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Irreversible Binding of Acetylethylcholine Mustard to Cardiac Cholinergic Muscarinic Receptors

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SUMMARY

The interaction of acetylethylcholine mustard (Aech-M) with cardiac muscarinic receptors was studied with radioligand binding techniques and with isolated beating atria. Aech-M and acetylcholine competed about equally for (−)-[^{3}H]quinuclidinyl benzilate (QNB)-binding sites and 5′-guanylylimidodiphosphate reduced the ability of both compounds to compete by 30-fold. Pretreatment of cardiac membranes with 0.25 μM Aech-M, followed by washing, reduced the [^{3}H]QNB binding capacity by 60% without changing the ligand affinity of the receptors left. In the same membranes, a similar fraction of receptors was lost as measured by [^{3}H]*N*-methyoscopolamine, but a greater fraction was lost when measured by [^{3}H]oxotremorine-M. The loss of [^{3}H]QNB binding capacity was dose, time, and temperature dependent, blocked by atropine and carbachol, and modulated by several guanine nucleotides but not affected by membrane pretreatment with several group-selective reagents. Superfusion of spontaneously beating atria with Aech-M (100 μM) initially reduced the beating rate which returned to control values by 17 min. Atropine blocked the initial reduction in beating rate. In contrast, both carbachol and acetylcholine produced sustained decreases in the beating rate. After pretreatment of atria for 30 min with 100 μM Aech-M alone or 10 μM Aech-M plus 10 μM edrophonium followed by washing, the EC_{50} value for carbachol inhibition of the beating rate was increased 15.8- and 10.3-fold, respectively, with no change in the ability of isoproterenol to increase the spontaneous beating rate. In addition, there was a 46–47% and a 37–41% reduction in the binding capacity of both [^{3}H]QNB and [^{3}H]oxotremorinel-M in the 100 and 10 μM Aech-M-pretreated atria, respectively. The data indicated that Aech-M is a muscarinic agonist which appears to irreversibly bind to the muscarinic receptor.

The interaction of agonists and antagonists with the muscarinic cholinergic receptor has been extensively studied in the past several years. In addition to initiating biochemical and physiologic responses (1, 2), agonist binding to the muscarinic receptor is modulated in some instances by guanine nucleotides (3–5), divalent cations (5, 6) and NEM (7). In contrast, antagonist binding either is not affected, is slightly affected, or shows effects opposite to that for agonists by the above modulators (3–8).

Irreversible muscarinic antagonists have been shown to be useful probes for the muscarinic receptor. They have been used to determine the concentration of receptors in some tissues (9), to study the relationship between the receptor concentration and tissue response (10), and to determine the binding subunit of the receptor by gel electrophoresis (11). In light of the differences between the binding of agonists and antagonists to the muscarinic receptor, it would be of interest to determine the effects of muscarinic agonists which bind irreversibly to the muscarinic receptor.

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In 1947, Hanby and Raydon (12) reported on the synthesis of ACh mustard which was subsequently shown to be a potent muscarinic agonist (13, 14). In several early studies using the contractile response of isolated tissues, little or no irreversible binding of ACh mustard was found (13, 14). However, Robinson *et al.* (15) reported that ACh mustard did bind irreversibly to the muscarinic receptor of intact longitudinal muscle strips from the guinea pig intestine by measuring the uptake of [^{3}H] propylbenzilylcholine mustard in intact tissue.

Recently, ethylcholine mustard aziridinium ion (AF-64A) has been used to irreversibly inactivate the presynaptic high affinity choline transport system (16). In the present study, a detailed examination on the interaction of the parent compound of AF-64A, Aech-M, with cardiac muscarinic receptors was undertaken. It was found that this compound is a muscarinic agonist that irreversibly bound to the receptor.

Experimental Procedures

Materials. (−)-[^{3}H]QNB (30.2 Ci/mmol), [^{3}H]Oxo-M (82 Ci/mmol), and [^{3}H]NMS (85 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA), Carbachol, ACh iodide, eserine, *N*-ace-

ABBREVIATIONS: NEM, *N*-ethylmaleimide; QNB, quinuclidinyl benzilate; Oxo-M, oxotremorine-M; NMS, *N*-methylscopolamine; ACh, acetylcholine; Aech-M, acetylethylcholine mustard; Gpp(NH)p, 5′-guanylylimidodiphosphate; S-A, sinoatrial; AChE, acetylcholinesterase.

tylimidazole, NEM, 5,5′-dithiobis(2-nitrobenzoic acid), 2-methoxy-5-nitrobenzylbromide, edrophonium, AChE (type VI-S), and atropine sulfate were obtained from Sigma Chemical Co. (St. Louis, MO). Aech-M hydrochloride was purchased from Research Biochemicals Inc. (Wayland, MA). All other chemicals were of analytical grade.

Membrane preparation. Male Sprague-Dawley rats (200–250 g) were killed by decapitation and the hearts were removed and rinsed in ice-cold saline. The atria and ventricles were dissected and cut into small pieces. The tissue was then homogenized (1 g/10 ml) in ice-cold 50 mM Tris-HCl buffer at pH 7.4 containing 0.32 M sucrose and 5 mM $MgCl_2$ for 20 sec with a Tekmar SDT-100EN homogenizer at setting 4. The homogenate was then diluted with 20 ml of homogenization buffer and centrifuged at 48,000 × *g* for 10 min. The supernatant was discarded and the pellet was resuspended (setting 3, 10 sec) in 30 ml of 50 mM Tris-HCl at pH 7.4 containing 5 mM $MgCl_2$, passed through nylon netting (30 mesh) to remove large pieces of connective tissue, and centrifuged at 48,000 × *g* for 10 min. The pellet was washed twice more by resuspension (setting 3, 10 sec) in 25 mM $NaPO_4$ buffer at pH 7.4 containing 2 mM $MgCl_2$ and centrifugation (48,000 × *g*). The final pellet was resuspended in $NaPO_4$ buffer (as above) for assays. The protein content was determined by the method of Lowry *et al.* (17) using bovine serum albumin as standard. AChE activity was assayed using acetylthiocholine as substrate by the method described by Ellman *et al.* (18).

