor-G protein coupling and the efficiency of different synaptic transmission pathways. We have established these assays in our laboratories, as described below.

Methods: Brian areas were dissected and homogenized in 50 mM Tris-HCl, pH 7.4, using a Teflon-glass tissue grinder. The homogenate is spun at 17,000 rpm for 20 min and the pellet resuspended and centrifuged again. The pellet is resuspended in the Tris buffer at a concentration of 5 mg/ml. A 10% SDS-PAGE gel (19:1 bis/acrylamide ratio) is loaded with 50 µg of protein and 5 µg of mid-range protein markers (Bio-Rad Mini-Protean apparatus). The gel is run at 25 mA (constant current) until the blue dye reaches the bottom of the gel. The protein is transferred to nitrocellulose membrane (9 x 5.5 cm) under the influence of a 30 mA current for 12 hours. After transferring, the membrane is temporarily stained with ponceau red while a pencil is use to indicate the location of the markers.

The membranes are blocked with 15 ml/blot of 5% non-fat dry milk in Tris (29 mM, pH 7.5) buffered saline (150 mM NaCl) with Tween 20 (0.05%) (TBST) for 1 hour, and then rinsed twice with TBST. The membrane are then incubated with the appropriate primary antibody (130 µl) for 1 hour at 37°C. Following 3 five min washes with TBST, the alkaline-phosphatase-conjugated, anti-rabbit secondary antibody is diluted 1:5000 in Tris buffered saline (20 mM Tris, pH 7.5, 150 mM NaCl; TBS) and added (15 ml) to each blot. Following a 30 min incubation at room temperature, the blots are washed 3 times with TBST and twice with TBS. The blots are developed with 15 ml of substrate for 1 hour; the reaction is then stopped by rinsing twice with distilled water.

Results: Each G protein α subunit was visualized and quantified using these protocols. A multiscreen apparatus (Bio-Rad) was used to process multiple samples and minimize antibody usage. The primary antibodies (anti-rabbit antisera) were purchased from NEN-Dupont). The blots were digitalized and analyzed on a Macintosh microcomputer using a digital camera and the Image program (NIH). Examples of blots from SK-N-SH cell membranes are presented below to demonstrate the feasibility of this approach.

SK-N-SH Cells

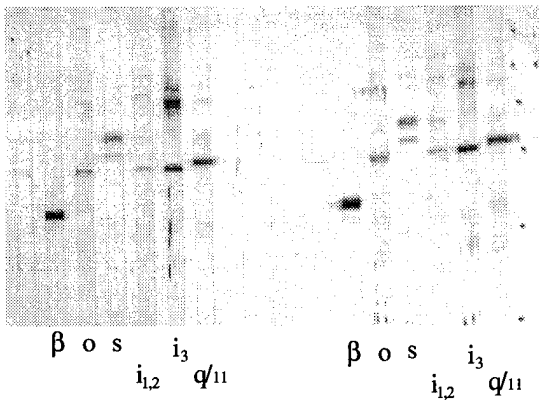


Figure 1. Western blot of G protein subunits expressed in SK-N-SH cells. Lanes were loaded with 50 µg protein as described above. Antibodies for the beta subunit and for the alpha subunits of G_o, $G_{i\ (1,2\ and\ 3)}$, G_s and $G_{q/11}$ were used, as indicated.

3. Molecular Analysis of the Influence of Ethanol on Muscarinic Receptor Expression.

A major goal of this research project is to determine the influence of chronic ethanol treatment on muscarinic receptor expression and synaptic transduction mechanisms. To this end, rats are being treated with ethanol in liquid diets (see above) and tissue is being harvested for ligand binding and signal transduction studies. It is highly desireable to incorporate molecular biological approaches to the evaluation of observed changes in receptor expression. Thus, the question is not simply the extent to which muscarinic receptor density is altered in animals chronically exposed to ethanol, but also how ethanol effects the modulation of muscarinic receptor expression.

Gene expression in eukaryotes is controlled in large part through events that activate pathways leading to the transcription of specific genes. Transcriptional proteins (factors) that bind to short *cis*-regulatory motifs comprising the promoter for a gene are central to these pathways. Specificity in gene activation is achieved through complex interactions of transcription factors with these regions of the DNA. A reasonable hypothesis is that *chronic ethanol exposure alters the synthesis or activation state of specific transcriptional factors that interact with the promoter elements for the muscarinic receptor genes.* Gene expression can also be controlled by posttranscriptional mechanisms that regulate the stability of the mRNA for specific genes. The increase or decrease in particular muscarinic receptor subtypes following chronic ethanol treatment may be a reflection of the stability of the mRNA for these receptor subtypes. Thus, a particular muscarinic receptor gene may be already constitutively activated, and a decreased stability of its mRNA following ethanol exposure would lead to a decrease in synthesis of the muscarinic receptor molecule.

Very little work has been performed on the molecular analysis of muscarinic receptor expression. Accordingly, we have analyzed the promoter regions of muscarinic receptor subtypes. We cloned 1-4 kilobase regions from each of the 5 receptor subtypes, and have completed sequencing of the m1 and m3 receptor promoters (see below); sequencing of the remaining promoters is in progress and will be completed shortly. We have analyzed these regions for known transcription factors by screening databases, and we have begun DNase I footprint analyses (in collaboration with Dr. John Noti) to identify protein binding sites. In order to perform functional analyses, we have cloned reporter gene constructs comprised of sequential regions of the promoter regions coupled to a luciferase reporter gene. These constructs will be used to identify sites important for the elaboration of ethanol's effects on muscarinic receptor expression. Thus, elimination of specific transcription factors binding sites may alter (enhance or suppress) the effect of chronic ethanol treatment on receptor expression.

To determine whether posttranscriptional mechanisms contribute to the expression of the muscarinic receptor genes, Northern blot analysis will be performed to quantitate and determine the stability of mRNA for each receptor subtype. Messenger RNA from the brains of rats chronically exposed to ethanol will be compared with mRNA isolated from unexposed rats. Cell lines that express various muscarinic receptor subtypes will be compared with non-expressing cell lines in an analogous manner. Nuclear run-on

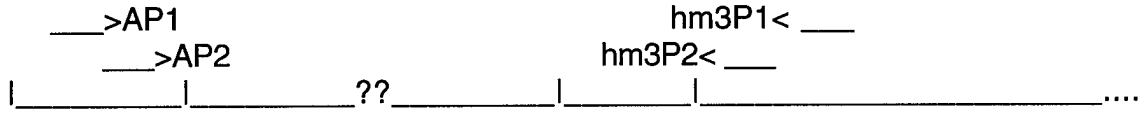
experiments will be performed in conjunction with the stability studies to determine whether chronic ethanol exposure alters the rate of transcription. These studies have not yet begun.

The hypothesis underlying this approach is that *chronic exposure to ethanol alters muscarinic receptor expression through transcriptional and/or posttranscriptional mechanisms.* Cis-regulatory elements that are essential for transcription of the muscarinic receptor genes and mediate the effects of ethanol exposure will be defined following functional analysis of discrete regions of the promoters using a luciferase reporter system. Essential functional regions of the promoter will be analyzed further for protein (transcriptional factor)-binding sites using DNase I footprint analysis, and the effect of ethanol on binding of protein to these sites will be assessed. The role that posttranscriptional mechanisms play in the expression of the muscarinic receptor subtypes will be correlated with these transcriptional studies.

Results from studies on the promoter analysis project are presented below.

Human m3 Muscarinic Receptor Promoter Studies

Ligation Mediated Polymerase Chain Reaction: The 5' untranslated region of the human m3 muscarinic receptor gene was cloned using the ligation mediated polymerase chain reaction protocol found in the PromoterFinder DNA Walking Kit (Clontech). Briefly, the kit includes five "libraries" comprised of human genomic DNA each cut with a different blunt end restriction endonuclease. The cut genomic DNA provided in the kit has been modified by the ligation of a short DNA adaptor that provides a region of known sequence to which PCR primers can anneal. Two PCR primers were provided by the kit, one for a primary reaction and another for a secondary or nested reaction. The sequence of the coding region and a short segment of 5' untranslated DNA was retrieved from Genbank, and two PCR primers were designed which were complimentary to sequences just inside the coding region and would extend back to the primers provided for the adaptor sequence. The primer scheme is illustrated in the diagram below.



Structure of Template and Orientation of Ligation Mediated PCR Primers

AP1 and AP2 represent the PCR primers included in the kit and hm3P1 and hm3P2 were primers designed from the protein coding sequence found downstream from the known untranslated region. The known untranslated (~100 base pairs) and the protein coding sequence were obtained from Genbank. The segmented line in the figure above represents a

possible PCR template which could be found in any or all of the restriction enzyme libraries. The double question marks in the unknown 5' untranslated segment indicate that this length will vary between libraries. The amplification process involved two successive PCR reactions, a primary reaction using AP1 and hm3P1 and a secondary or nested reaction with AP2 and hm3P2. The conditions of the PCR reactions allow amplification of templates up to 6 kb in length. The largest fragment produced following both rounds of PCR amplification resulted from the Sca I library and was about 1.7 kb. The other four libraries produced fragments from 250 base pairs to about 800 base pairs in length.

Cloning of the 5' Untranslated Region of the HM3 Gene: The 1.7 kb fragment was cloned into the Sma I site of the vector Bluescript II SK +/- (Stratagene). Briefly, the PCR product was isolated by precipitation with 2.5 volumes of 100% ethanol for 10 minutes at - 80°C, pelleted by centrifugation at 13,000xg for 15 min at 4°C, washed with 70 % ethanol and spun at 13,000xg for 2min. The microcentrifuge tube was inverted and the pellet was allowed to dry for about 15 min. The pellet was resuspended in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA), $MgCl_2$ and DTT were added to 5 mM, dNTP's were added to 0.2 mM and 1 unit of T4 DNA polymerase (NEB) was added. The reaction was performed at 11°C for 20 minutes followed by 10 minutes at 70°C to inactivate the enzyme. The DNA was precipitated with 2.5 volumes of 100% ethanol as described above, and resuspended in 1x T4 polynucleotide kinase buffer. ATP was added to 5 mM and 30 units of T4 polynucleotide kinase (USB) were added. The 20 µl reaction mix was incubated at 37°C for one hour and terminated by heating at 70°C for 10 minutes.

The cloning vector was prepared by overnight digestion of 5 µg of Bluescript (Stratagene) with Sma I (NEB) at 25°C in a 25 µl volume. The reaction was terminated by heating to 70°C for 10 minutes and the extent of cutting was determined by electrophoresis of 2 µl on a 1% agarose gel, followed by ethidium bromide staining. The remaining digestion mixture was diluted to 50 µl with distilled water and 5 µl of 10x calf intestine alkaline phosphatase (CIAP) buffer. Two units of CIAP (Promega) was added and the mixture was incubated at 37°C for one hour. The enzyme was inactivated and removed by heating at 70°C for 10 minutes, followed by extraction with phenol/chloroform/isoamyl alcohol (24:24:1), and the cut vector was precipitated with 2.5 volumes of 100 % ethanol as above. The pellet was washed with 70 % ethanol as described above and resuspended in 25 µl of TE. The ligation reaction mixture included the addition of 1 µl of the vector (~0.1-0.2 µg) to the 20 µl kinase reaction mixture, plus ATP to 2 mM and 200 units of T4 DNA ligase (NEB). The reaction was incubated overnight at 16°C.

Ten µl of the ligation reaction was added to 100 µl of competent JM109 *E. coli* cells (Promega). The mixture was incubated on ice for 10 minutes, heat shocked for 50 seconds at 42°C, and held on ice for another 2 minutes. SOC media was added (900 µl), and the tube was incubated at 37°C for 1 hr on a rotating wheel. Following this incubation, the cells were gently pelleted by centrifugation for 5 minutes at 4000xg. Eight hundred µl were removed, and the cells were resuspended and plated on LB plates containing 100 µg/ml ampicillin, 40 µg/ml X-gal, and 0.5 mM IPTG. White colonies were picked to inoculate 3 ml volumes of LB broth containing 100 µg/ml ampicillin, and the cultures were shaken at 37°C overnight. One ml of these overnight cultures was used to perform small scale plasmid isolations by the protocol of Sambrook et al. (1989). The isolated plasmids were cut with BamH I and EcoR I (which flank the Sma I site), and fragments were resolved on

a 1% agarose gel. Positive clones were characterized by a ~1.7 kb fragment following ethidium bromide staining and UV visualization. One ml of the remaining small overnight culture from which a positive clone was observed was used to inoculate 500 ml of LB (100 µg/ml ampicillin). This culture was shaken overnight at 37°C and used to perform a large scale plasmid isolation following the alkaline lysis method and cesium chloride gradient purification of Sambrook et al. (1989). A portion of this plasmid preparation was used to sequence the 1.7 kb fragment using the USB textbook method described below.

Sequence Determination of the 5' Untranslated Region of the HM3 Gene: Five micrograms (up to 20 µl total) of cesium chloride purified plasmid DNA was denatured by adding 2 µl of 2 M sodium hydroxide/1 mM EDTA and heating at 37°C for 30 minutes. After this incubation, the DNA was neutralized by adding 2.2 µl of 3 M sodium acetate pH 5. Subsequently, the DNA was precipitated by adding 75 µl absolute ethanol, and stored at -70°C for 20 minutes. The DNA was recovered by centrifugation at 16,000 X g for 15 minutes, washed once with 70% ethanol, and dried under vacuum.

The pellet was resuspended in 7 µl of water, 2 µl of 5X sequenase buffer, and 1 µl of 2.5 ng/µl sequencing primer. The tube was then labeled as "template/primer mix". The 10 µl volume was heated for two minutes at 65°C to re-denature, allowed to cool gradually (30 minutes) to 35°C, then placed on ice. During this 30 minute annealing step, 2.5 µl of dideoxy mix A was placed in a microfuge tube labeled "A". The same volume of dideoxy mix C, G, and T was placed in corresponding microfuge tubes labeled "C", "G", "T" respectively. Two µl of 1:5 diluted label mix, 1 µl of 100 mM DTT and 0.5–1.0 µl of alpha labeled ^{35}S (1000-1500 Ci/mmole) was placed in a tube labeled "labeling mix" and placed on ice.

The sequenase enzyme was diluted 1:8 with ice-cold enzyme dilution buffer and placed on ice. The 3.5 µl of label mix was added to the template/primer mix and placed on ice. The tubes with the dideoxy termination mixes were placed in a 42°C water bath in a stationary manner so reagents could be pipetted into the tubes without moving them. Two µl of enzyme mix were added to the denatured template/primer mix and incubated at room temperature for three minutes.

After the three minute labeling incubation, 3.6 µl of the labeled template/primer mix was added to tube "A". A new pipette tip was used to add 3.6 µl of template/primer mix to tube "C". The same procedure was used for tubes "G" and "T". Before the addition of the first aliquot of labeled template/primer mix to tube "A", a timer was set to count down to 5 minutes. After the 5 minute incubation at 42°C, 4 µl of stop solution were added to each of the four tubes. The tubes were stored at -20°C.

A 6% denaturing polyacrylamide sequencing gel was prepared by mixing 20 µl of 5X TBE buffer with 15 µl of a 40 % 19:1 acrylamide/bis-acrylamide mix in a 150 µl beaker. Distilled water was added to adjust the volume to 60 milliliters. Forty two grams of urea and 0.1 gram of ammonium persulfate were added to the gel solution and placed on a stir plate to dissolve.

