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HUMAN ERYTHROCYTE AS A MODEL FOR INVESTIGATING MUSCARINIC AGONISTS AND ANTAGONISTS

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Abstract—1. Human erythrocyte muscarinic receptor is further classified as M1 subtype by its binding of [^{3}H]pirenzepine (a specific M1 antagonist). Conversely, binding of [^{3}H]piperidinyl AF-DX116 (a specific M2 antagonist) is not detected.

2. There are high correlations between the binding efficacies of 25 known or putative cholinergic agents using either human erythrocyte membrane or mouse caudate homogenate. Also the *in vitro* data corresponds satisfactorily with *in vivo* data measuring the protective effect of these compounds against organophosphate poisoning.

3. The human erythrocyte membrane is an efficient model for investigating cholinergic agonists and antagonists.

INTRODUCTION

Cholinergic muscarinic receptors have been identified in the brain (Schiller, 1979), neuromuscular junctions (Yamamura and Snyder, 1974), pylorus (Gaginella *et al.*, 1980), cardiac aorta (Peterson and Schimerlik, 1982) and other tissues/organs by direct binding assays of radiolabeled cholinergic agonists or antagonists. It is well recognized that the cation channel, the anesthetic site, is coupled to cholinergic receptors (Aguilar *et al.*, 1980). The cholinergic muscarinic agonists either stimulate cGMP production or inhibit cAMP production (Greengard, 1975). Recently, numerous reports have alluded to the existence of four subtypes of muscarinic receptor that can be distinguished by their binding affinities and their amino acid sequences (Hirschowitz *et al.*, 1984; Giachetti *et al.*, 1986; Hammer *et al.*, 1986; Kubo *et al.*, 1986; Nathanson, 1987; Peralta *et al.*, 1987; Mutschler *et al.*, 1988; Rodrigues De Miranda, 1988). It has also been suggested that there are functional differences between the subtypes, M1 and M2. The M1 subtype is considered to be responsible for regulating receptor mediated cGMP production (Sokolovsky and Cohen-Armon, 1988), whereas the M2 subtype appears to have a role in facilitating secretion in exocrine glands (Giachetti and Micheletti, 1988).

The brain is known to possess a wide variety of neurotransmitter receptors. Both nicotinic and the muscarinic cholinergic receptors (together with their respective subtypes) have been identified in different regions of the brain (Rotter *et al.*, 1979; Dolly and Barnard, 1984; Sokolovsky, 1984). We have demonstrated the presence of a muscarinic receptor on the human erythrocyte membrane (Tang, 1986). This muscarinic receptor appears to regulate calcium entry into these cells via the ion channel. Calcium entry, in turn, stimulates guanylate cyclase activity (Tang *et al.*, 1981, 1984). In our earlier studies (Tang, 1986), the nicotinic receptor was not detected on the erythrocyte by direct binding assays using radiolabeled α-bungarotoxin, a nicotinic antagonist. Scatchard analysis of [^{3}H]QNB binding to the erythrocyte ghost yields a single component suggesting the presence of only one subtype of muscarinic receptor (Tang *et al.*, 1984). In order to further define the erythrocyte cholinergic receptors and to establish that the erythrocyte membrane provides an excellent model system for investigating cholinergic agonists and antagonists, we will perform radiolabeled binding assays and study over 25 known or newly synthesized putative cholinergic muscarinic agonists and antagonists. We will employ both human erythrocyte ghosts and mouse caudate nucleus homogenates as the sources of muscarinic receptors. We will attempt to correlate the receptor binding data with the results of *in vivo* cholinesterase inhibitor toxicity studies designed to assess the anticholinergic effects by studying protective effects in the presence of a cholinesterase reactivator. Our current studies can provide additional evidence that the muscarinic receptors on the surface of the erythrocyte are of the M1-subtype and are similar to their counterparts in the brain. These systems will provide a simple and effective method for determining the efficacies of newly synthesized chemicals against cholinergic toxicity.