Membrane pretreatments. Membrane protein (3 mg/ml) was resuspended in 25 mM $NaPO_4$ buffer at pH 7.4 containing 2 mM $MgCl_2$ and other additions as indicated in the text. When Aech-M was present, eserine (25 μM) was also added. The suspensions were then incubated for up to 30 min at 30°. At the end of the incubation, the suspension was diluted with ice-cold 50 mM Tris-HCl at pH 7.4 containing 5 mM $MgCl_2$ and centrifuged at 48,000 × *g* for 10 min. The pellets were washed three more times (by resuspension and centrifugation) in Tris buffer, and the final pellets were suspended in 1 volume of 50 mM Tris-HCl buffer at pH 7.4 containing 5 mM $MgCl_2$ for [^{3}H]QNB and [^{3}H]NMS assays or 10 mM Tris-HCl buffer at pH 7.4 containing 2 mM $MgCl_2$ for [^{3}H]Oxo-M assays.

Antagonist binding assay. Muscarinic receptors were determined by incubating membrane protein (ventricles, 0.1–0.25 mg; atria, 0.06–0.1 mg) in a total volume of 2.0 ml containing 50 mM Tris-HCl buffer at pH 7.4, 5 mM $MgCl_2$, 0.03–1.0 nM [^{3}H]QNB, and with and without 1 μM atropine for 60 min at 36°. At the end of the incubation, each suspension was diluted with 4 ml of ice-cold 50 mM Tris-HCl buffer at pH 7.4 containing 5 mM $MgCl_2$ and poured onto a Whatman GF/C glass fiber filter under reduced pressure. The filter was then washed with an additional 8 ml of buffer, placed in a scintillation vial containing 6 ml of Liquiscint (National Diagnostics, Somerville, NJ), and counted. Specific [^{3}H]QNB binding to the muscarinic receptor was calculated as the difference between total binding in the absence of atropine and the nonspecific binding determined in the presence of 1 μM atropine. Specific binding was from 85 to 95% of the total [^{3}H]QNB bound.

In some experiments, the ability of various compounds to inhibit specific [^{3}H]QNB binding was tested. Membrane protein (0.2–0.3 mg) was incubated in a total volume of 2 ml containing 25 mM $NaPO_4$ buffer at pH 7.4, 2 mM $MgCl_2$, 0.25 nM [^{3}H]QNB, varying concentrations of competitor as indicated in the text, and 25 μM eserine to inhibit AChE activity. Incubations were for 60 min at 36°, and the nonspecific binding and filtering (using phosphate buffer) were performed as described above. These competitive binding assays were performed in the absence and presence of 100 μM (Gpp(NH)p. All binding assays were performed in triplicate, the results varying by less than 5%.

Specific [^{3}H]NMS binding was determined in a total volume of 2 ml containing 50 mM Tris-HCl buffer at pH 7.4, 5 mM $MgCl_2$, 0.05–1 nM [^{3}H]NMS, and with and without 1 μM atropine for 45 min at 36°. At the end of the incubation, the suspensions were filtered on Whatman GF/C glass fiber filters. The filters were washed with 10 ml of ice-cold incubation buffer and counted, and the specific binding was calculated as described above for [^{3}H]QNB binding.

Agonist binding assays. The agonist high affinity binding state of the muscarinic receptor was determined by [^{3}H]Oxo-M binding. Membrane protein (ventricles, 0.3–0.4 mg; atria, 0.08–0.15 mg) was incubated in a total volume of 1 ml containing 10 mM Tris-HCl buffer at pH 7.4, 2 mM $MgCl_2$, 0.03–2 nM [^{3}H]Oxo-M, and with and without 1 μM atropine for 30 min at 25°. At the end of the incubation, each suspension was diluted with 4 ml of ice-cold incubation buffer and poured onto a Whatman GF/C glass fiber filter under reduced pressure. The filters were then washed with an additional 8 ml of ice-cold buffer, placed in a vial with scintillation fluid, and counted. Specific binding was calculated as described above for [^{3}H]QNB binding.

Beating atrial preparations. Rats were killed by decapitation and the hearts were quickly removed. The atria containing the sinus node (S-A) were immersed in modified Tyrode's solution (NaCl, 136.9 mM; KCl, 2.7 mM; $NaHCO_3$, 11.9 mM; NaH_2PO_4, 1.78 mM; $MgCl_2$, 0.5 mM; $CaCl_2$, 2.7 mM; glucose, 5.55 mM; bubbled with 98% O_2, 2% CO_2; pH 7.4). Tissue containing the S-A pacemaker cells, adjacent segments of the crista terminals and atrial appendage, was dissected free and mounted (endocardial surface up) in a constant temperature perfusion bath (37°). The preparation was superfused with modified Tyrode's solution and allowed to beat spontaneously. Changes in beating rate were measured by using standard microelectrode techniques to record transmembrane action potentials from the S-A node-atrial preparation as described previously (19).

The spontaneous beating rate was allowed to stabilize at least 1 hr, at which time the action potential configuration was observed and, if not normal, the preparation was not used. At the end of the equilibration period, cumulative carbachol response data were obtained by superfusing the drug for 3 min, at which time the beating rate was recorded. After the last dose of carbachol, the tissue was superfused with drug-free Tyrode's for 1 hr, at which time the beating rate was back to control values. The tissue was then superfused for 30 min with Tyrode's containing Aech-M followed by a 1-hr superfusion with Tyrode's alone. Carbachol dose-response experiments were then performed as described above.

Data analysis. The receptor concentrations and K_D values were determined from regression analysis of Scatchard (20) plots. The concentration of compounds which inhibited ligand binding by 50% (IC_{50}) was obtained from the Hill (21) slope of the competition data. The effective concentrations of drugs which gave 50% of a response (EC_{50}) were determined from the medium-effect equation using an Apple IIe microcomputer as described by Chou and Talalay (22). Statistical analysis of the data was performed using the Student's *t* test.