The volume was adjusted to 100 ml with distilled water and gravity filtered through a Whatman 1 qualitative filter. The gel was cast in a IBI sequencing apparatus using 0.44 mm spacers. The gel was electrophoresed at a constant power setting of 75 watts,

transferred to Whatman filter paper, dried and exposed to film (BioMax-Kodak) overnight. Autoradiograms were read manually.

Results: Human m3 muscarinic receptor, promoter region sequence

Our studies revealed the following sequence of the human m3 receptor promoter region:

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ACTTTATATA TGTTAAAACA TTTTGATGAG AAACGAAGCT TCTTTGGCCA   50
CTTATCGAAT TCTGGAAAGA ATTGATCAAC AATTGATTCT GTTATACAGA  100
AAATTATCAT TCCCATTTTT CAATGAGAAA ACTTGGACTC AAAGAAGTTA  150
AATATCTAAG TTCTCACCTC CAGTAAGTAG TCAAACAGAT TTGAACCTAG  200
GCCTTTTGAC CCCATAGTCT TCCTGCTATG CCAGAAAGCC TTTCCAGAAG  250
TCTCTGTGTG AATCTGACTA CTGCTTTGAG AGACACTCTG AGAAAACTCT  300
GGCATTCAAT ACCTAAAGGC TTGATTCAGG ACTCTTGCAA TGCACTTGCA  350
TATGTATGTT TATCTGTAAG AGTTTATTGC TGAAGATTTT TTTTAACCAA  400
GAGCATAAAT GTAGTAGCAG GGTACACAAT AGATTTTTGC TGGGATGATC  450
AAACTTTGAA CTCATAGAAT GACACTGATT GAGGAATGAT TTGCTTATCT  500
AGTGTACGGT GGCTGGCGGT GTGTGAATTC CTGAGCCAGT GGTGTCCCAG  550
AAGTACAGCC CTGGAGGCTC TGAGAGAACG GTACAGGTCA CAACCGAGGC  600
CAACAGTGAC ACCAAGGAGG TCTAACTGTG GCACCTTAAT TGTAATGCTT  700
CTTAGTTGTT CATATACCTG GCAACAAGTG TGTGAAAGCA CTAGTTGCAG  750
TTTTGCAGTT TTTATATGCA AATGTGTGTG GGTGTGTCCA TTTGTTCACA  800
TACGTGTGAC ACGCTAAAAT ACATTTTCAT GCTTGGATCT AAAATATCTA  850
AATACTAGAA ATATTTAAAG CTGGTTTGGT CCATTCAAAA CGTTCTGCTA  900
AAAGTTCATC TGCATGTTAT CAAGTAGAAG ATAGCTTTCC AGGAGATAGT  950
CGTGTTACCA GTCCTTACAA GGAACGGATG TCTTACTTTT TTGATCCTGT 1000
CTCAAAGAGG TAATATAGTG CGAATTGATT TACATTGGTT TTATCCCTTC 1050
TCCAGGTTGG CATTGCTCAT TTTAGTAAGA CTTAAATGGC TGGTATCACC 1100
TTAAAGTTGC GTATTTCTGA AGCCTCAGAT GGGTAAATGC CCAGATAGAT 1150
AAAATGCAAA TCAAGTCAAG TTATCAATTT AGCATGAAGA TAAAGCTGTT 1200
TGGATAATTG TAATAGAAAT GTCTGATATT TTCACCTTTA CAAAATAAGA 1250
GAGTAGGGCG ATTTTGTCTA GTAACAGAAT TACATGGAAT ATATTTAGAA 1300
ATGAGAAAAA TGTGGGAAAA AAGCACAATG TTCAGTGCCA TGCCACCAAT 1350
TGTATAATAC TATCTCAAAC AAATCGATGT CTGCCTGCCC TAGACTCCAC 1400
TTATTTAAAA TAAGAGAATG AACTCGATGT TTGGCTTCAT AGAGATTCAG 1450
CACCCTGTAA TAGGCCTTCC ATGTCTTTTA ACGTATGTAA TGCAAAGAAC 1500
AAACAAATAA AGGCAGAAAT TTTTCTAACT CTGTCTCTTC TCTCTTTCCC 1550
CCAGACTATG TCAGAGAGTC ACA ATG ACC TTG CAC AAT AAC AGT  1595
                          Met Thr Leu His Asn Asn Ser
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ACA ACC TCG CCT TTG TTT CCA AAC ATC AGC TCC TCC TGG ATA 1637
Thr Thr Ser Pro Leu Phe Pro Asn Ile Ser Ser Ser Trp Ile
CAC AGC CCC TCC GAT GCA GGG CTG CCC CCG GGA ACC GTC ACT 1679
His Ser Pro Ser Asp Ala Gly Leu Pro Pro Gly Thr Val Thr
CAT TTC GGC AGC TAC AAT GTT TCT CGA GCA GCT GGC AAT TTC 1721
His Phe Gly Ser Tyr Asn Val Ser Arg Ala Ala Gly Asn Phe
TCC TCT CCA GAC GGT ACC ACC GAT GAC CCT CTG GGA GGT CAT 1763
Ser Ser Pro Asp Gly Thr Thr Asp Asp Pro Leu Gly Gly His
ACC GTC TGG CAA GTG GTC TTC ATC GCT TTC TTA ACG GGC ATC 1805
Thr Val Trp Gln Val Val Phe Ile Ala Phe Leu Thr Gly Ile
CTG GCC TTG GTG ACC ATC ATC GGC AAC 1832
Leu Ala Leu Val Thr Ile Ile Gly Asn
```

Results: Human m1 muscarinic receptor, promoter region sequence

Essentially similar methods were used to sequence the promoter region of the human m1 muscarinic receptor. A total of 2,382 bases in the 5' unknown region was sequenced. This sequence stops at +464 bases (with respect to the start of the protein coding region), the extent of the previously known region.

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ATTTTTAGAA TTCGAATTAG CAAACTATGA TCCCCAAGGC TGGCCTTCGG
TTTTTGTAAA TGAAATTTCA CTGGAACAAA CTGCTTGGGC TCAAATCCTA
GTTCCTACCT TCCTTGCATG ATCTCGTACA ATTCCCTTGC TTTTCTCAGT
CTCAGCTTCC CTATCTATAA AATGGAGACA ATAGGCCAGG CGCGGTGGCT
CACGCCTGTA CCAGCACTTT GGGAGCCCGA GGCAGGCAGG CAGATCACAA
GGTCAGGAGA TCAAGACCAT CCTGGCTAAC ACTGTGAAAC CCCGTCTCTA
CTAAAAATAC AAAAATTTAG CCGGGCGTGG TGGTGGCAGG CGCCTGTAGT
CCCAGCTACT CAGGAGGCTG AGGCAGGAGA ATGGTGTGAA CCCGGAGGCG
GAGGTTGCAG TGAGCCGAGA TTGTGCCACT GCACTCCAGC CTGGGTGACA
CAGCGAGACT CCATCTCAAA AAAAGAAAGA AAAAGAAAAT GGAGACAATA
GCTGCAGTGA GCCGAGATCG CGCAACTGCA CTCCAGCCTG TATGACAGAG
CGAGACTCCA TCTCAAAAAA AAAAAAATGG AGACAACAGC CATCCCTTGA
TCCTAGGCTC CTGTTAAGAT TAAATAAGAT TATGCATATA ACCCTTTAAC
AGCAACTGGC ACTCAGGGAG CTCTCAATAA CAACAATGAG CATTATTATT
ACTAGTTGCT ATTGTCATAG TATCAGACAT TTCTTTTCTT TTTTTTTTTT
TGAGACAGAG TTTCACTCTT GTTGCCCAGG CTGGAGTGCA ATGGCACAAT
CTTGGCTCAC CACAACCTCT GCTTCCTGGG TTCAAGCAAT TCTCCTCAGC
CTCCAGAGTA GCTAGGGTTA CAGCCATGTA CCACCACCCC TGGCTAATTT
TGTATTTTTA GTAGAGACAG AGTTTCACTA TGTTGGCCAG GCTAGTCTTG
AACTCCCGAC CTCAGGCGAT CCACCCGCCT CGGTCTCCCA AAGGNGCTGG
GATTACAGGC GTGACAGCCG CACCCGGCCA AGAGGTAGAC ATTTCTAGGC
AGCCTAGCAC CCTCATTGCT ACACATCCTG AGAGTTAAAG GAGACCTACT
GTTTACCATG TGCCAGGGCC ATTCTAAGCA CCTTACATGC ATTGATTCAT
TTAATCCTCA TCACCTGAAA AGAGGTGACA GTCTTGCTAA TGGGTATTAT
CCCTTTTTCA ATTAAGGAAA CCTAGGCATA TAGAAGTTAT ATCACTAGCA
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ATAATCACAC ATCCAGTGAA CAGGATAGAC TAGATTCAAA TCCAGATAGT
CTGGCTCCAG AGTCCAGGTT GCACACTGCC CCCTCAAGTG CAAACTGTCC
CAGACACACA AGCACACACT GCCAGCCACA GTCCTTGCTC ATTCTGTCCC
CCACTGAACT CGCCACGCTG TCAGTGACTT GTACACACTG AGGGTTTACG
GCAAACCATG GAGATGCCCT CAGCCACAAA CCACCCAGGC CACCTATCAC
AGCAGCCTGT CACAGGCCAG GTGCCCAAGG AACACCAGGG CTCAGTGGGC
CTTNCTAACA TCTCTCCCTT GNGAATTTTT CCCAACAATA TAGCCCCTGG
AGGCCACAGC TGAGGGTCAG GGGATCCTCA ACCTCTTCAA TGGTCAGATG
GATTGCCTGA GGCCCAGAGC AGGAAGGGAC CCACCAAATC ACATGCAGNA
ACGCTCTGGC TGAGCTGCCT GGACCTTTAC CCTTCCATCT GGCTTCACTG
TGGCTGTGGG GCCTCCTGCC CAGCCAGCAG GTTGTTAAGG CCCCGGCCTT
TCTCTTTACC AGGCTTAGTG CCTGGAGCAG ATTCCAGATC CAAGAAAAGT
CTGGGGGATC TTCCCATTTC CCTTGCTTCT CCTCACTCAG CTGGGGCCCA
TTAACAGAGC CAGGAGAGGG AGGGAAGCCT CCCACACCCC ATGGGCCCAG
ACCCCCACTG AGCTGGGAGC TGAGGCCCTC ATCCCTGCTT CCTGCTTTTG
CCCCAGACCT TCTTTTTTTT CCTTTTGGTT TTTTTTTTTT TGAGATGGAG
TCTTGCTCTG TTGCCCANNT GGAGTAGCAG TGGCACAGTC TTGGCTCACT
GCAACCTCCG CCTCCCAGGT TCAAACGATT CTCCTGCCTC AGTCTCCCAA
GTAGCTGGGG CTACAGATCG TGCCACCACG CCCGGCTAAT TTTTTGTATT
TTTAGTAGAG ATGGGGTTTC ACCATGTTAG CCAGGATGGT CTCGATCTCC
TGACCTCGTG ATCCGCCTGC CTCTGCCTCC CAAAGTGCTG GGTTACAGGT
GTGAGCCACC ACGCCGGCCT GCCCCAGACC TTCCTACTAG CAACTGAGTG
TCTGAGTCCA GCACAATTTT CCCTGCTGCC CTCATAGAGA TTCAATTCAG
CCCCAAATAC CACCCCCTAC ATACGTATGT AAACCCGCAT GCGCAGAAAA
ACGTGCAGAA AAAAATACAT GCACGCACAC ACCCCTAAGG CCCCTCTGTT
CCCATCTCTC TCTGTTTATC TTCTCAGGAA CTCCCCTTTC TTCCCATCTC
TCCTTATTAG TGGAAGATCC CACTGGAGCA CCACATACCC GGGGAGAGTG
AGAATGGCTT CTTTTCTTTC TTTCCTGTTT TAGGACACAT TTCTGTTCTT
GCGGGTTCTT ATCATACTTA CTATCTCTGT CACCATGGCC AAGGCTTGCA
GCTGCTCAGA GCCACAGTTC CCTCACAGTG AGATGGGCAT AATAAGAGCC
CTTTCTCCTA GAGACGTACA GCAGTTAAAT TAGATAATTC ATTTAGGTGG
TTAGTACTGT GTCTGGCATG CAGTGACTAC TC +464

Human m2, m4 and m5 muscarinic receptors, promoter regions

Promoter regions from m2, m4 and m5 have also been clones. Twenty-five percent of m2, 52% of m4, and 50% of m5 unknown promoter regions have been sequenced.

Promoter deletion constructs for functional analysis

Sections of the cloned 1.7 kb 5' untranslated region of the m3 receptor gene were assayed for promoter function using a luciferase reporter system described below. The DNA constructs for this assay were prepared by PCR amplification of sections of the 5' untranslated region which had been cloned into the Bluescript (Stratagene) vector. These constructs are illustrated in Figure 2A.

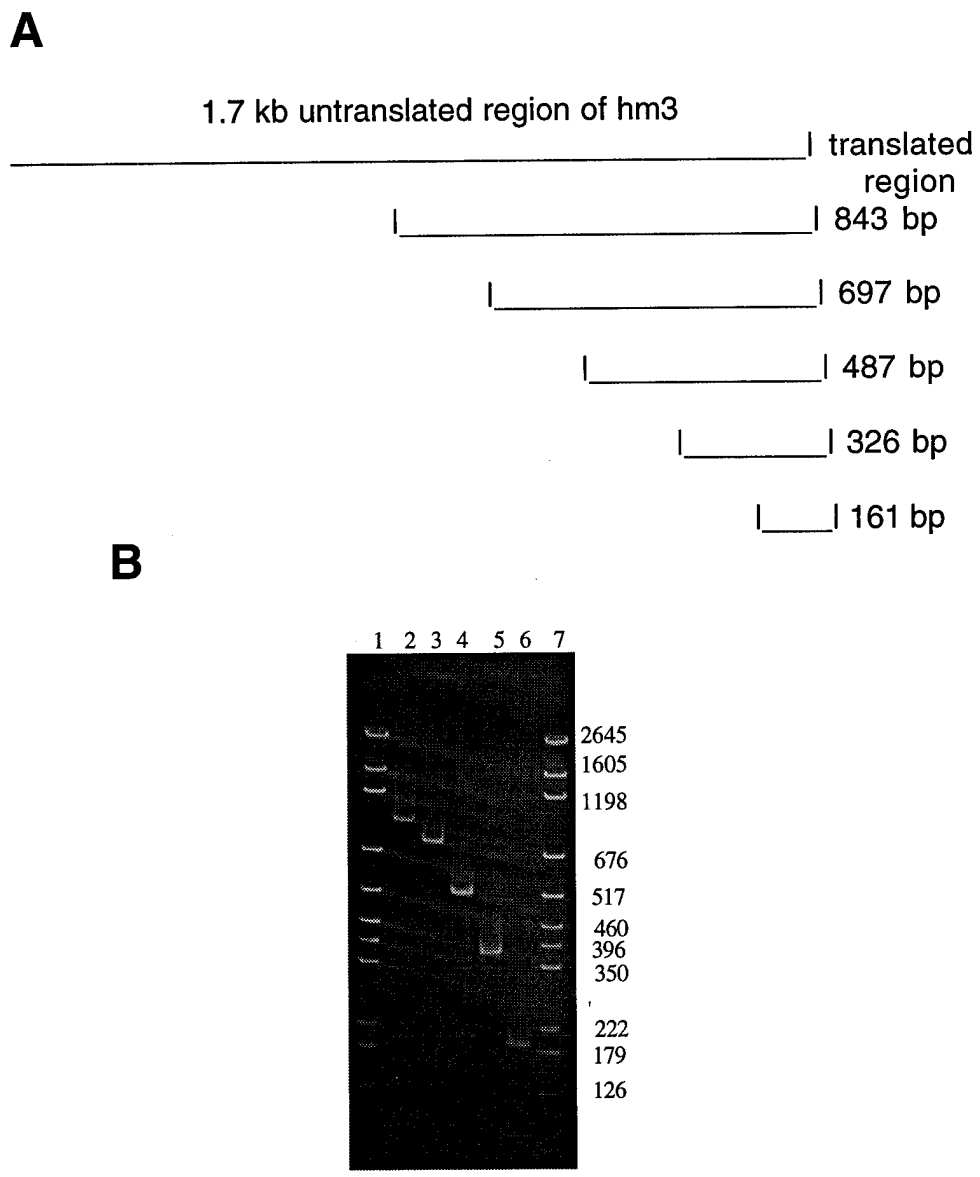


Figure 2: Construction of deletion mutants for luciferase assay studies. Panel A shows the size and location of constructs with respect to the translated regions of the human m3 gene. NOTE: sizes do not include the additional 24 base pairs present due to the incorporation of flanking HindIII restriction endonuclease sites. Panel B is a picture of the ethidium bromide stained agarose gel showing all five deletion constructs. Marker DNA (pGEM DNA markers-Promega) is shown in lanes 1 and 7 with the corresponding sizes indicated.

PCR primers were synthesized to produce various length PCR products with respect to a common downstream primer. The primers used for these reactions were synthesized with Hind III restriction endonuclease sites for subsequent cloning into a reporter vector. Following standard PCR amplification (Perkin-Elmer) the success of the reaction was verified by running 5 µl of the 100 µl PCR mix on a 2% agarose gel. The constructs were

precipitated with 2.5 volumes of cold 100% ethanol for 10 min at -80°C and centrifuged at 13,000xg for 15 min at 4°C. The pellets were washed with ice cold 70% ethanol and spun at 13,000xg for 3 min at 4°C. The pellets were resuspended in 34 µl distilled water after which 4 µl of 10x Hind III buffer was added followed by 2 µl (80 units) of HindIII (New England Biolabs). The reaction was performed at 37°C overnight and inactivated by incubation at 70°C for 10 min. Ten µl of a 5x loading dye was added to each tube and the mixtures were resolved on an 11x14 cm, 6% native polyacrylamide gel with 1x TAE as the running buffer. The gel was run until the bromophenol blue dye had reached the bottom. The gel was stained with ethidium bromide, and the fragments were visualized by UV illumination. Fragments were excised from the gel and crushed in the bottom of a microcentrifuge tube, after which the gel pieces were soaked in 800 µl of 500 mM ammonium acetate/ 1 mM EDTA overnight. The tubes were centrifuged at 13,000xg for 2 min in a microcentrifuge, and the supernatant was filtered through silanized glass wool to remove small pieces of acrylamide. The supernatant was divided into two aliquots (~350 µl each) and precipitated with 2.5 volumes of ethanol. The pellets were washed with 70% ethanol as described above, and the open tubes were left inverted for about 10 min to dry.

The vector pGL3 (Promega) was used as the reporter system. The basic vector contains the luciferase gene with an upstream multiple cloning region which contains the Hind III restriction site. This vector (5 µg) was cut with Hind III (40 units overnight in a 50 µl vol) to completion, as evident from agarose gel electrophoresis. Following inactivation of the HindIII enzyme as described above, the cut vector was dephosphorylated with 5 units of shrimp alkaline phosphatase (USB) for one hour at 37°C. The phosphatase was inactivated by incubation at 70°C for 10 minutes.