Abbreviations QNB, quinuclidinyl benzilate; 2-PAM, pralidoxime chloride; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; EDTA, ethylene diamine tetraacetic acid; LD_{50}, lethal dose; EC_{50}, effective concentration; IC_{50}, inhibition concentration; RBC, red blood cell

MATERIALS AND METHODS

Male, 6–8 week old mice weighing approx. 25 g were used for all *in vivo* and *in vitro* experiments. Blood was freshly

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drawn from healthy male human (non-smoker) volunteers and collected in heparinized tubes for erythrocyte membrane studies. The protein concentrations for the brain or erythrocyte membrane samples were determined according to the procedure of Lowry *et al.* (1951).

In vitro studies

Preparation of erythrocyte membrane (ghost). Erythrocyte ghost were prepared as described earlier (Tang *et al.*, 1984; Tang, 1986). Briefly, fresh male human blood was centrifuged at 4°C for 20 min at 800 *g* to separate the red blood cell (RBC) from the plasma, white blood cells and platelets. The packed RBC were washed 3 times with ice-cold 10 mM sodium phosphate buffer (containing 140 mM NaCl), pH 7.4. These cells were then lysed to remove the hemoglobin by washing with 10 × vol of ice-cold 10 mM sodium phosphate buffer (without NaCl), pH 7.4, followed by centrifugation at 20,000 *g* for 40 min at 4°C. The washing and centrifugation were repeated at least 4 times or until the sediment (the RBC membrane) became colorless. It is critical that the erythrocyte membrane suspension contain no hemoglobin. Hemoglobin interferes with the binding of the radiolabeled cholinergic ligands.

Preparation of caudate nucleus homogenates. Mice were killed by decapitation. The brains were removed within 30 sec after decapitation and placed on an ice-cooled watch glass. The roof of the lateral ventricles was removed. Both caudate nuclei were dissected and homogenized in ice-cold 50 mM Tris buffer, pH 7.4 in a glass homogenizer. The homogenates were kept at 4°C and until they were employed for radiolabeled ligand binding assays (no longer than 3 days).

Muscarinic receptor binding assay. RBC membranes were suspended in 20 mM sodium phosphate buffer, pH 7.4 with 0.1 mM ethyline diamine tetraacetic acid (EDTA) in 1:4 of the original whole red blood cell volume or approx. 1 mg protein/ml.

The caudate nucleus homogenates were suspended in 50 mM Tris–HCl buffer, pH 7.4 at approx. 1 mg protein/ml.

[^{3}H]QNB (muscarinic antagonists) binding assay. 200 µl aliquots of either the RBC membrane suspension or the caudate nucleus homogenates were placed in disposable 10 mm diameter 4 × 12 cm glass tubes and incubated with 1×10^{-4} M [^{3}H]QNB with or without addition of atropine, or with one of the chemicals listed in Table 1, at room temperature for 40 min. At least 5 different concentrations of each compound (ranging from 1×10^{-15} to 10^{-4} M) were employed in studies to determine the EC_{50}. The total volume of each incubation was 1.5 ml. The binding was terminated by the addition of ice-cold 20 mM sodium phosphate or 50 mM Tris HCl buffer and filtering the suspension through a Whatman GF/B glass filter under reduced pressure. The filter was then washed with at least 15 ml of ice-cold sodium phosphate buffer, pH 7.4 containing 0.1 mM EDTA or 50 mM Tris–HCl buffer, pH 7.4 to get rid of the unbound radiolabeled QNB. Specific binding was determined by blocking the [^{3}H]QNB binding with atropine or pirenzepine (1×10^{-4} M). For evaluating the binding affinity of the compounds tested, the compounds were used to inhibit the [^{3}H]QNB binding instead of atropine. All assays were performed in triplicate.

The compounds investigated are listed in Table 1. Various concentrations of each chemical were used, ranging from 1×10^{-15} to 1×10^{-4} M. The compounds were either obtained from commercial sources or synthesized by various laboratories as listed in Table 1.