Results

Effect of Aech-M on muscarinic receptors in cardiac membranes. Fig. 1 shows the ability of ACh and Aech-M to compete for [^{3}H]QNB-binding sites in cardiac ventricular membranes. Eserine (25 μM) was also present to inhibit any cholinesterase activity. In the absence of Gpp(NH)p, the competition curves were shallow and the IC_{50} values for ACh and Aech-M were 0.8 ± 0.2 and 0.5 ± 0.12 μM, respectively. The Hill slope for ACh was 0.59 ± 0.06 and for Aech-M was 0.56 ± 0.07. In the presence of 100 μM Gpp(NH)p, both compounds were less potent at inhibiting [^{3}H]QNB binding, with the IC_{50} values shifted about 30-fold (ACh, 30 ± 6 μM; Aech-M, 15 ± 4 μM). In addition, the competition curves in the presence of Gpp(NH)p steepened somewhat with Hill slopes of 0.74 ± 0.02 for ACh and 0.78 ± 0.04 for Aech-M. In the absence of a cholinesterase inhibitor, there was a small decrease in the ability of Aech-M to inhibit [^{3}H]QNB binding (IC_{50}, 0.82 μM), which may have been due to hydrolysis of the Aech-M by the enzyme. The

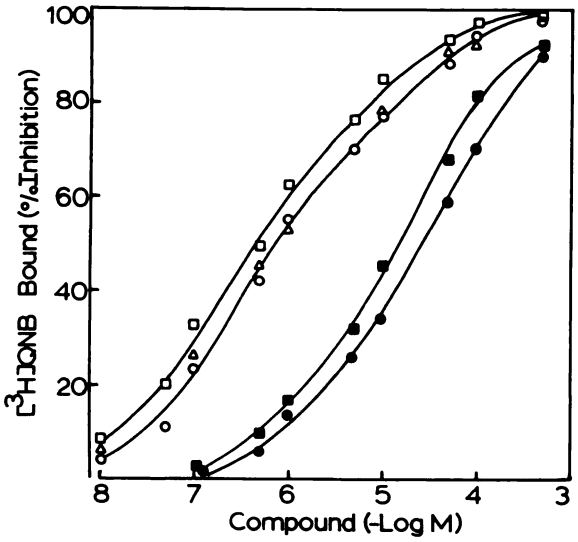

Fig. 1. Competition of ACh and Aech-M for [^{3}H]QNB binding in cardiac membranes. Membrane protein was incubated in 25 mM sodium phosphate buffer, pH 7.4, containing 2 mM $MgCl_2$, 0.25 nM [^{3}H]QNB, 25 μM eserine, and the indicated concentrations of ACh (○), Aech-M (□), and Aech-M incubated without eserine (△) for 60 min at 36°C. Competition assays were also performed with the indicated concentrations of ACh (●) and Aech-M (■) in the presence of 100 μM Gpp(NH)p as described under Experimental Procedures. The control [^{3}H]QNB binding was 137 ± 6 and 122 ± 8 fmol/mg of protein for the competition assays with ACh and Aech-M, respectively. Each point on the graph is the mean of three to four determinations performed in triplicate.

cardiac membrane preparation used hydrolyzed acetylthiocholine at a rate of 0.4 ± 0.16 nmol/min/mg of protein (mean ± SE, n = 4). Thus, even though the cholinesterase activity was low, a cholinesterase inhibitor was used in most experiments to reduce any possible hydrolysis of the compounds.

Fig. 2A shows the effect of temperature and time on the ability of 1 μM Aech-M pretreatment to reduce [^{3}H]QNB binding. At the end of a 30-min preincubation period, followed by membrane washing, there was a 13, 20, 59, and 76% loss of binding sites when the preincubations were performed at 4°, 15°, 25°, and 30°, respectively. At each temperature, the binding site loss was time dependent. Over a 20-min preincubation period at 30°, 72% of the [^{3}H]QNB binding sites were lost, after which little further loss was observed. In control preincubations (no Aech-M present), less than 5% of the sites were lost at the 30-min time point for each temperature studied. Fig. 2B shows a Scatchard plot of [^{3}H]QNB binding to cardiac membranes after pretreatment with Aech-M. After a 30-min preincubation with 0.1 and 1 μM Aech-M, followed by washing, there was a 40 and a 74% decrease in the number of specific [^{3}H]QNB-binding sites, respectively. There was no change in the K_D value (68–70 pM) for [^{3}H]QNB binding to the receptors left as compared to the control.

Table 1 shows the effect of a 30-min preincubation of cardiac membranes with 0.25 μM Aech-M on [^{3}H]QNB, [^{3}H]NMS, and [^{3}H]Oxo-M binding. There was a similar 53 and 58% loss of sites for [^{3}H]QNB and [^{3}H]NMS binding, whereas an 83% loss was observed with [^{3}H]Oxo-M binding. Interestingly, the absolute loss of sites in fmol/mg of protein was similar (58–64) for [^{3}H]QNB and [^{3}H]Oxo-M, whereas the loss for [^{3}H]NMS was 38 fmol/mg of protein. As shown in Table 2, the addition of atropine (2 μM) or carbachol (200 μM) largely protected [^{3}H]QNB-binding sites from an Aech-M induced loss. Table 2 also