One µl of the vector (100 ng) was added to each tube containing the PCR fragments, followed by 7 µl of distilled water. The tubes were incubated at 45°C for 10 minutes to dissociate any annealed ends, and the tubes were transferred to an ice bath, at which time 1 µl of 10x T4 DNA ligase buffer, and 1 µl (about 200 units) of T4 DNA ligase (NEB) was added. The ligation reactions were incubated at 16°C for 12 hours.

After 12 hours the entire ligation mixture was transformed into competent JM109 *E. coli* cells (Promega protocol). Briefly, the ligation mix was added to 100 µl of competent cells incubated for 10 min on ice, heat shocked at 42°C for 50 sec, incubated on ice for 2 more min, mixed with 900 µl SOC media, mixed gently for one hour at 37°C, and plated on LB plates containing 100 µg/ml ampicillin. The colonies visible after incubation at 37°C overnight were picked and used to inoculate 3 ml of LB. These samples were shaken overnight at 37°C, and 1 ml was used to perform small scale plasmid isolations by the protocol of Sambrook et al. (1989). The resulting plasmids were assayed for insert content by cutting with HindIII and resolving the resulting fragments on a 6% native polyacrylamide gel. Plasmids containing inserts were sequenced to determine if the inserts were in the proper orientation with respect to the luciferase gene. The remaining culture of the positive minipreps was used to inoculate 500 ml cultures for large scale plasmid isolations. Large scale plasmid isolations were performed using the protocol of Sambrook et al. (1989). with plasmid DNA purified by cesium chloride gradient fractionation. This purified plasmid was quantitated by UV spectrophotometry (260 nm) and used to transfect various human cell lines as described below.

Cell culture: SK-N-SH and HeLa cells were purchased from ATCC. CHO cells stably tranfected with the genes for human muscairnic receptor subtypes were provided by Dr. Mark Brann (University of Vermont). SK-N-SH and HeLa cells were grown in Dulbeccos's Modification of Eagles Media (DMEM: Mediatech, Fisher Scientific) with 10% fetal bovine serum (Biofluids Inc.), Penicillin/Streptomycin solution (100X; Sigma Chemical) to equal a 1X solution, and Non-essential Amino Acids (Mediatech 100X; Fisher Scientific) to equal a 1X solution. CHO cells were grown in HAM'S F-12 (MOD) with L- glutamine (Mediatech, Fisher Scientific), 1X Penicillin/Streptomycin, and 5% fetal bovine serum.

Cell culture protocols: All cell culture was done in a laminar flow hood to maintain sterility. The cell lines, received packed in dry ice to maintain their viability, were placed in a 37°C water bath until thawed, and then placed into separate 25 cm^2 flasks with 6 ml of the appropriate complete media. The cells were kept in a 37°C incubator that had a constant flow of CO_2 at 5%, and allowed to grow overnight. The flasks were removed and the cells were observed with an inverted microscope to ascertain their viability. The media was removed, replaced with fresh complete media, and the cells were placed back in the incubator to continue to grow for a few days.

Cell growth was checked on a daily basis. When the cells had reached about 80% confluency they were divided into larger flasks as follows. The media in the flasks was removed and discarded and 1 ml of Viokase (a buffered solution of porcine pancreatin) was added to the flask to dislodge the cells. After an additional 10 minutes in the incubator, the flasks were removed and gently tapped to completely dislodge the cells. Four ml of sterile 1X HANK'S buffer (Mediatech; Fisher Scientific) were added to rinse the inner side of the flask where the cells were attached, and the cell mixture was removed from the flask and placed into large, 150 cm^2 flasks (Corning) (1 ml per flask), which contained 25 ml of the appropriate complete media. The cells were placed in the incubator overnight and the media was replaced the following day. The flasks were returned to the incubator and allowed to grow to the desired confluency.

Applications: *SK and HeLa* were grown to between 50-60% confluency, this insured that the cells maintained an optimum growth state. The media was removed and discarded as waste, 4 ml of Viokase was added to the large flask, spread over the entire flask surface. The flasks were placed in the incubator for 10 min, and then gently tapped to dislodge the cells. The flasks were rinsed with 5-8 ml of the 1X HANK'S buffer and contents of like cell lines were combined. The cell solution was inoculated into new large flasks (about 1 ml/flask into 4-8 new flasks), and they were allowed to grow to between 50-60% confluency. Some flasks (2-4) allowed to grow to 100% confluency were used to harvest cells for storage in liquid nitrogen.

CHO cells were grown to full confluency and removed from the flasks by gently scraping with a cell scraper (Fisher Scientific). The media and cells were removed by pipetting and this mixture was centrifuged at 1100 RPM for 10 minutes. The supernatant was discarded and the remaining cell pellet was resuspended in a minimal amount of 50 mM Tris (about 5 ml), and stored at -80°C until further use. Another 25 ml of complete media was added to the flasks that were scraped and they were placed back into the incubator. Some of these

cells were washed with 1X HANK'S buffer and used for storage in liquid nitrogen as described below.

Cryostorage: SK-N-SH, HeLa, and CHO cells were all stored in liquid nitrogen using the same protocol. Several flasks (2-4), were grown to 100% confluency. The cells were removed by the use of viokase. The cell lines were spun down at 1100 rpm for 10 min and the supernatant was discarded as waste. Like cell lines were resuspended and combined in a total of 10 ml of HANK'S buffer and spun down at 1100 rpm for 10 min. The supernatant was discarded and the cells were resuspended in serum free media (2-4 ml). An equal volume of Cryoprotective Media (Whitaker Bioproducts) was added to each cell line and gently mixed. The mixture was transferred to cryostorage vials (Nalgene) in 1 ml aliquots and placed in the -80°C freezer for 1 hour. The tubes were then submerged in liquid nitrogen for prolonged storage. This storage procedure was repeated about every two months, or as needed. Cells were brought out of storage approximately every two months, or as needed.

Counting cells: SK and HeLa were grown to between 50-60% confluency in several 150 cm^2 flasks (12-20) and the cells were removed by treatment with Viokase. The cells were spun down and all like strains were combined into one centrifuge tube in 1X HANK'S buffer and mixed gently. The cells were spun at 1100 rpm for 10 min. The supernatant was removed and discarded and the pellet was resuspend in 10 ml of 1X HANK'S buffer. Twenty µl of cell solution was added to 20 µl of Trypan Blue (Fisher Scientific) and mixed gently. A cover slip was placed on a hemocytometer (Fisher Scientific), and enough cell dye solution was added to cover the reading area of the slide. The 16 grids of the hemacytometer were read (only the cells that weren't stained blue were counted). The number of cells counted was multiplied by 1×10^4 which gives the number of cells that were contained in 1 ml of the solution. This number was then multiplied by 10 to give the total number of cells in the 10 ml of 1X HANK'S buffer.

Transfecting cells: A total of 3.0×10^6 cells were needed for each transfection. A plasmid DNA vector, PGL2 (Promega), which contains the SV40 promoter site and a gene coding region for the protein luciferase, was purified and quantitated. A total of 30 µg of DNA was used per set of cells to be electroporated. The cells and DNA were mixed together and incubated at room temperature for 10 min in electroporation cuvettes (Bio Rad). Electroporation was performed on a BioRad Gene Pulser system. The SK cell line was electroporated at the following settings; 960 µF and 160 v, the HeLa cells were electroporated at 960 µF and 200 v. After the cells were electroporated, they were allowed to incubate at room temperature for 10 min. The cells were then removed from the cuvettes and placed into petri dishes (Sarstedt 100 x 20 mm) which contain 10 ml of the appropriate complete media. The petri dishes were then placed into the incubator and allowed to grow for 2 days. The cells were then assayed for luciferase content.

Luciferase assay: The petri dishes were removed from the incubator and the supernatant was removed and placed into a centrifuge tube. The cells were then scraped from the surface of the petri dish with a cell scraper and added to the respective supernatant. The cell supernatant was centrifuged at 3500 RPM for 10 min, and the supernatant was removed and discarded. The pellet was resuspended in 300 µl of 1X lysis buffer (Luciferase Assay Kit; Promega), transferred to a 1.5 ml Eppendorf tube, and

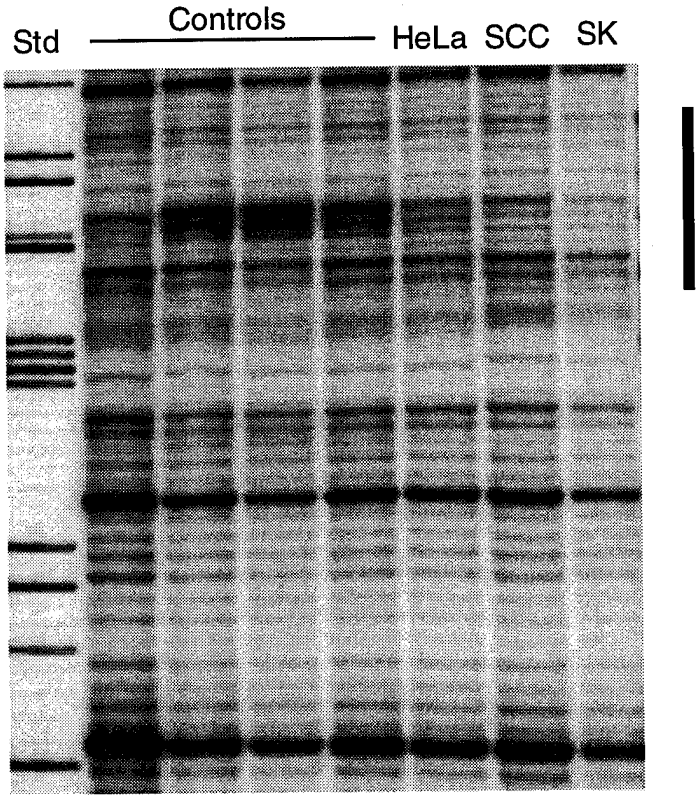
allowed to incubate at room temperature for 10 min. The lysed cells were pelleted for 10 min at high speed in a microcentrifuge, and the supernatant was transferred to a new 1.5 ml tube. The sample was stored at -80°C until assayed.

To run the assay, the tops of enough Eppendorf tubes were cut off to equal 1 tube/sample. Twenty µl of sample were placed in labeled trimmed microcentrifuge tubes and placed into scintillation vials. Enough luciferase substrate (Luciferase Assay Kit Promega) was thawed to provide 100 µl for each sample, a standard control composed of 20 µl of 1X lysis buffer and 100 µl of substrate and negative controls. The samples were read for luminescence in a scintillation counter (Beckman). The program was set to read the sample for 0.50 minutes at 0.10 minute intervals. The 100 µl of substrate was added to the samples at precisely the 0.20 interval (one sample at a time was read, and all were set up exactly in this fashion). The luciferase substrate was added to each sample and the trimmed tubes were mixed thoroughly and placed back into the scintillation vials. Negative controls which lacked added DNA were run as well. These negative controls were simply cells that had been electroporated but no DNA had been added to the mix. These were used for a comparison to see how much luminescence the cells have without added PGL2 vector. Several replications of each sample were run and the results were averaged and tabulated into graph form for further comparisons.