[^{3}H]pirenzepine (a specific M1 antagonist) and [^{3}H]piperidinyl AF-DX116 (a specific M2 antagonist) binding assays. 200 µl aliquots of the RBC suspension were used for the binding assays. The method is basically the same as for the [^{3}H]QNB binding except the radiolabeled ligand was [^{3}H]pirenzepine or [^{3}H]piperidinyl AF-DX116 instead of [^{3}H]QNB. The specific binding was determined by adding either unlabeled atropine, pirenzepine or gelamine (1×10^{-8} M).

In vivo toxicity studies

The *in vivo* studies were performed at the Institute of Chemical Defense. The LD_{50} of all the compounds investigated were obtained using Swiss albino ICR male mice.

The animals were injected with 2 LD_{50} of soman (a cholinesterase inhibitor) and a standard dose (25 mg/kg) of pralidoxime chloride 2-PAM (a cholinesterase reactivator) in conjunction with 11.2 mg/kg of atropine, a muscarinic antagonist, or with different dosages of the compounds listed in Table 1, and observed for 24 hr. The dosages of each compound evaluated were 1/8, 1/16, or 1/32 or its LD_{50}. The survival rate of at least 6 animals was recorded for each dose of each compound tested. From these data the protection of the muscarinic receptor against cholinesterase inhibitors intoxication (the antimuscarinic effect) was determined for these compounds by comparisons with the results obtained using the standard treatment for soman: that is, 2-PAM and atropine.

Statistical analysis

Correlation coefficient for the binding affinities of the muscarinic cholinergic receptors using erythrocyte membrane or caudate nucleus and the protective index against chemical poisoning were computed using probit analysis and linear regression analysis. The EC_{50} or the IC_{50} of the chemicals investigated were determined by probit analysis.

RESULTS

Table 1 is a list of the compounds, including their chemical structures, that have been tested for their antimuscarinic effects using both erythrocyte membrane and mouse caudate nucleus homogenates (i.e. *in vitro* assays) and in their protective effects against soman organophosphate toxicity *in vivo*. The results of the binding affinities of the compounds studied are presented as EC_{50} or IC_{50} in Table 2.

The correlation between the ranking of the binding affinities using erythrocyte membrane and the ranking of binding affinities using caudate nucleus is shown in Fig. 1. The 14 compounds that show effects in all three types of assay systems, i.e. radiolabeled binding assays using erythrocyte membrane or caudate nucleus homogenates and the *in vivo* toxicity studies were ranked in order of 1 to 14 according to their efficacies (1 being the strongest and 14 the weakest). In Fig. 1, the binding affinities of the chemicals in the experiments using the caudate homogenates or the RBC membrane were correlated according to their rank orders. A satisfactory correlation coefficient of 0.85 was obtained. This high correlation between the results when using either caudate homogenates or erythrocyte membrane suspensions establish that the RBC membrane can be used instead of brain tissue to test compounds for antimuscarinic activity.

Figure 2 denotes the correlation between the *in vitro* binding efficacies of the experiments using caudate homogenates and the *in vivo* data collected from the studies on the protective effects of the 14 compounds according to their rank order. The correlation coefficient of the *in vivo* and the *in vitro* data on toxicities and binding affinities, of these compounds when analyzed in the same manner as the *in vitro* data is 0.94 (Fig. 2).

Table 1. Chemical structures of known and putative muscarinic agonists and antagonists. Compounds Nos 1–13 and 22–24 were synthesized by Franklin Research Center and Nos 14–21 were synthesized by Stanford Research Institute. Compound No. 25 was purchased from Sigma.