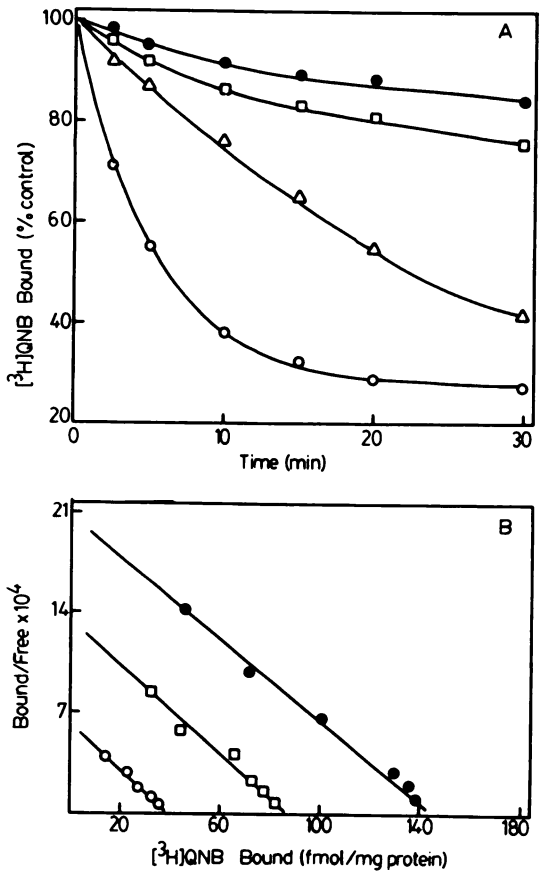

Fig. 2. Time course of [^{3}H]QNB binding loss (A) and Scatchard analysis of [^{3}H]QNB binding (B) after pretreatment with Aech-M. In A, membrane protein (3 mg/ml) was incubated in 25 mM sodium phosphate buffer containing 2 mM $MgCl_2$, 25 μM eserine, 1 μM Aech-M at 4° (●), 15° (□), 25° (△), and 30° (○). At the times indicated, samples were removed, washed three times with buffer, and assayed with 0.25 nM [^{3}H]QNB. Less than 5% of the [^{3}H]QNB sites were lost in control incubations at each time point. The control [^{3}H]QNB binding was 118–131 fmol/mg of protein. In B, membrane protein (3 mg/ml) was incubated in 25 mM sodium phosphate buffer at pH 7.4 containing 2 mM $MgCl_2$, 25 μM eserine, and without (●) and with 1 μM (○) or 0.1 μM (□) Aech-M for 30 min at 30°. At the end of the incubation, the membranes were washed three times and assayed with [^{3}H]QNB as described under Experimental Procedures. The data are plotted as the ratio of the amount specifically bound ligand (pmol/mg of protein) to free ligand (pmol/liter) versus the amount of specifically bound ligand/mg of protein. Data points are the means of triplicate determinations and are representative of three experiments.

TABLE 1

Effect of Aech-M pretreatment on the loss of ventricular muscarinic receptors

Cardiac membranes (3 mg/ml) were preincubated without and with 0.25 μM Aech-M for 30 min at 30°. At the end of the incubation, the membranes were washed three times and assayed as described in Experimental Procedures.

	Control	Aech-M pretreated
[^{3}H]QNB bound (fmol/mg protein)	120 ± 3[a]	56 ± 8[b] (53)[c]
K_D (pM)	101 ± 9	94 ± 7
[^{3}H]NMS bound (fmol/mg protein)	66 ± 6	28 ± 4[b] (58)
K_D (pM)	220 ± 29	220 ± 36
[^{3}H]Oxo-M bound (fmol/mg protein)	70 ± 10	12 ± 6[b] (83)
K_D (nM)	1.5 ± 0.2	1.3 ± 0.1

[a] Values are means ± standard errors, n = 3–4.
[b] Significantly different from the respective control ($p < 0.001$).
[c] Numbers in parentheses, percentage loss of control values.

TABLE 2

Protection from Aech-M-induced muscarinic receptor loss and the effect of AChE on the ability of Aech-M and ACh to inhibit [^{3}H]QNB binding

In Part A, cardiac membranes (3 mg/ml) were preincubated with the additions indicated for 30 min at 30°. At the end of the incubation, the membranes were washed three times and assayed with 0.25 nM [^{3}H]QNB. In Part B, Aech-M (1 mM) and ACh (1 mM) were incubated in 25 mM $NaPO_4$ buffer at pH 7.4, and without and with 1.0 unit of AChE for 10 min at 30°. At the end of the incubation, the solution was diluted 100-fold with buffer containing 10 μM eserine, and the ability of an aliquot of the diluted solution (calculated to give 1 μM final concentration) to inhibit 0.25 nM [^{3}H]QNB binding to cardiac membranes was determined as described in Experimental Procedures.

Preincubation additions	[^{3}H]QNB bound
	fmol/mg protein
A. None	115 ± 4[a]
Aech-M (1 μM)	38 ± 5
Atropine (2 μM) + Aech-M (1 μM)	104 ± 8
Carbachol (200 μM) + Aech-M (1 μM)	110 ± 10
B. Control	133 ± 4
Aech-M (1 μM)	45 ± 3
Aech-M + AChE	85 ± 2[b]
ACh	41 ± 5
ACh + AChE	110 ± 7[b,c]

[a] Values are means ± standard errors, $n = 3$–4.
[b] Significantly different from the respective Aech-M and ACh group ($p < 0.001$).
[c] Significantly different from the Aech-M + AChE group ($p < 0.005$).

shows that, after 1 mM stock solutions of ACh or Aech-M were preincubated with AChE, a dilution of these stock solutions has a reduced ability to inhibit [^{3}H]QNB binding. The reduced inhibition of the AChE-treated compounds was greater for ACh (control, 69%; AChE-treated, 17%) than for Aech-M (control, 66%; AChE-treated, 36%).

As shown in Fig. 3A, the ability of a range of Aech-M concentrations to induce a loss of [^{3}H]QNB-binding sites is reduced in the presence of 100 μM Gpp(NH)p. After a 30-min preincubation with Aech-M followed by washing, the concentrations of Aech-M that induced a 50% loss of receptors were 0.15 and 2.5 μM in the absence and presence of Gpp(NH)p, respectively. It should be noted, however, that Gpp(NH)p treatment will not reverse the binding site loss after incubation with Aech-M alone (data not shown). The effect of several guanine nucleotides to modulate the ability of Aech-M to induce a loss of [^{3}H]QNB sites is shown in Fig. 3B. The half-maximal concentrations of the nucleotides required to reduce the Aech-M-induced loss of [^{3}H]QNB binding sites were 0.08, 12, and 73 μM, and > 1 mM for Gpp(NH)p, GTP, GDP, and GMP, respectively.