In Vitro DNase I footprinting analysis: Nuclear extracts were prepared from Hela, SCC9, and SKW cells. Briefly, 5-10 x 10^8 cells were washed with phosphate buffered saline, resuspended in 5 vol of 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM phenylmethysulfonyl flouride (PMSF), 2 ug per ml each of antipain, leupeptin, pepstatin A, and lysed in a Dounce homogenizer. The nuclei were collected by centrifugation at 1200 x g for 10 min, and resuspended in 0.5 to 1 ml of 20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 2 ug per ml antipain, leupeptin, pepstatin A. Nuclear proteins were extracted for 30 min at 4°C after adjusting the KCl concentration to 0.7 M. The nuclei were removed by centrifugation at 32,000 x g for 60 min at 4°C, and the supernatant containing the extracted proteins was dialyzed for 1-2 hr against a binding buffer consisting of 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF.

Double-stranded probes were prepared by the PCR with appropriate primer pairs. One primer was labeled with (γ-^{32}P)ATP. The probes were purified by spin-dialysis through Millipore ultrafree 30,000 MC filters according to the manufacturer's instructions (Millipore, Bedford, MA), extracted once with an equal volume of phenol(1:1), and ethanol precipitated. Approximately 1-2 x 10^5 cpm of probe (1-2 ng), 100 µg of crude nuclear extract or 1-10 µg of purified c-jun or CREB protein, 5 µg poly (dI-dC), and $MgCl_2$ (6.25 mM final concentration) were incubated in a total volume of 50 ul for 15 min at room temperature. Then 50 µl of 5 mM $CaCl_2$, 10 mM $MgCl_2$, and 0.15 units of DNase I were added. After 1 min at room temperature, the reaction was stopped with 90 ul of 0.2 M NaCl, 0.03 M EDTA, 1% SDS, 10 µg *E. coli* tRNA. Following phenol extraction and ethanol precipitation, the products were analyzed on a sequencing gel.

Figure 3. An example of DNase I footprinting of the m3 receptor promoting is shown in the accompanying Figure. The first column contains standards, the next four columns contain controls (i.e., different amounts of material with no nuclear extracts). The next 3 lanes contain nuclear extracts from HeLa, SCC-9 and SK-N-SH cells. One region (approximately 1 kb from the translation start site) contains an apparent protein binding region and is indicated by the solid line.



RNA detection and quantitation

Messenger RNA transcribed from the muscarinic promoter genes will be detected and quantitated from a variety of tissue and cell samples using the RNase Protection Assay System from Promega. This kit utilizes RNA probes synthesized *in vitro*, therefore, the first step was to transfer a portion of the cloned segment of the hm3 gene to a vector containing transcription start sites such as SP6 or T7 sites. The determined sequence of hm3 was analyzed for common restriction sites and HindIII and KpnI sites were found to be present on opposite ends of the cloned region. The figure below depicts a partial restriction map of the cloned 1.7 kb fragment of the hm3 receptor gene.

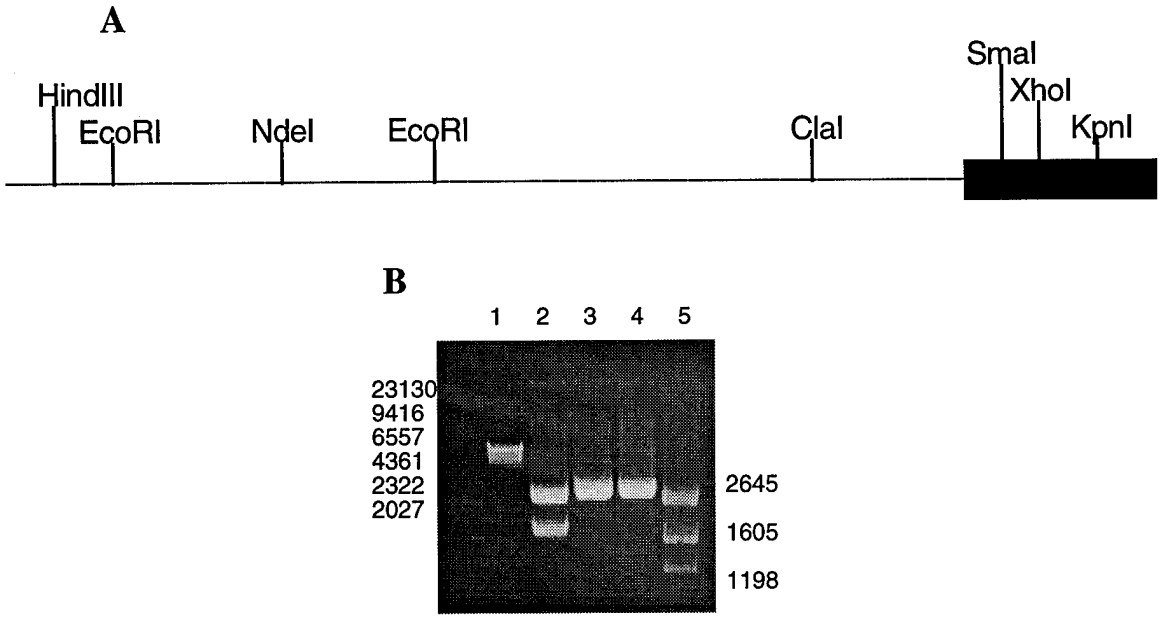


Figure 4: Construction of DNA template for RNA probe synthesis via *in vitro* transcription. Panel A): Partial restriction enzyme map of 1.7 kb fragment in Bluescript (Stratagene) sequencing vector. Panel B): Restriction screening of plasmid isolates following cloning of 1.6 kb Hind III to Kpn I fragment in pGEM-3Z for *in vitro* transcription. Lane 1 and 5 are markers, Hind III digested lambda DNA and pGEM DNA markers respectively. Lane 2, 3 and 4 are plasmid isolates derived from small scale preps of single colonies. Lane 2 contains a ~1.6 kb fragment (bottom band) in addition to the ~3 kb fragment from the linearized pGEM-3Z.

The1.6 kb fragment corresponding to the region between the Hind III site and the Kpn I site (shown in lane 2 of Figure 4 panel B, bottom band) was excised from the Bluescript (Stratagene) vector, resolved on a preparative 6 % native polyacrylamide gel, extracted from the gel by crush and soak, and cloned into the vector pGEM-3Z (Promega) that had been cut with Hind III and Kpn I and dephosphorylated. Following transformation into JM109 cells and plating on LB plates supplemented with ampicillin (100ug/ml), IPTG and X-gal for blue/white screening, white colonies were chosen and used to innoculate small cultures for restriction enzyme screening. Successful cloning was determined by recutting plasmids isolated by the small scale method of Sambrook et al (1989) with Hind III. Protocols used for this cloning were similar to those described in the cloning of the promoter deletion fragment constructs for use in the luciferase functional studies described earlier.

The pGEM-3Z construct will be used as template for *in vitro* transcription via the Riboprobe *in vitro* Transcription System from Promega. The kit contains all reagents necessary to produce RNA probes from a linear DNA template via run off transcription. The pGEM-3Z construct will be linearized with Cla I (NEB) and transcription will be

performed with T7 RNA polymerase. T7 polymerase will be used since this polymerase will transcribe in the opposite direction with respect to the production of messenger RNA. This opposite direction will, therefore, produce a probe complimentary to the mRNA to be analyzed. The probe will be about 350 bases in length and radiolabel will be incorporated during transcription.

The probe produced above will be used to hybridize to mRNA isolated from a variety of sources to determine the presence and quantity of the message. The presence and quantity of mRNA will be determined using the process of nuclease protection via the RNase Protection Assay System from Promega. This kit provides all necessary reagents to detect and quantify mRNA from an aqueous solution. The mRNA will be initially isolated using the MicroFastrack mRNA isolation kit from Invitrogen. The labeled RNA probe will be hybridized to the mRNA and the amout of probe protected from subsequent RNase digestion will be related to the quantity of mRNA present in the cell or tissue sample. The different quantities of mRNA from various samples may reflect differences in the expression of hm3 in the various cell or tissue types from which the samples were obtained.

Publications and presentations supported in whole or part by this grant:

Aronstam, R.S., R.L. Dennison, R. Ravindra and D.C. Martin, Ethanol disruption of muscarinic acetylcholine receptor-stimulated G protein GTPase activity and guanine nucleotide binding in rat brain, *in revision.*

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*Presentation at scientific meeting.

7. Conclusions

The results to date provide partial support for our hypothesis that *acute ethanol administration disrupts synaptic transmission by interfering with receptor-G protein interactions*. However, these effects are not universal; for instance, disruption of receptor-G protein interactions by ethanol was not apparent with each receptor examined, and not all muscarinic receptor subtypes were equally affected by ethanol. The reasons for these differences are not yet apparent.

In ligand binding studies, receptor-G protein interactions were more readily visualized in m2 and m4, compared to m1, m3 and m5, muscarinic receptors. This agrees with our previous findings in intact tissue that these interactions were most readily visualized in brainstem, which expresses mostly m2 receptors. Similarly, we have shown a large effect of guanine nucleotides on atrial muscarinic receptors, which are almost exclusively of the m2 subtype. In these studies ethanol decreased the sensitivity of agonist binding to inhibition by guanine nucleotide in a manner which is consistent with our hypothesis that ethanol disrupts receptor-G protein interactions (Aronstam et al., 1993). However, certain observations inconsistent with our hypothesis, and with our results in intact tissues, have been made on muscarinic receptors expressed in CHO cells. For example, the interaction of m4 receptors expressed by CHO cells display a sensitivity to ethanol by one measure ([^{3}H]antagonist saturation) but not by another (agonist affinity). These points are the focus of continued study. A number of challenges present themselves when dealing with muscarinic receptors expressed by cultured cell lines, most of which stem from a relative paucity of experimental material. Novel physical and chemical mechanisms for disrupting receptor-G protein interactions (i.e., heat and alkylation) are being used to gain further insight into ethanol's actions and the nature of muscarinic receptors expressed in foreign systems.

We have obtained further evidence that ethanol interferes with muscarinic receptor control of the G protein cycle in muscarinic cholinergic systems. Thus, we have demonstrated an inhibition by acute ethanol exposure of muscarinic receptor control of G protein GTPase activity and guanine nucleotide binding in brain tissues. These effects are consistent with the effects of ethanol on the guanine nucleotide sensitivity of agonist binding, and are similar to the effects of volatile anesthetics on the same system. These findings raise the possibility that interference with receptor-G protein interactions is a common mechanism of action of compounds which nonspecifically depress synaptic transmission.