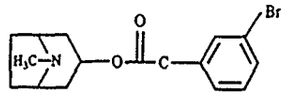
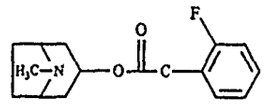
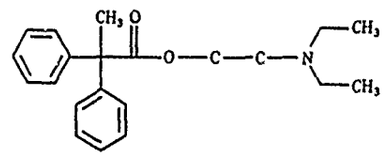
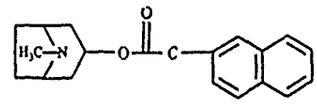
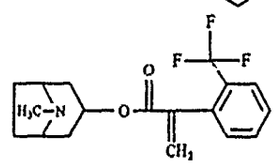
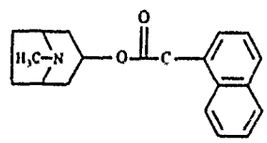
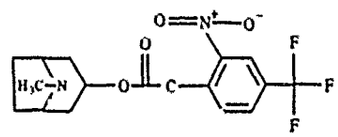
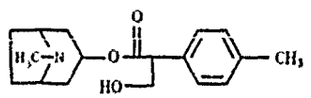
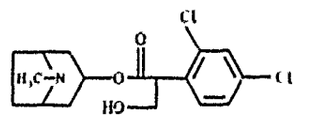
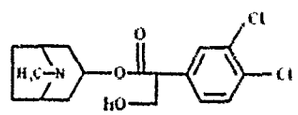
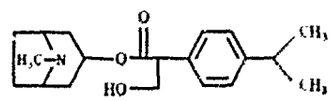
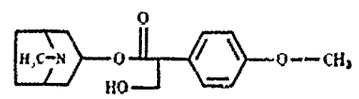
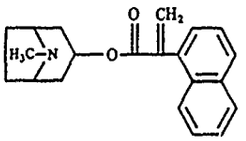
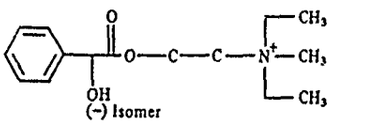
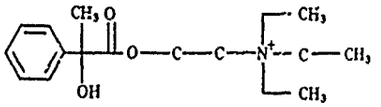
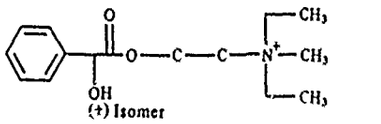
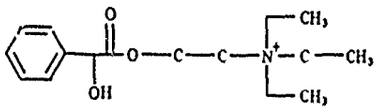
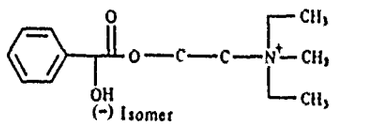
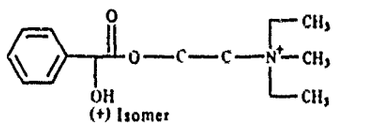
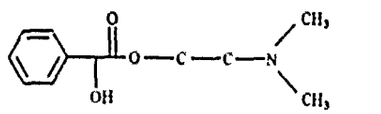
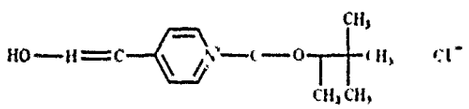
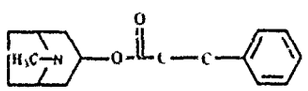
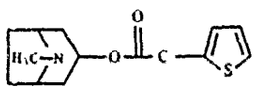
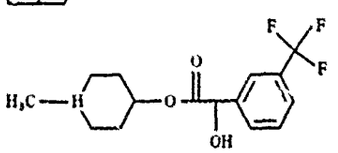
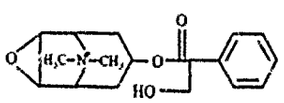
1		HCl
2		HCl
3		HCl
4		HCl
5		HCl
6		HCl
7		HCl
8		HCl H_2O
9		H_2SO_4 H_2O
10		H_2O HCl
11		HCl
12		HCl H_2O
13		HCl
14		I^- (−) Isomer
15		I^-
16		I^- (+) Isomer
17		I^-
18		I^- (−) Isomer
19		I^- (+) Isomer
20		HCl
21		Cl^-
22		HCl
23		HCl
24		HCl
25		Br^-

Table 2. IC_{50} or EC_{50} of the 25 compounds listed in Table 1 were determined using caudate homogenates or RBC membrane