Attempts to prevent the Aech-M-induced irreversible loss of cardiac [^{3}H]QNB-binding sites by several group-selective reagents were unsuccessful. For example, pretreatment of cardiac membranes for 20 min at 30° with 100 μM NEM followed by washing had no effect on subsequent [^{3}H]QNB binding but reduced [^{3}H]Oxo-M binding by 73%, indicating that NEM pretreatment selectively inhibited the agonist high affinity binding state ([^{3}H]Oxo-M binding). This effect of NEM on muscarinic receptors has been previously reported (7). In addition, NEM pretreatment also reduced the [^{3}H]QNB-binding site loss induced by 1 μM Aech-M from 70% to 26%, which was similar to the reduction induced by Gpp(NH)p (Fig. 3A). However, when membranes were pretreated with 100 μM NEM, washed, pretreated for 30 min at 30° with 10 μM Aech-M, and washed again, there was a 78% decrease in [^{3}H]QNB-binding sites (control, 132 ± 4; treated, 29 ± 3 fmol/mg of protein). This was the same as a 77% loss of [^{3}H]QNB-binding sites

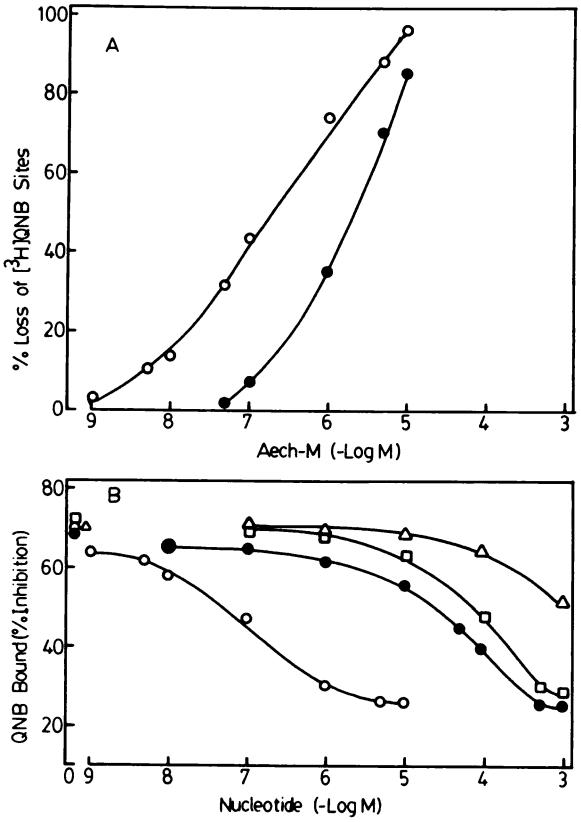

Fig. 3. Effect of guanine nucleotides on the Aech-M induced loss of [^{3}H]QNB binding to cardiac membranes. In A, cardiac membranes (3 mg/ml) were incubated in phosphate buffer at pH 7.4 containing 2 mM $MgCl_2$, 25 μM eserine, the indicated concentration of Aech-M, and with (●) and without (○) 100 μM Gpp(NH)p for 30 min at 30°. At the end of incubation, the membranes were washed three times and assayed with 0.25 nM [^{3}H]QNB. In B, membrane protein (3 mg/ml) was incubated in phosphate buffer at pH 7.4 containing 2 μM $MgCl_2$, 0.5 mM Aech-M, and without or with the indicated concentrations of Gpp(NH)p (○), GTP (●), GDP (□), or GMP (△) for 30 min at 30°. At the end of the incubation, the membranes were washed three times and assayed with 0.25 nM [^{3}H]QNB as described in Experimental Procedures. Each point on the graphs is the mean of three determinations performed in triplicate. The control values for [^{3}H]QNB binding ranged from 125 ± 6 to 138 ± 8 fmol/mg of protein.

after a preincubation with 10 μM Aech-M plus 100 μM Gpp(NH)p to parallel the NEM effect (control, 132 ± 4; treated, 31 ± 3 fmol/mg of protein). A similar effect was observed when membranes were pretreated with the thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid) (100 μM), the tyrosine-selective reagent *N*-acetylimidazole (100 μM) (23), and the tryptophan-selective reagent 2-methoxy-5-nitrobenzylbromide (100 μM) (24).

Effect of Aech-M on isolated beating atria and atrial muscarinic receptors. Fig. 4 shows the effect of Aech-M (100 μM) on the spontaneous atrial beating rate as a function of superfusion time. Initially, Aech-M caused a decrease in beating rate which was maximally suppressed by 38% after 7 min of superfusion. Thereafter, the beating rate slowly returned to the control level after 17 min of superfusion. In contrast, during superfusion with 1 μM carbachol, the beating rate dropped to 50% of the control value and remained there throughout the 30-min superfusion period. Similar to carbachol, superfusion with 10 μM ACh reduced the beating rate to 37% of the control value during the first 10 min and the rate remained constant for the rest of the 30-min superfusion period. The Aech-M-

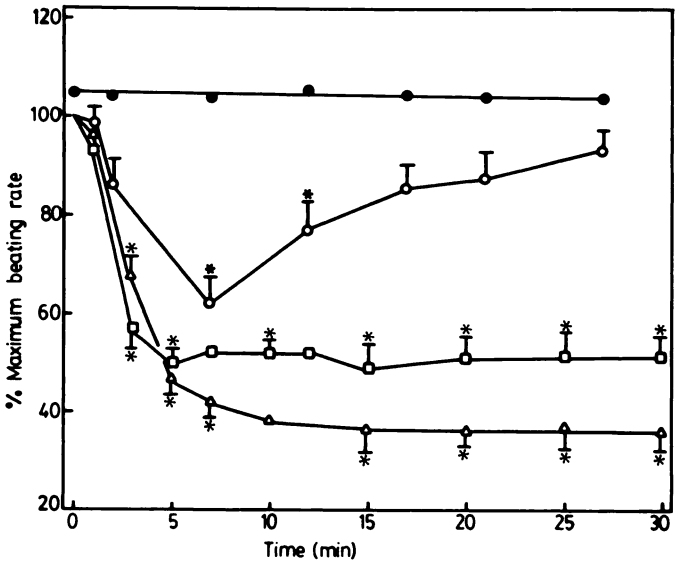

Fig. 4. Time course effect of Aech-M, ACh, and carbachol on the spontaneous atrial beating rate. Spontaneous atrial beating atria were superfused with 100 μM Aech-M (○), 1 μM carbachol (□), or 10 μM ACh (△) in modified Tyrode's solution and, at the times indicated, the beating rate was determined as described in Experimental Procedures. Beating atria were also superfused for 5 min with 5 μM atropine after which 100 μM Aech-M was added (●). Each point on the graph is the mean ± standard error of four to six determinations.

TABLE 3

Ability of (−)-isoproterenol to increase spontaneous atrial beating rate after pretreatment with Aech-M

Spontaneously beating atria were superfused without and with 100 μM Aech-M for 30 min at 37°. The atria were then superfused for 1 hr after which cumulative doses of (−)-isoproterenol were superfused. Each dose of the agonist was superfused for 5 min at which time the beating rate was determined.