We have demonstrated multiple acute effects of ethanol on the control of adenylate cyclase. Basal activity was not consistently affected, suggesting that ethanol's actions reflect effects on the regulatory mechanisms (i.e., receptors and/or G proteins) rather than the catalytic subunit per se. Ethanol was found to increase acetylcholine inhibition of forskolin-stimulated cyclase activity. Further experiments suggested that the effect of ethanol reflects an increase in the ability of guanine nucleotides (e.g., GTP) to stimulate adenylate cyclase activity, rather than an effect on cholinergic signal transduction mechanisms. It is likely that there is a balance between inhibitory (Gi-mediated) and stimulatory (Gs-mediated) influences on adenylate cyclase activity that is disrupted by ethanol. At low guanine nucleotide concentrations it seems likely that a stimulatory effect of ethanol on Gs predominates; this may change at higher nucleotide concentrations. Thus, it may not be possible to isolate ethanol's effects on cholinergic transmission using this approach. Accordingly, in subsequent experiments, we gave increased attention to other measures and aspects of cholinergic signaling, particularly measurements of intracellular calcium concentration regulation.

We examined muscarinic control of intracellular calcium levels utilizing microfluorescence techniques, as a general method for examining synaptic signal transduction. The responses of SKN-SH cells and CHO cells expressing different muscarinic receptor subtypes were characterized. Several parameters of the calcium response were characterized, including concentration dependence, pharmacology, dependence on calcium from intracellular and extracellular sources, stimulation of extracellular divalent cation entry, and desensitization. Clear differences were observed in the responses of m2/m4 and m1/m3/m5/SK-N-SH cells. A novel strategy was adopted to deal with inter-experiment variability, including grouping cells according to nature of the response and using each cell as its own control. Our results indicate that ethanol inhibits the response of m1 and m5-expressing receptors to muscarinic stimulation. Experiments currently underway will extend this analysis to each parameter in the different cell types.

We also examined the acute influence of ethanol on muscarinic signaling in a non-neuronal cell line, the muscarinic receptor-mediated stimulation of cell-adhesion activity in small cell lung carcinoma (SCLC) cells. This response involves m3 receptor-mediated increases in E-cadherin binding activity. Ethanol disrupted muscarinic receptor-mediated adhesion processes at concentrations well within concentrations achieved during intoxication. Thus, ethanol can disrupt muscarinic signal transduction in non-neuronal cells.

We initiated studies on the influence of chronic ethanol treatment on neurotransmitter receptors and their interactions with G proteins. These studies required 1) equipment modification of the cages to handle the liquid diet and 2) the establishment of baseline protocol measurements before utilizing this expensive material. We established Western blot assays to determine the levels of G proteins and Northern blots to determine the levels of receptor mRNA in brain tissues following chronic treatment with ethanol. To analyze the control of receptor expression, we analyzed the promoter regions of the 5 human muscarinic receptor subtypes. We cloned, sequenced and characterized the promoter regions by sequence analysis and footprinting using nuclear extracts. Reporter constructs incorporating luciferase and portions of the promoter regions were prepared and cloned to assist in this analysis.

In all, approximately 40% of the proposed research has been completed and the study has been extended in a number of new directions to incorporate emerging technologies (especially in the molecular analysis of gene expression). The entire team of researchers has been recruited and trained, and it is anticipated that all of the proposed work will be completed within the specified granting period. A number of publications have come from the supported work, although the majority of work has yet to be prepared for publication.

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Ethanol Disruption of Muscarinic Acetylcholine Receptor-Stimulated G Protein GTPase Activity and Guanine Nucleotide Binding in Rat Brain

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This work was supported by Department of the Army Grant Number DAMD17-94-J-4011. The content of this paper does not necessarily reflect the positionor policy of the U.S. government..

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Summary Paragraph

The influence of ethanol on the low K_m GTPase activity and guanine nucleotide binding associated with the G proteins coupled to muscarinic acetylcholine receptors was studied in membranes isolated from rat striatum. Acetylcholine stimulated low K_m GTPase activity measured in the presence of 1 μM GTP by up to 78% with an EC50 of 1 μM. Stimulation of this activity was completely blocked by atropine (IC50 ≈ 0.8 μM). In the presence of ethanol (10 - 1000 mM), basal GTPase activity was increased by 10 – 15%, while stimulation of GTPase activity by 100 μM acetylcholine was inhibited by up to 80% (range = 50 – 90% in 6 preparations). The IC50 for this effect was approximately 40 mM, while a maximal effect required 400 mM ethanol. Carbamylcholine also stimulated high affinity [^{35}S]GTP binding to striatal membranes by up to 18% at 1 mM. Ethanol inhibited carbamylcholine-stimulated [^{35}S]GTP binding by up to 80% with an IC50 of approximately 70 mM; basal [^{35}S]GTP binding was much less sensitive to ethanol. These findings suggest that ethanol disrupts transmission at muscarinic synapses by interfering with receptor-G protein interactions. In this action ethanol resembles several of the volatile anesthetics.

Key Words: Ethanol, G Proteins, Muscarinic Receptors, GTPase Activity

Introduction

Acute ethanol administration produces a wide range of depressant effects on neuronal excitability [1,2], although the precise mechanisms underlying ethanol intoxication, tolerance, and dependence have not been clearly defined. However, it is noteworthy that ethanol inhibits synaptic transmission at lower concentrations than it disrupts axonal conductance or the electrical properties of nerve cells (e.g., refs 2,3).

The cholinergic system is affected by ethanol in several ways [3]. Perhaps the most consistent neurochemical effect of acute ethanol administration is a decrease in acetylcholine release from brain tissue *in vivo* and *in vitro* [4-8]. Moreover, acetylcholine release is more sensitive to ethanol than is the release of other brain monoamines [9,10]. Variable effects of ethanol on brain acetylcholine levels, turnover, and cholinergic enzyme activities have also been reported [11-14]. Cholinergic involvement in ethanol intoxication is also suggested by physostigmine antagonism of ethanol-induced sleep time in mice [4], as well as choline potentiation of ethanol-induced hypomotility in rats [15].

Several acute effects of ethanol on postsynaptic muscarinic processes have been described [2]. Systemic administration of ethanol selectively enhances the sensitivity of hippocampal pyramidal cells to acetylcholine (as well as somatostatin) [16]. Ethanol inhibits antagonist binding to muscarinic receptors (but only at very high concentrations, IC50's >1 M), and the potency of short chain alcohols in inhibiting antagonist binding is directly related to the length of the carbon chain [17]. Ethanol, at physiologically tolerable concentrations, selectively decreases agonist binding affinity to muscarinic receptors by decreasing hydrophobic binding effects [18,19]. Ethanol has been reported to inhibit muscarinic receptor-mediated stimulation of

[^{32}P] incorporation into synaptosomal phosphatidic acid [20], but not muscarinic receptor-mediated inhibition of adenylate cyclase activity in the striatum [21].

Recent evidence has implicated guanine nucleotide-dependent transducer proteins (G proteins) in the actions of ethanol. Ethanol increases the activation of adenylate cyclase by G_s (e.g., refs. 22, 23). Hoffman and Tabakoff [24] have proposed that ethanol selectively enhances the rate of activation of G_s (an action that is normally catalyzed by an interaction with receptors) as well as the interaction of $G_{s\alpha}$ with guanine nucleotides. On the other hand, Bauché et al. [25] have presented evidence that ethanol also disrupts G_i-mediated control of adenylate cyclase in rat brain. Charness et al. [26] have demonstrated differential regulation by ethanol of the expression of both $G_{i\alpha}$ and $G_{s\alpha}$ in several neuronal cell lines.

We have demonstrated that compounds in a second class of global central nervous system depressants, the volatile general anesthetics, inhibit muscarinic neurotransmission by disrupting receptor coupling to transducer G proteins [27-32]. This disruption was initially detected as a depression in the guanine nucleotide sensitivity of agonist binding to the receptors [28,32]. Similar effects were observed with a second G_i-coupled receptor, the α_2-adrenergic receptor of rat cerebral cortex [33]. In the present study we explored the possibility that ethanol has similar effects on muscarinic receptor-G protein interactions in rat brain by examining the effect of ethanol on muscarinic regulation of two aspects of G protein function, GTPase activity and guanine nucleotide binding.

MATERIALS AND METHODS

Materials:

[γ-^{32}P]Guanosine triphosphate ([^{32}P]GTP; 6000 Ci/mmole) and [^{35}S]guanosine 5'-(γ–thio)triphosphate ([^{35}S]GTP; 1000 Ci/mmole) were purchased from DuPont-NEN (Boston, MA).

Tissue Preparation:

Adult, male Wistar rats (Harlan Sprague-Dawley, Indianapolis, IN) were decapitated and the striatum dissected and homogenized in 20 volumes of TED buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol) containing 10% sucrose using a Teflon-glass tissue grinder. The homogenate was spun at 1,000 x *g* for 10 min. The pellet was resuspended in the same buffer and spun under the same conditions. The supernatants were combined and centrifuged at 17,000 x *g* for 20 min. The pellet was resuspended in TED buffer (without sucrose) and washed twice by centrifugation. The resulting pellet was suspended in TED buffer adjusted to pH 8.0 and incubated on ice for 30 min. This suspension was then centrifuged at 17,000 x *g* for 20 min, and the pellet resuspended in TED buffer, pH 7.4, and used without further treatment. Low Km GTPase activity was quite sensitive to elevated temperatures, so tissue preparation was carried out at 0-4°.