Chemical No.	EC_{50} or IC_{50} Caudate homogenates	RBC membrane
1	0	0
2	5×10^{-6} M	6×10^{-6} M
3	1×10^{-6} M	3×10^{-6} M
4	0	0
5	2×10^{-7} M	5×10^{-7} M
6	2×10^{-7} M	7×10^{-7} M
7	5×10^{-7} M	1×10^{-7} M
8	2×10^{-8} M	1×10^{-8} M
9	3×10^{-7} M	9×10^{-7} M
10	2×10^{-6} M	1×10^{-6} M
11	1×10^{-7} M	5×10^{-8} M
12	8×10^{-6} M	8×10^{-6} M
13	N	5×10^{-6} M
14	0	0
15	0	0
16	5×10^{-6} M	0
17	5×10^{-8} M	1×10^{-7} M
18	7×10^{-8} M	3×10^{-7} M
19	N	1×10^{-7} M
20	4×10^{-7} M	7×10^{-7} M
21	0	0
22	1×10^{-5} M	1×10^{-5} M
23	0	0
24	8×10^{-7} M	8×10^{-8} M
25	1×10^{-9} M	1×10^{-9} M

0 denotes no effect and N not tested.

The results obtained from the *in vivo* and *in vitro* assay appear to correlate exceedingly well as seen in Table 3. The compounds that possessed anticholinergic muscarinic properties were marked positive in the table. Chemicals that inhibit the binding of [^{3}H]QNB to an erythrocyte membrane of the caudate nucleus homogenates at the concentration of 1×10^{-4} M or smaller are considered and given the term "positive", whereas those that have no effect at the level of 1×10^{-4} M on the binding of the radioactive ligand are considered as "negative". Table 3 demonstrates that the results from the *in vitro* experiments using either erythrocyte membrane or the mouse caudate nucleus homogenates agree with those obtained in the *in vivo* studies.

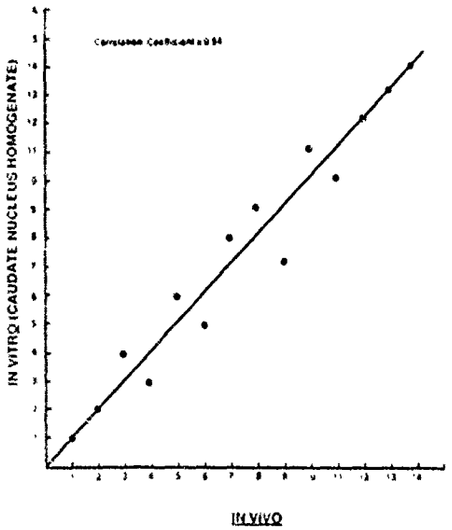


Fig 1 The positive results of binding efficacies of the chemicals using caudate homogenates plotted against the *in vivo* (protecting effect against organosphosphate poisoning) according to their rank order.

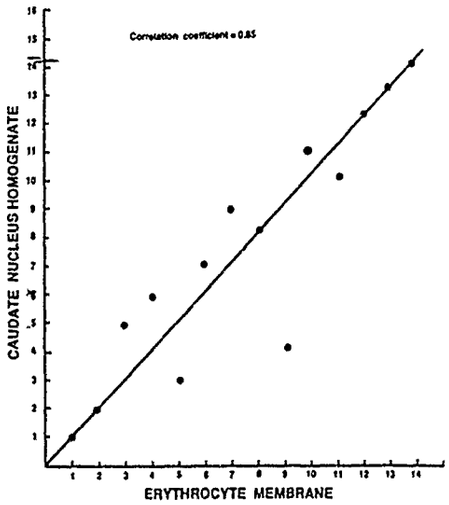


Fig. 2. The positive results and correlation of the 2 *in vitro* tests (the binding efficacies of the chemicals using caudate homogenates and erythrocyte membrane as samples) according to their rank order.