Dose of (−)-isoproterenol	Beating rate (beats/min)	
	Control	Aech-M (100 μM) pretreated
None	216 ± 17[a]	187 ± 10
1 nM	229 ± 11 (106)[b]	229 ± 16 (122)
10 nM	259 ± 17 (119)	253 ± 14 (135)
100 nM	302 ± 14 (140)	274 ± 24 (146)
1000 nM	299 ± 15 (138)	275 ± 14 (147)

[a] Values are means ± standard errors, $n = 3$.
[b] Numbers in parentheses, percentage of control values.

induced decrease in beating rate was completely blocked by the concurrent superfusion of 5 μM atropine (Fig. 4). Although superfusion with atropine alone (5 μM) did not significantly increase the spontaneous beating rate from the average of several preparations, the drug did cause a 5–15% increase in the spontaneous rate in each individual preparation. No cholinesterase inhibitor was present in these experiments as several of them (eserine, neostigmine) either greatly reduced or completely stopped the spontaneous rate. In addition, when the tissue was superfused with Aech-M above 250 μM, the beating rate decreased but was not or was only partly reversed as a function of time or tissue superfusion in the absence of the compound (data not shown). Table 3 shows the ability of (−)-isoproterenol to increase the spontaneous beating rate in control atria and after atria had been superfused for 30 min with 100 μM Aech-M, followed by a 1-hr superfusion in the absence of drug. There was no significant change in the ability of (−)-isoproterenol to increase the beating rate or the maximum rate obtained between the control and Aech-M-treated atria.

The ability of carbachol to decrease the spontaneous atrial beating rate before and after treatment with Aech-M is shown in Fig. 5. After the initial tissue equilibration period, carbachol decreased the beating rate in a dose-dependent manner with an EC_{50} value of 0.94 μM. The initial control carbachol dose response curve did not subsequently alter the tissue's response to carbachol since a second control dose response to carbachol 2.5 hr after the first was the same as the initial control. At the end of the initial dose response experiment, the tissue was superfused for 1 hr with Tyrode's solution alone and then superfused with 100 μM Aech-M for 30 min or with 10 μM edrophonium for 5 min followed by 10 μM Aech-M for 30 min. At this time, the EC_{50} for carbachol inhibition of the beating rate was increased to 14.9 and 9.7 μM for the 100 μM Aech-M-pretreated and the 10 μM edrophonium plus 10 μM Aech-M-pretreated atria, respectively. Edrophonium was chosen as the cholinesterase inhibitor since it appeared to wash out of the tissue faster than eserine (based on the time for the beating rate to recover to within the control value).

The effect of pretreating intact atria with Aech-M on [^{3}H]QNB and [^{3}H]Oxo-M binding is shown in Table 4. After pretreatment of atria with 100 μM Aech-M for 30 min followed by membrane washing, there was a 46–47% decrease in both [^{3}H]QNB and [^{3}H]Oxo-M binding. In the atria pretreated with 10 μM edrophonium plus 10 μM Aech-M, there was a 37–41% decrease in [^{3}H]QNB and [^{3}H]Oxo-M binding. In both treated groups, there was no change in the K_D values for either ligand

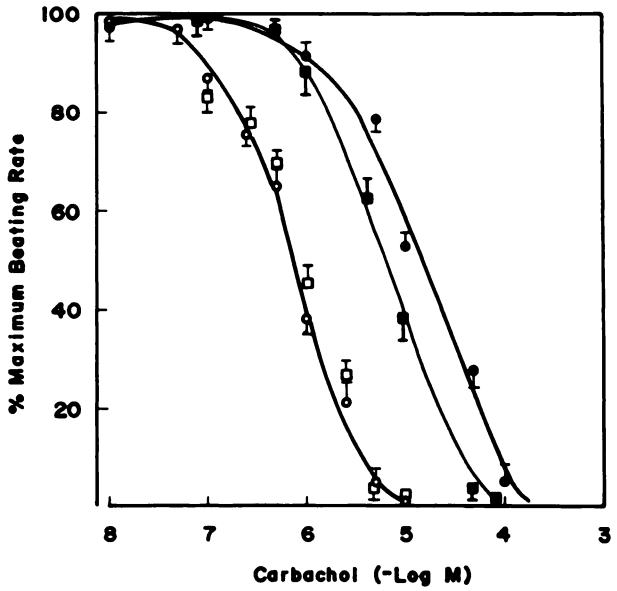

Fig. 5. Effect of carbachol on the spontaneous beating rate after treatment with Aech-M. Spontaneously beating atria were allowed to stabilize for 1 hr after which they were superfused with the indicated concentrations of carbachol for 3 min (○). After the last concentration, the atria were superfused for 1 hr and then treated for 30 min with 100 μM Aech-M alone or for 5 min with 10 μM edrophonium, and then with 10 μM Aech-M plus 10 μM edrophonium for 30 min. The atria were then washed for 1 hr and the carbachol superfusions were repeated with atria pretreated with 100 mM Aech-M (●) and 10 μM Aech-M plus 10 μM edrophonium (■). Carbachol dose response curves were also performed in control atria and then repeated (□) 1.5 hr after superfusion with Tyrode's solution alone. Each point on the graph is the mean ± standard error of four to six determinations. The spontaneous beating rates in the control, after 100 mM Aech-M and 10 μM edrophonium plus 10 μM Aech-M pretreatments were 190 ± 8, 184 ± 12, 178 ± 9 beats/min, respectively.

TABLE 4

Effect of Aech-M pretreatment on the carbachol EC_{50} value for inhibition of atrial beating rate and atrial muscarinic receptors

The carbachol EC_{50} values were taken from Fig. 5. Intact whole atria were incubated for 30 min at 37° in 10 ml of modified Tyrode's solution alone, with 100 μM Aech-M, or with 10 μM Aech-M plus 10 μM edrophonium. The atria were transferred to fresh Tyrode's solution with and without the compounds every 10 min. At the end of the incubation the atria were quickly rinsed in ice-cold saline and the membranes were isolated. The washed membranes were then assayed for specific [^{3}H]QNB and [^{3}H]Oxo-M binding sites as described in Experimental Procedures.