G Protein Activity:

GTPase activity was determined by the method of Cassel and Selinger [34]. The reaction mixture (100 µl) contained 75 mM Tris-HCl, pH 7.4, [γ-^{32}P]GTP (70-100,000 cpm), 1 µM unlabeled GTP, 2 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM 5'-adenylylimidodiphosphate, 5 mM phosphocreatine, 50 units/ml creatine phosphokinase, 50 µg bovine serum albumin, 0.1 mM EDTA, 0.2 mM EGTA, 1 mM

cAMP, 100 mM NaCl, and 8-20 μg membrane protein. The reaction was initiated by the addition of protein, and was continued for 5 min at 37°. The reaction was quenched by the addition of 5% activated charcoal in 20 mM phosphoric acid (pH 2.5). The samples were kept on ice for 10 min and then centrifuged at 7,000 x *g* for 10 min. The radioactivity content of the supernatant was determined by liquid scintillation counting. Low K_m GTPase activity was calculated by subtracting [^{32}P] released in the presence of 100 μM unlabeled GTP. Receptor-stimulated activity was measured by including acetylcholine and 10 mM physostigmine in certain assays. The muscarinic nature of this stimulation was established by including 10 μM atropine in a parallel series of incubations. Measurements in each experiment were performed in quadruplicate.

The binding of [^{35}S]GTP was measured using a filtration procedure. Striatal membranes (15 μg protein) were incubated with 0.4 nM [^{35}S]GTP in TED buffer containing 2 $MgCl_2$ at pH 8.0 in a final volume of 1 ml. Nonspecific binding was measured in the presence of 10^{-5} M GTP. After a 30 min incubation at 30° the suspension was filtered through a glass fiber filter (#32; Schleicher and Schuell; Keene, NH). The tubes and filters were washed twice with 5 ml TED buffer, and the radioactivity content of the filters was determined by liquid scintillation counting. Carbamylcholine and atropine were included in certain assays to identify muscarinic receptor-stimulated [^{35}S]GTP binding.

Results

G Protein GTPase Activity

A time course of the low K_m GTPase activity of striatal membranes is shown in Fig. 1. Activity was linear over at least 20 min under these conditions. Basal activity in the presence of 1 μM GTP was 60 ± 12 pmol/mg/min (n=12) over a standard incubation period of 5 min at 37°.

Inclusion of 100 μM acetylcholine in the assay increased the low K_m GTPase activity at all time points tested (Fig. 1). A concentration-response curve revealed a maximal stimulation of about 75% with an EC50 of about 1 μM (Fig. 2). The stimulation by acetylcholine was completely prevented by preincubating the protein with 10 μM atropine (Fig. 2). A concentration response curve for atropine inhibition of acetylcholine-stimulated GTPase activity is presented in Fig. 3. The relatively high concentrations of atropine required to inhibit acetylcholine stimulation (IC50 ≈ 0.8 μM) probably reflects the slow onset of atropine's action (the tissue was not preincubated with atropine in this experiment).

Ethanol specifically inhibited acetylcholine-stimulated GTPase activity by up to 80% with an IC50 of ≈ 40 mM (Fig. 4). Ethanol slightly increased basal activity measured in the absence of acetylcholine (10-15%). Selective inhibition of muscarinic receptor-stimulated GTPase activity was observed at several GTP concentrations from 0.5 – 2.0 μM (Fig. 5A). Double reciprocal plots (Fig. 5B) indicate that ACh increases the maximum enzymatic rate; this increase is largely reversed by ethanol.

[^{35}S]GTP Binding

Carbamylcholine caused a small but consistent increase in GTP binding to striatal membranes (Fig. 6A). In the presence of 2 mM $MgCl_2$, carbamylcholine increased

the binding of 0.4 nM [^{35}S]GTP by up to 18%. This increase was completely blocked by preincubating the membranes with 10 μM atropine, or by treating the membranes with pertussis toxin under conditions which favored ADP-ribosylation of G proteins.

Ethanol strongly suppressed carbamylcholine stimulation of [^{35}S]GTP binding (Figs. 6,7). The IC50 for ethanol's inhibition of carbamylcholine-stimulated [^{35}S]GTP binding was about 80 mM (Fig. 7). [^{35}S]GTP binding measured in the absence of carbamylcholine was much less sensitive to ethanol; 200 mM ethanol inhibited this binding by less than 20% (Fig. 7).

Discussion

The present results indicate that ethanol disrupts muscarinic regulation of G protein activity in striatal membranes as evidenced by its inhibition of agonist-stimulated G protein GTPase activity and guanine nucleotide binding. The concentrations of ethanol required to produce this inhibition support the notion that these actions contribute to the intoxicating effects of ethanol.

Agonist binding to muscarinic (and many other hormonal and neurotransmitter) receptors is thought to engender an interaction of the receptor with the α subunit of transducer G proteins [35,36]. This interaction triggers a release of GDP from the α subunit. GTP then binds to the α subunit, initiating a dissociation of G protein subunits. The α_{GTP} subunit then interacts with an effector mechanism (e.g., enzyme or ion channel) altering its activity. This interaction is terminated by an intrinsic GTPase activity of the α subunit. The $G\alpha\beta\gamma$ complex then reassociates, completing the cycle. By catalyzing GDP release from the subunit, the receptor interaction accelerates turnover of the G protein cycle, seen in the present study as an increase in guanine nucleotide binding and hydrolysis. Muscarinic receptor-mediated stimulation of both [^{35}S]GTP binding and [γ-^{32}P]GTP hydrolysis has been reported by others [37-39].

Concentrations of ethanol associated with impairment of reaction time in humans are about 4 - 6 mM. Ethanol concentrations associated with gross intoxication range from 30 - 50 mM, while lethal concentrations are generally above 80 mM [40]. The IC50's associated with ethanol inhibition of muscarinic receptor-stimulated GTPase activity and GTP binding were approximately 40 to 80 mM, respectively. Thus, the present actions of ethanol might contribute to the physiological disturbances observed in ethanol intoxication.

The present results are consistent with evidence derived from ligand binding studies [41], in which ethanol diminished the guanine nucleotide sensitivity of agonist binding to muscarinic receptors at concentrations as low as 25 mM. This effect was seen in both carbamylcholine/[^{3}H]*N*-methylscopolamine competition curves and in direct measurements of high affinity [^{3}H]oxotremorine-M binding. The guanine nucleotide sensitivity of agonist binding is an indication of receptor-G protein interaction insofar as guanine nucleotides engender dissociation of receptor-G protein complexes, leaving the receptor in a state characterized by low affinity for agonists.

In these actions, ethanol resembles a second class of CNS depressants, the volatile general anesthetics [27]. The general anesthetics interfere with receptor-G protein interactions in (at least) muscarinic and α_2-adrenergic systems, thereby depressing the guanine nucleotide sensitivity of agonist binding, receptor control of G protein function, and receptor regulation of adenylate cyclase activity [27,29,33]. This similarity raises the possibility that disruption of synaptic transmission by a number of hydrophobic depressant compounds (i.e., compounds with access to interior membrane structures) involves an interference with receptor-G protein interactions. In this regard, it is noteworthy that the majority of neurotransmitter receptors couple to transducer G proteins, although we have found considerable variability in the susceptibility of different G protein-coupled receptors to the actions of volatile anesthetics. For example, adenosine A_1 and serotonin 5-HT-$_{1A}$ receptors were not affected by a volatile anesthetic [42]. It will be interesting to see if ethanol displays a similar specificity of action.

In summary, we have presented further evidence that ethanol interferes with receptor control of the G protein cycle in muscarinic cholinergic systems in rat striatum. This is seen as an inhibition of muscarinic receptor control of G protein GTPase activity and guanine nucleotide binding. These effects are consistent with

the effects of ethanol on the guanine nucleotide sensitivity of agonist binding, and are similar to the effects of volatile anesthetics on the same system. These findings further suggest that interference with receptor-G protein interactions is a common mechanism of action of compounds which nonspecifically depress synaptic transmission.

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Figure Legends

Fig. 1. Time course of low K_m GTPase activity in rat striatal membranes: stimulation by acetylcholine. GTPase activity was measured with 1 μM GTP in the absence (O) and presence (●) of 100 μM acetylcholine. Each point and bar represents the mean and standard deviation from 3 experiments.

Fig. 2. Acetylcholine stimulation of low K_m GTPase activity in rat striatal membranes. The percent stimulation of basal GTPase activity (i.e., activity measured in the absence of acetylcholine) was determined in the presence of acetylcholine at the concentrations indicated on the abscissa (O). In a parallel set of experiments the membranes were pretreated with 10 μM atropine for 30 min at 4° before GTPase activity was measured (●). Each point and bar represents the mean and standard deviation from 3 experiments.

Fig. 3. Atropine inhibition of acetylcholine-stimulated GTPase activity in rat striatal membranes. Atropine was included in the GTPase assay medium (i.e., the membranes were not preincubated with the atropine). Each point and bar represents the mean and standard deviation from 3 experiments.