The data obtained from the experiments using [^{3}H]pirenzepine to evaluate the binding efficacy of M1 subtype antagonist to the erythrocyte muscarinic receptors are not significantly different from the binding affinities of [^{3}H]QNB (Tang, 1986) those erythrocyte receptors. Also, the experiments on determining the specific binding of both [^{3}H]QNB or [^{3}H]pirenzepine by applying either atropine or non-radiolabeled pirenzepine yielded similar results. No detectable binding was observed when using the

Table 3 The summary of the results of all 3 tests *in vivo* (protecting effect against organophosphate poisoning) and *in vitro* (efficacies in binding competition using erythrocyte membrane or caudate homogenates)

	In vivo	*In vitro* Erythrocyte	*In vitro* Brain
1	–	–	–
2	+	+	+
3	+	+	+
4	–	+	+
5	+	+	+
6	–	+	+
7	–	+	+
8	+	+	+
9	+	+	+
10	+	+	+
11	+	+	+
12	+	+	+
13	+	+	N
14	–	–	–
15	–	–	–
16	+	–	+
17	+	+	+
18	+	+	+
19	+	+	N
20	+	+	+
21	–	–	–
22	+	+	+
23	–	–	–
24	+	+	+
25	+	+	+

+ Means positive effect, – represents no effect and N means not determined.

radiolabeled [^{3}H]piperidinyl AF-DX116 for the study of M2 subtype muscarinic receptor on the RBC ghost.

DISCUSSION

In this study chemicals that are considered positive exhibit cholinergic activity either as agonists or antagonists. They either inhibit with or displace the radiolabeled QNB from the membrane of RBC and caudate nucleus homogenates. The results on the experiments of direct radiolabeled binding assays of all the compounds studied agreed except compound 16 yielded positive results only in those experiments using caudate homogenates but not with the RBC membrane. We postulate that this compound may have effect on other subtypes of muscarinic receptors than those that exist on the RBC. the M1 subtype. The *in vivo* data also appear to be in accord with those of the *in vitro* findings. Compounds numbered 4, 6 and 7 show negative results in the *in vivo* experiments which differ from those obtained from both using RBC and caudate homogenates. We have considered the possibility that these compounds may not be able to cross the gut and therefore are not being absorbed. Alternatively, these compounds may not bind to the M1 subtype on the RBC or they do not cross the blood brain barrier.

The data obtained by using [^{3}H]pirenzepine instead of [^{3}H]QNB for binding efficacy using erythrocyte membrane and applying pirenzepine or atropine to determine the specific binding affinities are similar. The M2 muscarinic subtype is not manifest on the erythrocyte membrane since binding of [^{3}H]piperidinyl AF-DX116 (a specific M2 subtype antagonist) is not observed. Thus these experiments demonstrate the muscarinic receptors on the RBC membrane are exclusively of one subtype, namely the M1.

In all different areas of the brain both nicotinic and muscarinic receptors are found. Also, the receptors in the brain are of mixed subtypes. There have been difficulties in acquiring a good source of pure muscarinic receptor in brain or other tissues/organs. It is unique that the muscarinic receptors on the RBC membrane are only of one cholinergic muscarinic subtype, M1. This provides a convenient model for differentiating the functions and characteristics of the various subtypes of muscarinic receptors. The results presented in this study further validate that the RBC membrane is an ideal model for investigating potential muscarinic agonists and antagonists.

SUMMARY

Human erythrocyte muscarinic receptor is classified as an M1 subtype by its specific binding of the radiolabeled M1 antagonist, pirenzepine. That the M1 subtype is the only type of cholinergic receptor on the RBC membrane is demonstrated by the similar results obtained from the experiments using atropine (an antagonist for all subtypes of muscarinic receptors) and from using non-radioactive pirenzepine for evaluating binding specificities. The M2 subtype muscarinic receptor is not detected on the erythrocyte membrane by direct binding assay using radiolabeled [^{3}H]piperdinyl AF-DX 116, an M2 specific antagonist. Human erythrocyte muscarinic receptors appear to be similar in pharmacological responses to those existing in the brain. The responses of the human erythrocyte membrane receptor to 25 known and putative cholinergic agonists and antagonists resemble the responses of the mouse caudate nucleus homogenates to these same compounds. An excellent correlation is also observed between the binding affinities of the muscarinic receptors for the various chemicals and the degree of protection in mice against organophosphate poisoning. These results indicate that the human erythrocyte membrane can serve as a model system for investigating the structure and function of cholinergic agonists and antagonists.

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