	Control	Aech-M (100 μM) treated	Aech-M (10 μM) + edrophonium (10 μM) treated
Carbachol EC_{50} (μM)	0.94 ± 0.13[a]	14.9 ± 1.3[b]	9.7 ± 1.8[b]
[^{3}H]QNB bound (fmol/mg protein)	472 ± 23	254 ± 26 (54)[b,c]	297 ± 18 (63)[b]
K_D (pM)	93 ± 5	93 ± 4	102 ± 5
[^{3}H]Oxo-M bound (fmol/mg protein)	330 ± 21	175 ± 18 (53)[b]	195 ± 13 (59)[b]
K_D (nM)	1.1 ± 0.2	0.9 ± 0.05	1.4 ± 0.4

[a] Values are means ± standard errors, n = 4–6.
[b] Significantly different from the control group ($p < 0.001$).
[c] Numbers in parentheses, percentage of control values.

to the receptors left as compared to the control. In contrast, pretreatment of atria for 30 min with 200 μM carbachol did not change the maximal binding of [^{3}H]QNB or [^{3}H]Oxo-M as compared to control ([^{3}H]QNB control, 453 ± 13; treated, 447 ± 21 fmol/mg of protein; [^{3}H]Oxo-M control, 298 ± 11; treated, 315 ± 17 fmol/mg of protein, n = 3).

Discussion

The data presented using isolated cardiac ventricular membranes showed that Aech-M was a relatively potent muscarinic ligand. Indeed, Aech-M was about equal to or slightly more potent than ACh in competing for specific [^{3}H]QNB-binding sites. The slight reduction in the potency of Aech-M to compete for [^{3}H]QNB binding in the absence of eserine suggested that Aech-M was hydrolyzed by the residual cholinesterase activity in the membrane preparation. That Aech-M is hydrolyzed by AChE was strongly indicated by the large reduction in [^{3}H]QNB inhibition observed after pretreatment of a stock drug solution with the enzyme. Interestingly, there was a greater reduction of [^{3}H]QNB inhibition with ACh than with Aech-M after AChE treatment, suggesting that ACh may have been hydrolyzed faster. In comparison, it has been reported that ACh mustard is hydrolyzed by bovine erythrocyte AChE (13).

The ability of Aech-M to irreversibly bind to the muscarinic receptor was established by washout experiments. Receptor loss by Aech-M was time and temperature dependent, not reversed by extensive membrane washing but prevented by concurrent treatment with atropine and carbachol. Furthermore, Scatchard analysis of [^{3}H]QNB binding after incubation with Aech-M and washing indicated a noncompetitive interaction as the receptor capacity decreased, whereas the K_D for [^{3}H]QNB binding did not change. These results suggested that, initially, Aech-M binds to the muscarinic receptor in a competitive manner, after which the compound changes into a form which covalently binds to the receptor. Similar to the present study using Aech-M, it has been reported that ACh mustard irreversibly binds to the muscarinic receptor from intestinal tissue (15), and, more recently, a mustard analogue of oxotremorine has been shown to bind irreversibly to muscarinic receptor from intestinal and brain tissue (25, 26).

The mechanism of irreversible Aech-M binding is probably an initial conversion to acetylethylcholine aziridinium ion which could then react with several nucleophilic groups in or around the receptor. Aziridinium ion formation has been shown to occur with several other types of mustard compounds (25, 27). In the present study, attempts were made to determine the group through which Aech-M covalently attached to the receptor. Although the group-selective reagents that reacted with thiol, tyrosine, and tryptophan groups modulated the ability of low concentrations of Aech-M to induce a receptor loss in a manner similar to that of Gpp(NH)p, they did not prevent a receptor loss under conditions to account for their modulating effect. However, it is possible that these reagents, at the concentrations used, did not react with the groups in the receptor that Aech-M interacts with. Alternatively, Aech-M may interact with groups not blocked by these reagents. Further work will be necessary to determine the receptor group that Aech-M covalently attaches to.

Previous studies using cardiac membranes have shown that agonists can discriminate several affinity states of the muscarinic receptor and the high affinity states are modulated by guanine nucleotides (3–5). Furthermore, the muscarinic agonist [^{3}H]Oxo-M has been shown to bind to an agonist high affinity state of the receptor, the binding of which is reduced or abolished by Gpp(NH)p (4, 5). In contrast, the antagonist [^{3}H]QNB appears to bind to all of the receptor sites with little or no nucleotide modulation (4, 5, 8). In the present work, the ability of Aech-M to compete for specific [^{3}H]QNB-binding sites and to induce a loss of binding capacity was greatly reduced by Gpp(NH)p (Figs. 1 and 3). Furthermore, the potency series of the guanine nucleotides Gpp(NH)p>GTP>GDP>GMP for reducing the Aech-M-induced loss of binding sites is similar to that reported for their ability to reduce the affinity of agonists for the muscarinic receptor (3, 5). Although guanine nucleotides can modulate the loss of binding sites induced by Aech-M, it should be pointed out that, at high enough concentrations, both in the presence and absence of Gpp(NH)p, the majority of binding sites are lost (Fig. 3). Recent evidence suggests that guanine nucleotide-sensitive high affinity agonist binding represents a ternary complex of agonist, receptor, and the inhibitory guanine nucleotide-binding protein (5, 28). Presumably, the lower maximal binding reported for agonists as compared to antagonists indicates that a fraction of the receptors may not form the ternary complex, is in an agonist low affinity state, and is not modulated by guanine nucleotides (5, 29). Based upon this, careful control of the Aech-M concentrations may lead to a selective loss of those receptors able to form the agonist high affinity binding state (Figs. 1 and 3). Selective loss of those receptor populations forming the different affinity states has been reported using both Oxotremorine mustard (26) and a combination of a reversible agonist and an irreversible antagonist (30).