Fig. 4. Ethanol inhibition of acetylcholine-stimulated GTPase activity in rat striatal membranes. **A.** GTPase activity (1 μM GTP) was measured in the presence (●) and absence (O) of 100 μM acetylcholine in the presence of the concentration of ethanol indicated on the abscissa. Each point and bar represents the mean and standard deviation from 6 experiments. **B.** the percent inhibition of acetylcholine-stimulated GTPase activity by each concentration of ethanol is plotted; information is derived from the data in **A.**

Fig. 5. Influence of ethanol on basal and acetylcholine-stimulated GTPase activity determined at several concentrations of GTP. **A.** GTPase activity was measured in the absence (circles) and presence (triangles) of 200 mM ethanol in the absence (open symbols) and presence (closed symbols) of 100 μM acetylcholine. The GTP concentration of the assay medium is indicated on the abscissa. Each point represents the mean from 3 experiments which varied by up to 15%. **B.** Double reciprocal plots of the data presented in **A**. Lines are drawn according to linear regression analysis. (The control/ACh (Δ) line was essentially similar to the control/no ACh (▲) line, and is not shown for reasons of clarity.)

Fig. 6. Muscarinic stimulation of high affinity [^{35}S]GTP binding to rat striatal membranes: influences of atropine and ethanol. The binding of 0.4 nM [^{35}S]GTP was measured in the presence of the indicated concentrations of carbamylcholine in the absence (O,●) or presence (Δ) of 100 mM ethanol. In one series of experiments, the membranes were preincubated with 10 μM atropine for 30 min at 4° (●). Binding is expressed as fraction of total specific binding measured in the absence of carbamylcholine, atropine and ethanol. Each point and bar represents the mean and standard deviation from 3 experiments.

Fig. 7. Ethanol inhibition of [^{35}S]GTP binding to rat striatal membranes. Total (O) and carbamylcholine (100 μM)-stimulated (●) [^{35}S]GTP binding was measured in the presence of the concentration of ethanol indicated on the abscissa. Binding is expressed as fraction of binding (basal or carbamylcholine-stimulated) measured in the absence of ethanol. Each point and bar represents the mean and standard deviation from 3 experiments.

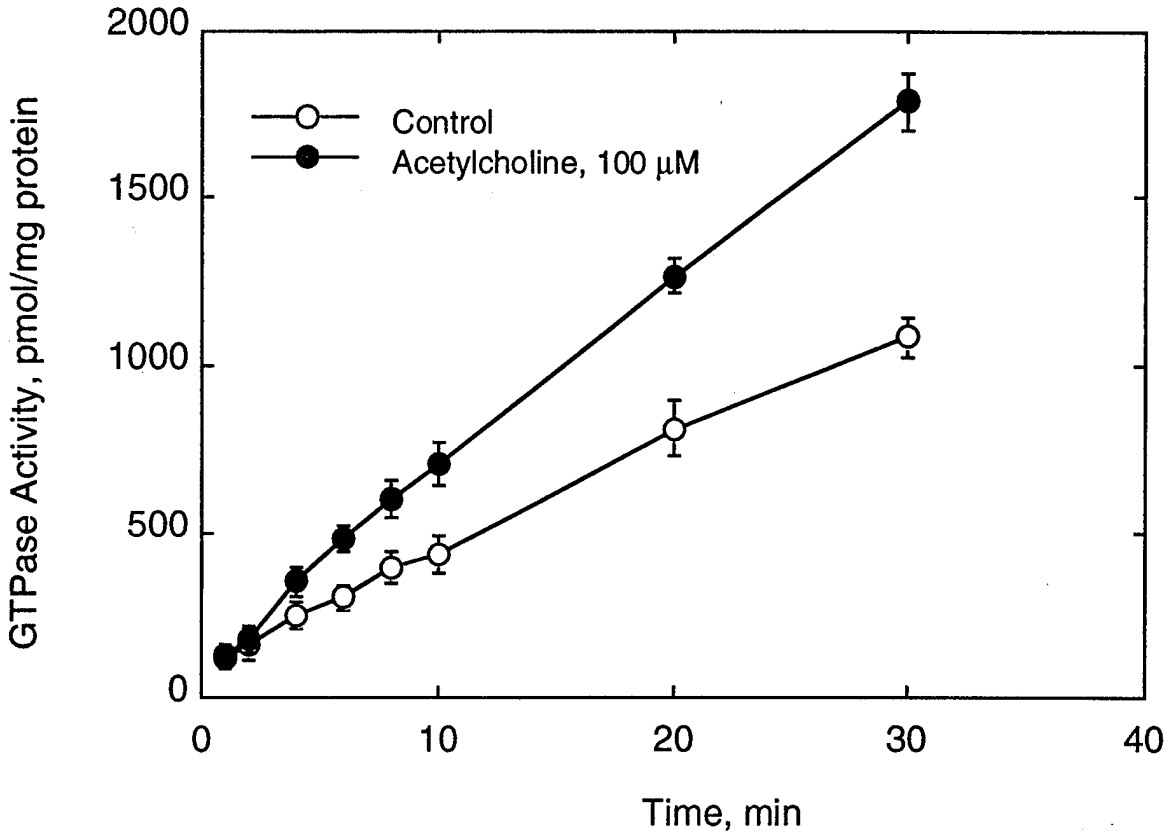
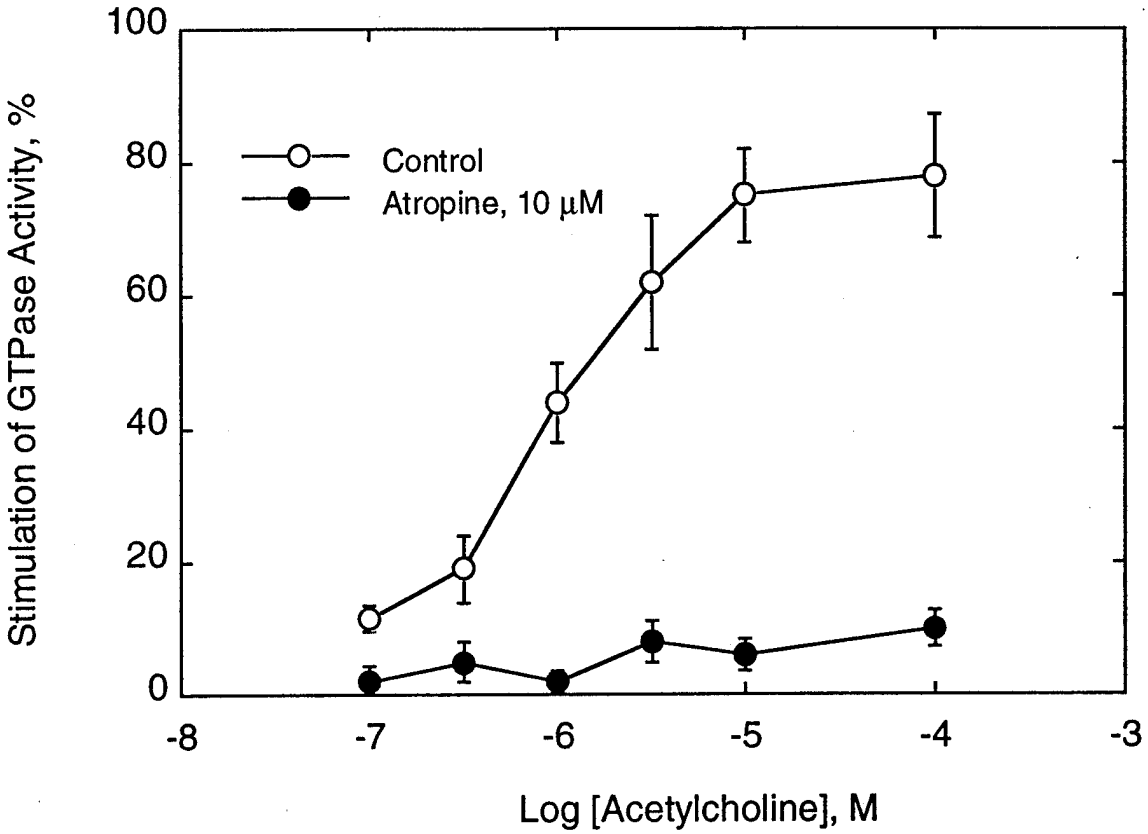


Figure 1. Aronstam et al.



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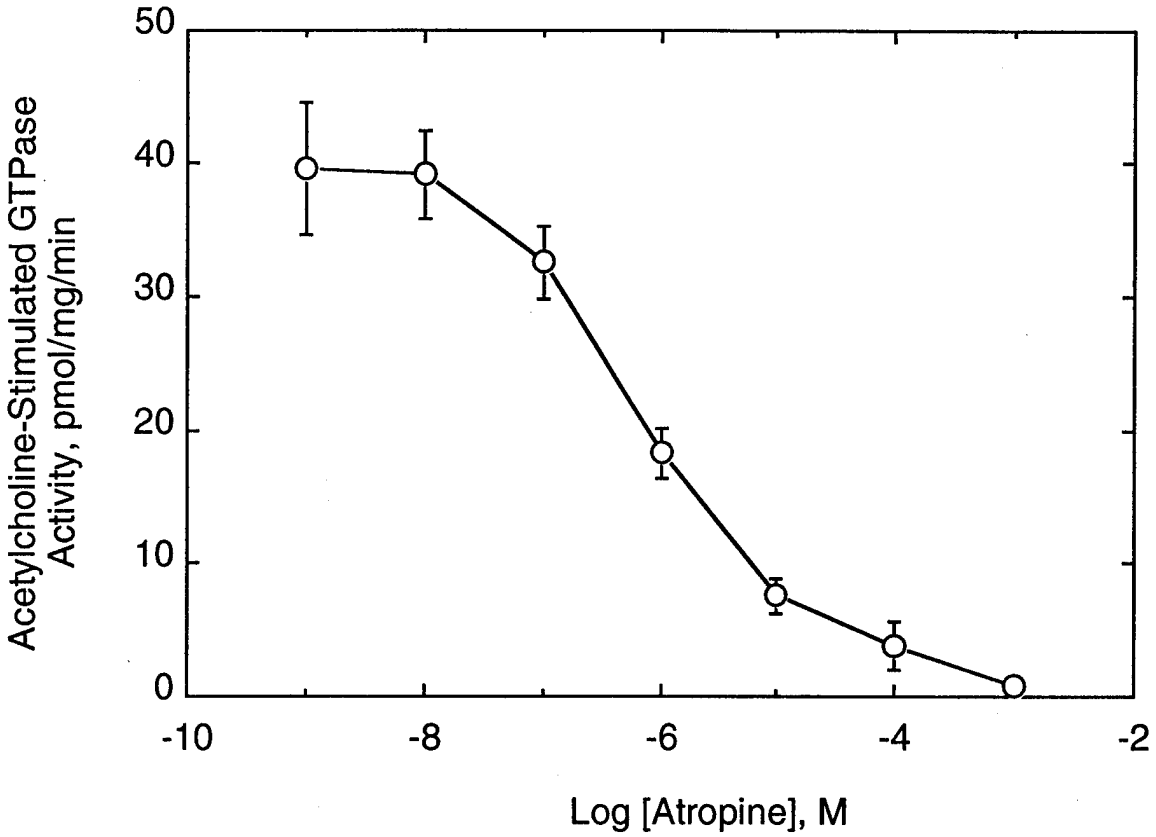

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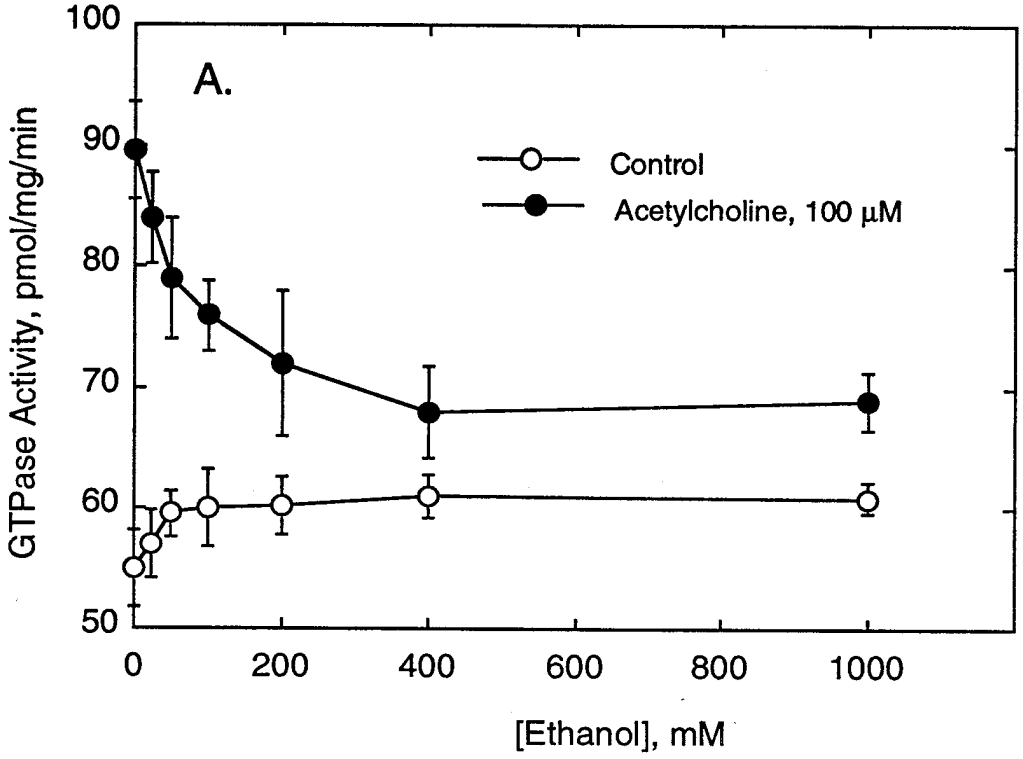

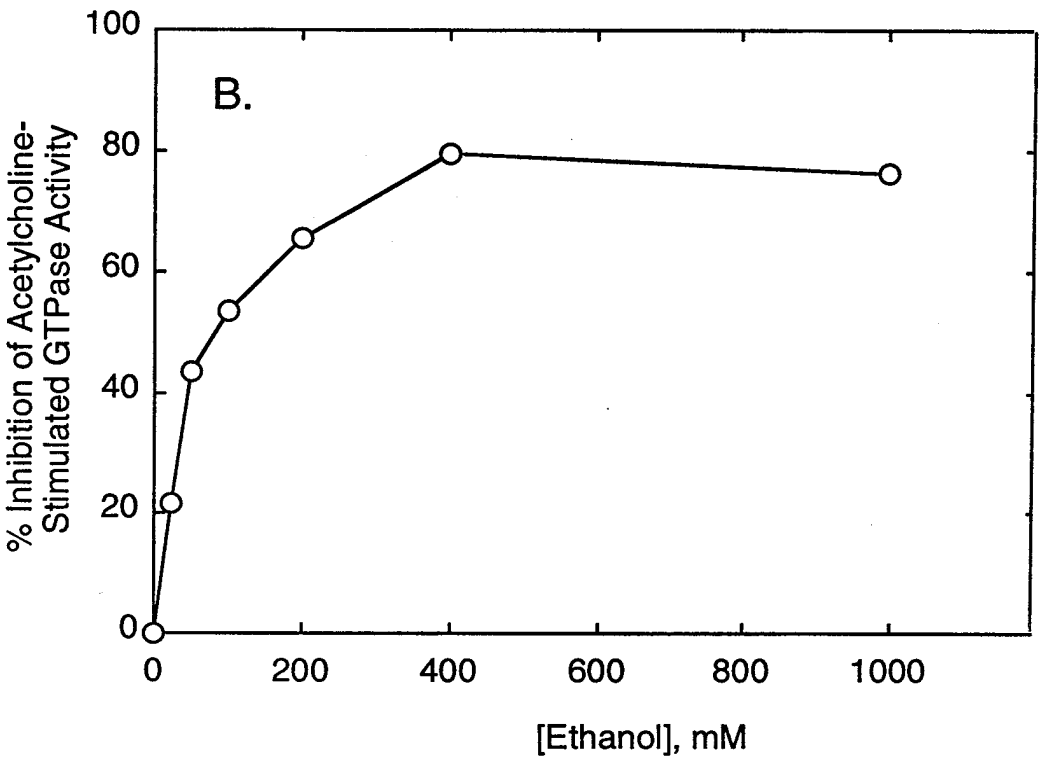

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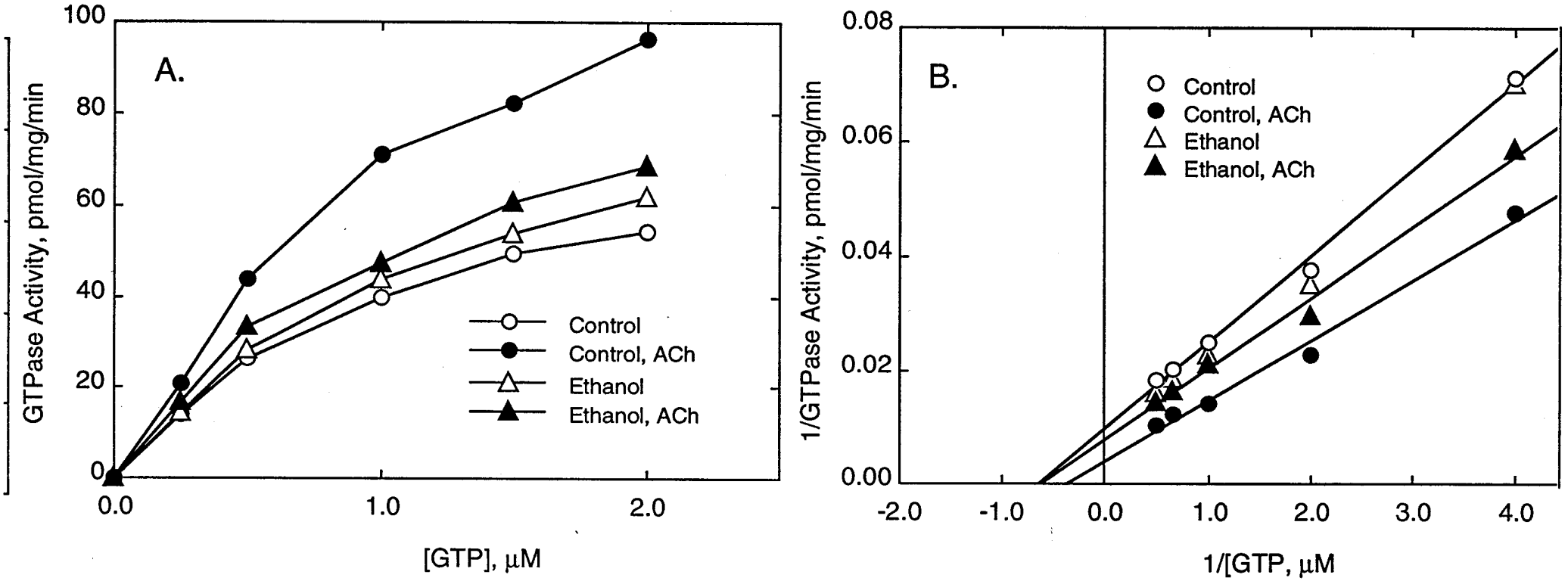

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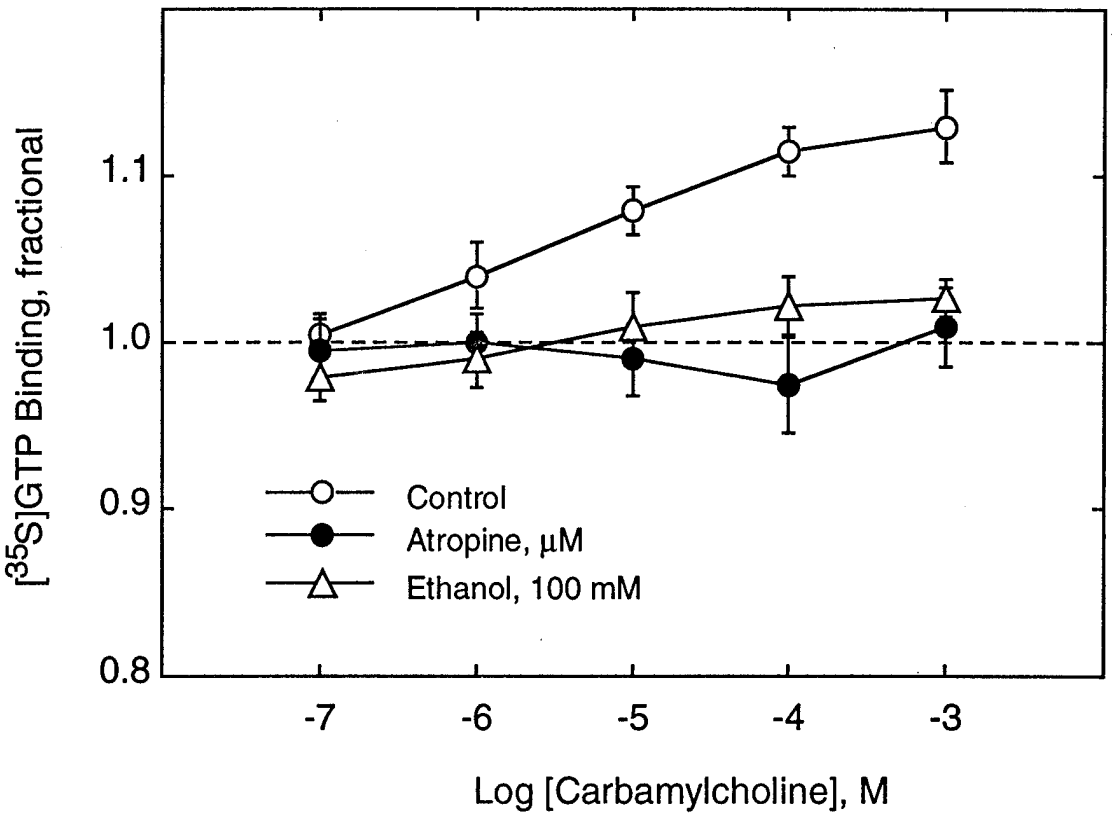

Figure 6. Aronstam et al.

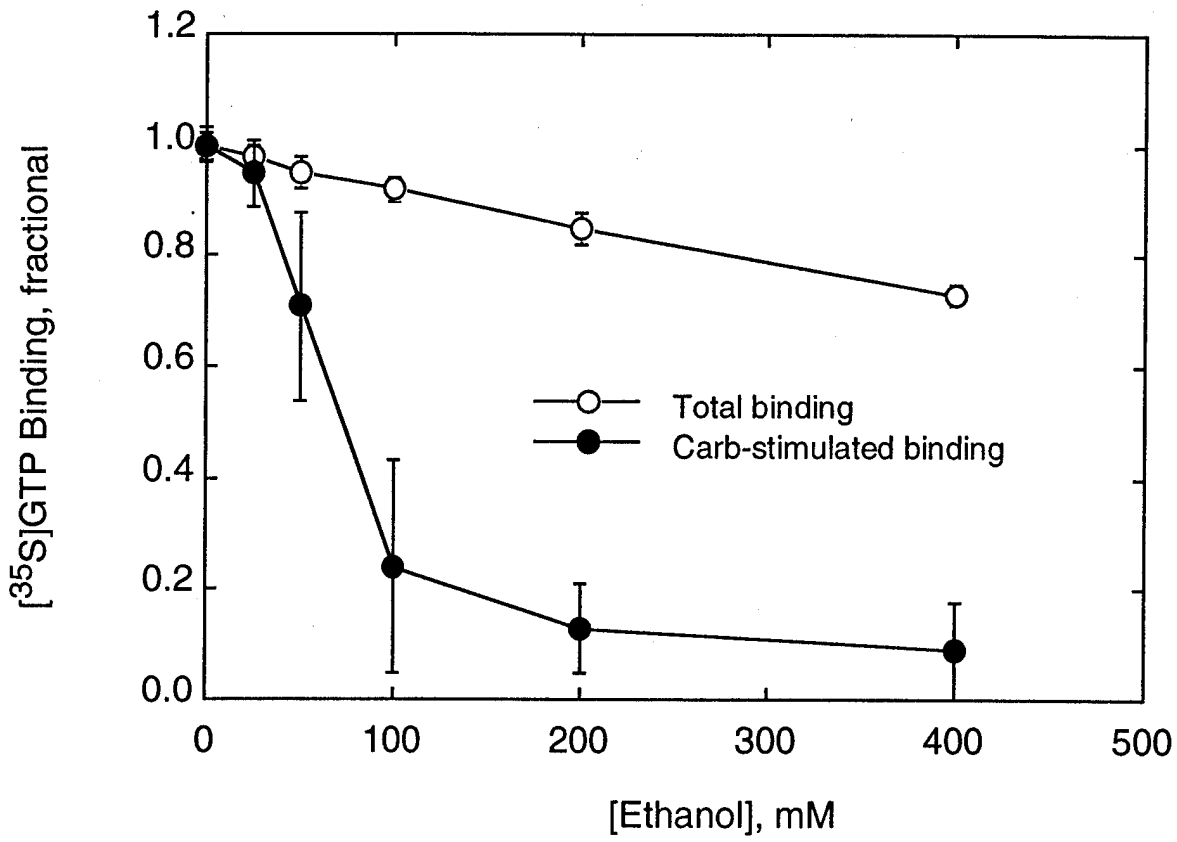

Figure 7. Aronstam et al.

Muscarinic Acetylcholine Receptors

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This work was supported by Department of the Army Grant Number DAMD17-94-J-4011. The content of this paper does not necessarily reflect the positionor policy of the U.S. government.

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Muscarinic acetylcholine receptors

Muscarinic receptors comprise one of the two classes of receptors for the neurotransmitter acetylcholine, nicotinic receptors comprising the other class. Muscarinic receptors are selectively activated by the alkaloid muscarine from the mushroom *Amanita muscaria,* and are blocked by belladonna alkaloids, such as atropine and scopolamine (Figure 1). Muscarinic receptors are widely involved in the transduction of cholinergic signals in the central nervous system, autonomic ganglia, smooth muscles, and other parasympathetic end organs.

The history of muscarinic systems is intimately associated with the development of receptor theory and the discovery of neurotransmitter transmission. Muscarinic receptors are members of the superfamily of G protein-coupled receptors. Muscarinic receptors are related to the ion channel-containing nicotinic acetylcholine receptors only insofar as their physiological agonist is acetylcholine; muscarinic and nicotinic receptors share little similarity in their structure, physiological functions and pharmacology (except for a few close analogues of acetylcholine itself).

The genes for 5 subtypes of muscarinic receptors, m1–m5, were identified, cloned and sequenced between 1986 and 1990. These receptor subtypes differ in their distribution, pharmacology and signal transduction pathways (Table 1). This heterogeneity raises the possibility of selectively affecting specific muscarinic functions in the brain and other organs; accordingly, the pharmacology of muscarinic receptor subtypes has been the subject of intensive investigation. Unfortunately, the selectivity of antagonists for receptor subtypes rarely exceeds 10 fold. Properties of the different subtypes are summarized in Table 1. A schematic diagram of the human m2 receptor is presented as Figure 2.

Receptor Structure. Muscarinic receptors are comprised of single polypeptides of 440-540 amino acids with an extracellular N-terminus and an intracellular C-terminus. Receptor genes contain a single exon. Hydropathic analysis of the protein sequence suggests 7 regions of 20-24 amino acids which are likely to form membrane spanning α-helical structures. The amino acid composition of the membrane-spanning regions is highly conserved (90% sequence similarity) among the 5 subtypes. Between the 5th and 6th membrane spanning units is a large intracellular loop that is highly variable in composition and size. Several consensus sites for phosphorylation are located on the 3rd intracellular loop, as well as on the C terminal chain. A disulfide bridge is formed between a conserved cysteine adjacent to the third transmembrane segment and a cysteine in the middle of the 2nd extracellular loop. Two to four consensus sites for N-glycosylation are present in the extracellular N-terminal domain, and 25% of the receptor mass may be contributed by these oligosaccharides. While this carbohydrate component probably plays a role in the processing and orientation of the receptor, it plays no discernible role in ligand binding or signal transduction processes.

Chimeric receptors and mutational analysis have revealed sites on the receptors proteins that are specifically involved in ligand binding and coupling to transducer G proteins. Acetylcholine binds to a site within a pocket formed by the circularly arranged transmembrane domains. An aspartate moiety in the third transmembrane region participates in an ionic interaction with the quaternary nitrogen of acetylcholine, while a series of tyrosine and threonine residues located in the membrane-spanning segments approximately 1/3 of the distance through the membrane probably form hydrogen bonds with muscarinic ligands. In agreement with classical pharmacological analyses, the antagonist binding site is thought to overlap the acetylcholine recognition site, but additionally involve adjacent hydrophobic areas of the receptor protein and membrane. Muscarinic receptors also possess a site for allosteric regulation by compounds, including gallamine (Figure 1), which decrease the dissociation of cholinergic ligands. Gallamine

binding appears to involve the sixth transmembrane domain as well as the third extracellular loop.

A number of regions have been identified that participate in the interactions of muscarinic receptors with transducer G proteins. In particular, the 2nd intracellular loop and the N and C terminal portions of the 3rd intracellular loop are involved in coupling to G proteins. Desensitization of muscarinic receptors probably entails phosphorylation of conserved threonine residues on the C terminal portion of the receptor, as well as other sites on the 3rd intracellular loop.

Functions. Muscarinic receptors subserve a number of physiological functions. Muscarinic receptors are present in autonomic ganglia and on the target organs of postganglionic parasympathetic fibers. Thus, muscarinic receptors mediate such parasympathetic effects as smooth muscle contraction, blood vessel dilation, decreases in heart rate and cardiac contractility, and glandular secretion.

In the central nervous system, cholinergic fibers arise in nuclei in the midbrain and pons to diffusely innervate muscarinic receptors on neurons in the thalamus and other diencephalic structures, as well as the reticular formation and other brainstem and cranial nerve nuclei. A second major projection system arises from basal forebrain cholinergic system (which includes the medial sepal nucleus, diagonal band nuclei, and nucleus basalis) and innervates muscarinic receptors on neurons throughout the telencephalon. Cholinergic interneurons innervating muscarinic receptors are located in several brain structures, including the cerebral cortex, nucleus accumbens and striatum. Muscarinic receptor density is particularly high in the striatum, cerebral cortex, hippocampus and other specific nuclei. Muscarinic receptor subtypes display striking regional and laminar heterogeneity (Table 1). Central muscarinic systems are prominently involved in arousal, attention, learning and memory. Other functions involving central muscarinic actions include movement, analgesia, and temperature regulation.

A subset of m2 and m4 receptors have a presynaptic localization and play a role in the control of neurotransmitter release; the other receptors are predominantly postsynaptic in location.

Signal Transduction. Muscarinic receptors alter the activity of receptive cells by a number of different signal transduction pathways. The biochemical pathways activated depend on the nature and quantity of the receptor subtype, transducer proteins, effector molecules, and protein kinase substrates expressed in the tissue, as well as the potential for cross talk between the various transduction pathways. The odd-numbered receptor subtypes, m1, m3 and m5, efficiently couple through pertussis toxin-insensitive G proteins of the Gq/G11 family to a stimulation of phospholipase C. Phospholipase C releases the second messengers diacylglycerol and inositol triphosphate from phosphatidylinositol. Diacylglycerol activates protein kinase C, while inositol triphosphate releases Ca^{2+} from intracellular stores. The even numbered receptors, m2 and m4, inhibit adenylate cyclase through actions involving Gi.