In addition to muscarinic agonist binding selectivity, it has been shown that the muscarinic antagonist [^{3}H]NMS binds to a smaller proportion of the muscarinic receptors labeled by [^{3}H]QNB (31). The more hydrophobic nature of [^{3}H]QNB may allow it to measure sites in the membrane that [^{3}H]NMS does not have access to at low concentrations. In the present work, the binding capacity of [^{3}H]NMS was only 55% of that measured by [^{3}H]QNB in cardiac membranes. Although pretreatment of membranes with Aech-M, which is quaternary in structure, produced a greater absolute loss of [^{3}H]QNB sites as

compared to [^{3}H]NMS sites, the percentage loss of sites was similar, suggesting that Aech-M had equal access to the sites measured by both ligands. In contrast, Aech-M induced a similar absolute loss of sites when measured by the agonist [^{3}H] Oxo-M and the antagonist, [^{3}H]QNB, although, on a percentage basis, more [^{3}H]Oxo-M sites were lost (Table 1). This further supports the selective Aech-M-induced loss of agonist high affinity binding sites as discussed above.

The observation that Aech-M decreased the atrial spontaneous beating rate and the decrease was blocked by atropine indicates that Aech-M is a muscarinic agonist (Fig. 4). However, full dose response curves to determine the efficacy and potency of Aech-M would be difficult to perform. Although it is tempting to label Aech-M as a partial agonist because, even at higher concentrations, it reduced the beating rate less than ACh or carbachol, this may not be correct because of a possible race between receptor activation and receptor loss (alkylation) when Aech-M interacts with the receptor. Furthermore, as discussed previously, it is likely that Aech-M is hydrolyzed by cholinesterases. Since no enzyme inhibitor was present when Aech-M was superfused to the atria, the precise concentration in the tissue was not known. Hydrolysis of Aech-M would partly account for the higher concentrations of Aech-M needed to reduce the atrial beating rate as compared to inhibition of [^{3}H]QNB binding in the presence of an inhibitor. A cholinesterase inhibitor was not used in the beating rate as a function of time experiments because in the presence of one, variable reductions in the beating rate occurred. This suggested that some spontaneous release of ACh was occurring which has been previously reported for isolated atria (32).

Although, initially, Aech-M (100 μM) decreased the spontaneous beating rate, after several min of superfusion, the beating rate returned to control levels. However, at higher Aech-M concentrations (above 250 μM), the spontaneous beating rate decreased to a highly variable extent which was not reversed, even by extensive tissue superfusion in the absence of Aech-M. This may suggest that, at high Aech-M concentrations, tissue components other than the muscarinic receptor may have been alkylated, resulting in a nonspecific depression of the tissue response. Significant nonspecific tissue depression after superfusion with 100 μM Aech-M was unlikely since no difference in the ability of isoproterenol to increase the beating rate was observed before and after treatment with the drug. At the lower Aech-M concentration used (100 μM), the recovery of the beating rate could be accounted for by possibilities including responsiveness desensitization, agonist-induced receptor down-regulation, or the initial action of Aech-M as an agonist, whereas after receptor alkylation Aech-M acts as an antagonist. Responsiveness desensitization and an agonist-induced down-regulation of receptors may be unlikely explanations since, as previously reported (19) and found in the present study, the sensitivity of beating atria did not change after repeated doses of carbachol (Fig. 5) or during continuous superfusions with ACh or carbachol (Fig. 4). Furthermore, no change was detected in atrial [^{3}H]QNB or [^{3}H]Oxo-M binding after a 30-min exposure to a high concentration (200 μM) of carbachol. However, the above observations were made with reversible agonists, and it is possible that Aech-M with its complex reversible and irreversible binding components could induce a densentization and/or receptor down-regulation. Nonetheless, if Aech-M acted as an agonist, even after receptor alkylation, then one might expect a sustained decrease in the spontaneous beating rate as observed with ACh and carbachol. Since this was not observed with Aech-M, it is possible that, after receptor alkylation, Aech-M was acting as an antagonist. That Aech-M did induce a receptor loss was indicated by a 47% decrease in both [^{3}H]QNB and [^{3}H]Oxo-M binding sites in atria pretreated with Aech-M under conditions similar to that used in the beating atrial experiments.

After pretreating atria with either Aech-M alone (100 μM) or with Aech-M plus edrophonium (10 μM each) followed by washing, there was a parallel shift to the right of the carbachol dose response curve for inhibition of the atrial beating rate with no change in the maximal response (Fig. 5). The similar shifts by both treatments suggests that, by inhibiting cholinesterase activity, the concentration of Aech-M could be reduced from 100 to 10 μM to produce similar effects. The shift in the dose response curves for carbachol inhibition of the atrial beating rate was probably related to the 46–47% decrease in [^{3}H]QNB and [^{3}H]Oxo-M binding after treatment with 100 μM Aech-M and the 37–41% decrease in [^{3}H]QNB and [^{3}H]Oxo-M binding after treatment with 10 μM Aech-M plus edrophonium. The loss of muscarinic receptors coupled with a parallel shift in the carbachol dose response curve indicated that all of the receptors were not required to produce a maximal response, i.e., there may be a substantial receptor reserve or a relatively high efficiency of coupling between the receptor and its coupled tissue response. However, it should be pointed out that the beating rate originates from S-A nodal cells, whereas the ligand binding assays measured the receptors from all of the atrial cells. Thus, it is not known whether the receptor loss from the whole atria parallels the loss of receptors from the cells of the S-A node. Nonetheless, a high coupling efficiency has been previously reported for muscarinic receptors in the guinea pig ileum and for β-adrenoreceptors in the heart using irreversible antagonists to reduce the receptor concentrations (10, 33, 34). Interestingly, the similar percentage loss of [^{3}H]QNB- and [^{3}H] Oxo-M-binding sites in intact atria is in contrast to the unequal loss observed in isolated membranes. This could be due to intracellular guanine nucleotides of the intact atria maintaining most of the muscarinic receptors in a single affinity state and is supported by the recent report showing that agonist binding in isolated cardiac membranes shows several affinity states, whereas, using intact cardiac cells, only a single low affinity state was observed (35).

The present experimental results show that, initially, Aech-M interacts with the muscarinic receptor in membranes in a manner similar to that of other muscarinic agonists and produces an agonist-like action in spontaneously beating atria. Over time, Aech-M appears to alkylate the receptor, and the data are consistent with a long-term antagonist-like effect. Thus, in addition to the recently introduced oxotremorine mustard (25, 26), Aech-M may be a useful tool for investigating agonist-receptor interactions, the relationship of receptor-effector coupling, and the turnover of muscarinic receptors.

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