This simple classification has been extended by more recent demonstration of pathways involving additional transducer proteins, including the $\beta\gamma$ subunits of G proteins, as well as an appreciation of secondary effects and cross talk between the pathways. Other actions include 1) stimulation of adenylate cyclase mediated by the $\beta\gamma$ subunits of G proteins, 2) inhibition of phosphodiesterase, 3) stimulation of phospholipase A2 (m1, m3, m5), and 4) stimulation of phospholipase D (m1, m3). Muscarinc receptor stimulation activates a number of depolarizing and hyperpolarizing currents through both direct and indirect mechanisms. Prominent effects include 1) stimulation of inwardly rectifying potassium conductances by m2 and m4 receptors, 2) activation of calcium-dependent potassium, chloride and cation conductances by m1, m3 and m5 receptors, 3) inhibition of the M-current, a voltage- and time-dependent potassium conductance, by m1 and m3 receptors, and 4) inhibition of a calcium conductances by m2 and m4 receptors, directly via Go and indirectly via reductions in cAMP.

Table 1. Properties of muscarinc receptor subtypes.

Property	Subtype				
	m1	m2	m3	m4	m5
Molecular weight	51,240	51,715	66,127	53,058	60,186
amino acids	460	466	590	479	532
G protein coupling	Gq, G11	Gi, Go	Gq, G11	Gi, Go	Gq, G11
effectors	IP3/DAG	cAMP (-)	IP3/DAG	cAMP (-)	IP3/DAG
tissues	brain, autonomic ganglia, secretory glands, vas deferens, sympathetic ganglia	brain, heart, sympathetic ganglia, lung, ileum, uterus and other smooth muscles	brain, secretory glands, smooth muscles	brain, lung	
brain regions	cerebral cortex, hippocampus and dentate gyrus, striatum, olfactory bulb and tubercle, amygdala	cerebellum, medulla, pons, basal forebrain, olfactory bulb, diencephalon	cerebral cortex, thalamus, piriform cortex, olfactory bulb, brainstem nuclei	occipital cortex, caudate and putamen, visual nuclei, olfactory tubercle, hippocampus	
subtype-selective antagonists	pirenzepine telenzepine mamba venom-toxins	methoctramine AF-DX-116 gallamine, himbacine	4-DAMP hexahydro-sila-difenidol	tropicamide himbacine	

Molecular weights are calculated for the polypeptide contribution only.

Antagonist-selectivity of the subtypes is relative; antagonists generally differ in their interactions with the different subtypes by a factor of less than 20.

IP3/DAG: Stimulation of phospholipase C to release the second messenger inositol triphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol.

cAMP(-): Inhibition of adenylate cyclase, thereby lowering cellular content of the second messenger cAMP.

Only low levels of m5 receptor expression have been detected in tissues.

Further Reading

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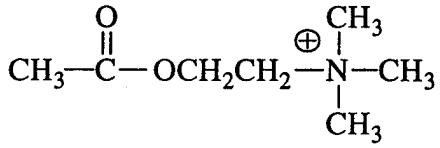
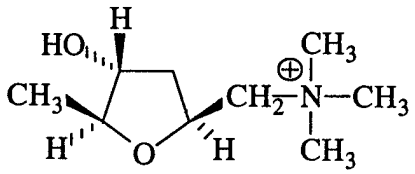
Figure Legend

Figure 1. Structures of muscarinic ligands. Acetylcholine is the physiological agonist; muscarine and atropine are the prototypical agonist and antagonist which define the receptor class; gallamine is an allosteric receptor antagonist.

Figure 2. Schematic diagram of a human m2 muscarinic receptor showing the primary amino acid sequence. Four consensus sites of for glycosylation at asparagine residues are shown on the extracellular N-terminal domain. The aspartate and tyrosine and threonine residues in the membrane-spanning regions that are thought to be involved in agonist binding are highlighted. Regions of the third intracellular loop involved in G protein recognition and coupling are indicated by shading. Consensus sites for threonine phosphorylation in the C terminal domain are indicated by highlighting.



Acetylcholine



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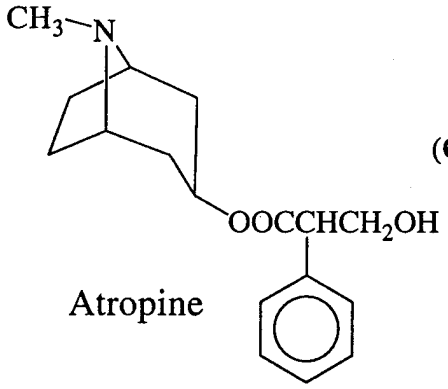
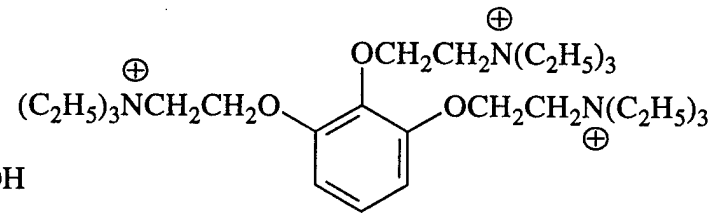


Atropine



Gallamine

Figure 1. Muscarinic Ligands

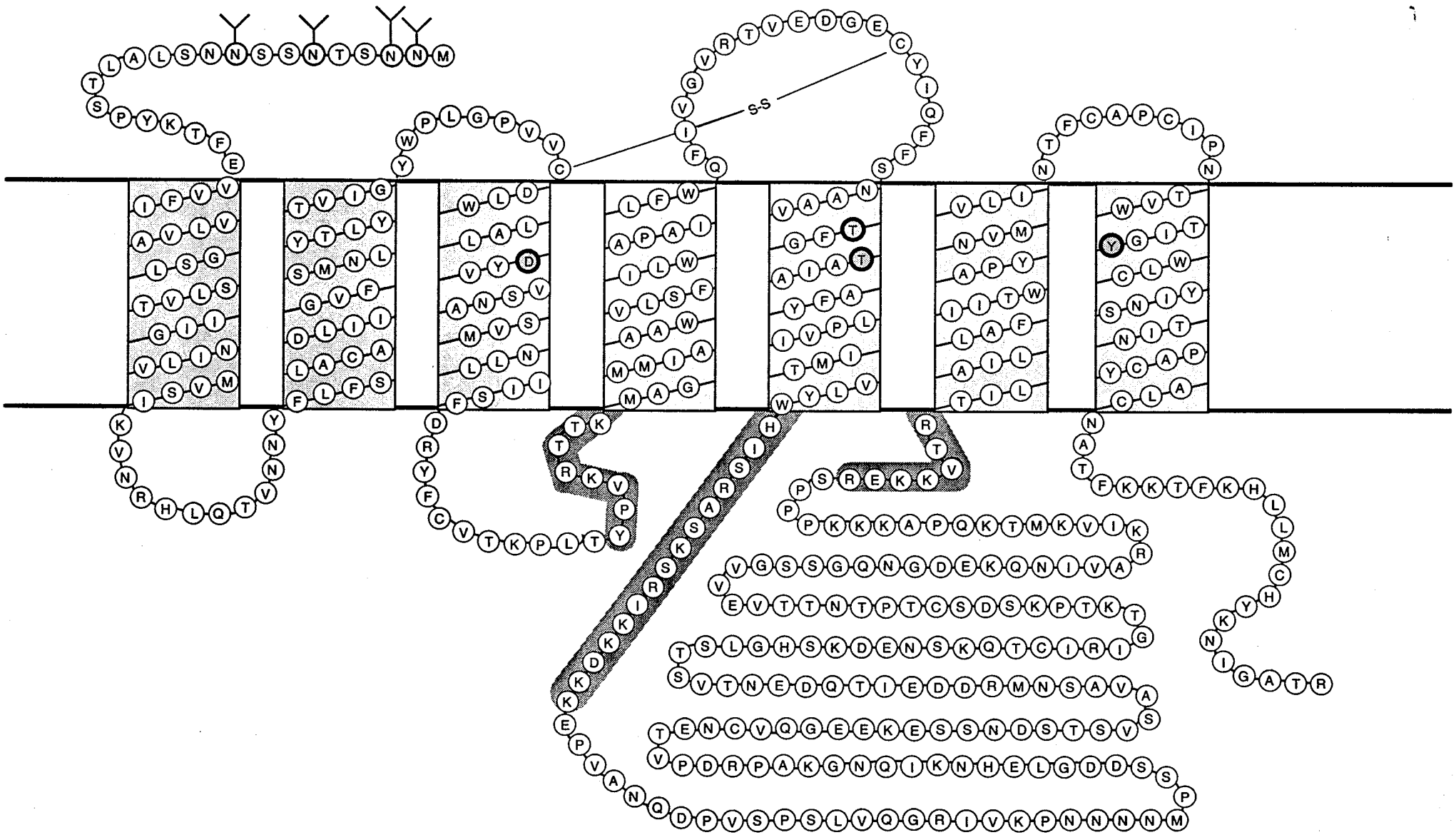


Figure 2. Human m2 Muscarinic Receptor

Reprinted from Diversity of Interacting Receptors
Volume 757 of the *Annals of the New York Academy of Sciences*
May 10, 1995

S-Nitrosylation of m2 Muscarinic Receptor Thiols Disrupts Receptor–G-Protein Coupling[a]

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Muscarinic acetylcholine receptors possess nine cysteine residues which are conserved among all five receptor subtypes: four are in membrane-spanning helices; one is in the cytoplasmic NH_2-terminal tail and is probably palmitoylated; two are in the third extracellular loop; and two cysteines located in the first and second extracellular loops participate in disulfide bond formation.[1,2] In addition, each receptor has a variable number (1–6) of cysteine residues in the large third intracellular loop. Although biochemical manipulation of these groups has little effect on [^{3}H]antagonist binding, it has been known for at least 10 years that the redox state of these sulfhydryl moieties influences the functional coupling of receptors to transducer G-proteins.[3,4] However, natural mediator(s) that may act on these groups to regulate receptor activity have not been identified. The work of Lipton and co-workers[5] raises the possibility that *S*-nitrosylation of reactive sulfhydryl centers may be a common pathway for molecular control of protein function. Nitric oxide (NO) group transfer (of NO^+) from nitric oxide depends on the redox chemistry intrinsic to the NO molecule.[5] Sodium nitroprusside (SNP) has a strong NO^+ character and nitrosylates protein thiols. In the present study, we examined the possibility that nitrosylation of sulfhydryl groups by NO^+ affects receptor–G-protein coupling.

Rat atrial membranes contain m2 muscarinic receptors whose binding of [^{3}H]*N*-methylscopolamine increases dramatically in the presence of Gpp(NH)p and after physical treatments (heat, low pH) that engender m2 dissociation from G-proteins.[4,6] Exposure of atrial m2 receptors to SNP (1–5 mM for 30 minutes at room temperature) produced a moderate (18–30%) increase in [^{3}H]MS binding (FIG. 1A). Gpp(NH)p (10 μM) increased the apparent density of muscarinic binding sites by 60% (FIG. 1B). However, marked stimulation of [^{3}H]MS binding by Gpp(NH)p was moderated by SNP (FIG. 1B). Fully two-thirds of the increase in binding engendered by Gpp(NH)p was eliminated by 5 mM SNP, so that [^{3}H]MS binding was reduced to a level lower than that measured after exposure to 5 mM SNP alone. Under redox conditions (i.e., exposure to ascorbate) which promoted $NO\cdot$, as opposed to NO^+, formation from SNP, m2 binding and activity were severely depressed or eliminated

[a]This work was supported by grants GM46408, NS25296, Department of the Army contract DAMD17-94-J-4011, and the Research Service of the Veterans Administration.
[c]Corresponding author.

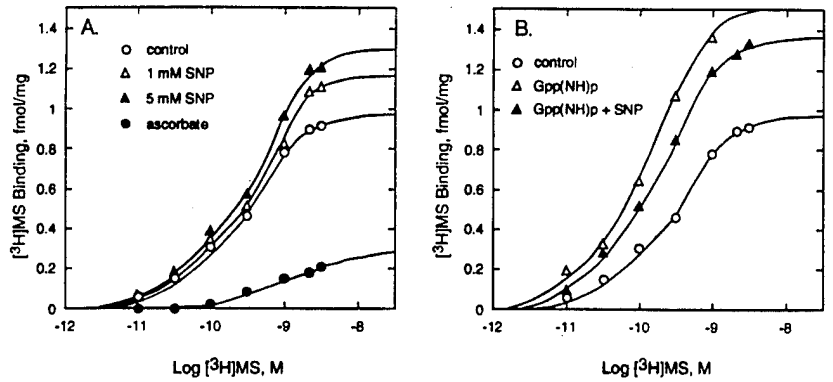

FIGURE 1. Influence of sodium nitroprusside (SNP) on the binding of [^{3}H]MS to atrial m2 receptors. Each point represents the mean from 3–6 experiments. (**A**) The binding of the indicated concentrations of [^{3}H]MS was measured in the absence of SNP (○) or after exposure to 1 (△) or 5 (▲) mM SNP. In one series of experiments, atrial membranes were treated with 1 mM ascorbate for 10 min at room temperature before being exposed to 1 mM SNP (●). Nonlinear regression analyses using a single receptor population model indicated no change in [^{3}H]MS affinity but increases in apparent receptor density from 0.99 ± 0.07 to 1.18 ± 0.12 and 1.30 ± 0.16 fmol/mg protein after exposure to 1 and 5 mM SNP, respectively. (**B**) The binding of [^{3}H]MS was measured in the absence of modulating agents (○) or in the presence of 10 μM Gpp(NH)p (△) or 10 μM Gpp(NH)p after exposure to 1 mM SNP (▲). Nonlinear regression analyses using a single receptor population model indicated an increase in receptor density in the presence of Gpp(NH)p (from 0.99 ± 0.07 to 1.57 ± 0.05 fmol/mg protein) with no change in [^{3}H]MS binding affinity. The apparent receptor density in the presence of Gpp(NH)p was reduced to 1.39 ± 0.11 and 1.19 ± 0.08 after exposure to 1 (▲) and 5 (not shown) mM SNP, respectively.

(FIG. 1A). Exposure to ascorbate alone did not alter [^{3}H]MS binding. Sodium nitroprusside (5 mM) decreased surface and total thiol content by 27% with an IC_{10} of 1.0 ± 0.2 mM (n = 3) (FIG. 2). These effects of SNP were not observed under conditions (i.e., heat and low pH) in which receptor–G-protein coupling was eliminated. Thus, *S*-nitrosylation appears to uncouple m2 receptors from transducer

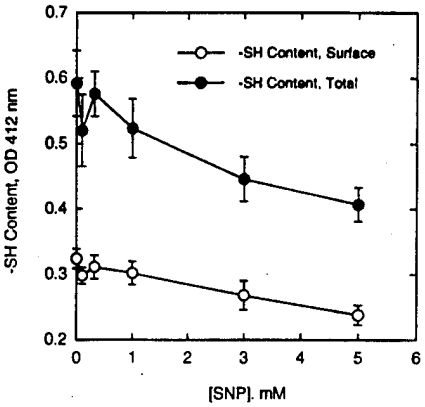

FIGURE 2. Influence of sodium nitroprusside (SNP) on the sulfhydryl content of atrial membranes. Sulfhydryl content was measured after a 10-min exposure to SNP in undisrupted membranes (○) or in SDS-solubilized (●) membranes. Measurements were performed spectroscopically (412 nm) using 5,5′-dithio-*bis*-2-nitrobenzoate as a specific probe.

G-proteins. It is interesting that SNP effects were not observed in cultured human neuroblastoma cells (Sk-N-SH), which predominantly express m3 muscarinic receptors. Preliminary experiments also indicate that treatment with 5 mM SNP disrupts m2 receptor control of G-protein GTPase activity, although the nature of this effect (i.e., a receptor, G-protein, or coupling effect) is not yet clear.

These findings suggest a mechanism for modulation of muscarinic receptor function that depends on both the redox milieu and nitric oxide production. Although the identity of the relevant reactive sulfhydryl center awaits confirmation by mutational analysis, the fact that m2 but not m3 receptors are selectively affected might indicate involvement of the cysteine moiety on the second intracellular loop, which is only present in m2 and m4 receptors. This work supports the suggestion by Lipton and co-workers[5] that nitrosylation of reactive sulfhydryl groups is a common biochemical mechanism for the control of protein function.

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