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METHOCTRAMINE BINDING SITES SENSITIVE TO ALKYLATION ON MUSCARINIC RECEPTORS FROM TRACHEAL SMOOTH MUSCLE

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Abstract—The binding of L-[benzilic-4,4′-^{3}H]quinuclidinyl benzilate was studied in the plasma membrane fraction of bovine tracheal smooth muscle treated with the alkylating agent *N*-ethylmaleimide (NEM). It was found that NEM (2.5 mM) reduced significantly the B_{max} from 1116 to 853 fmol/mg protein and increased the K_D values of the muscarinic acetylcholine receptor (mAchR) activity from 36 to 61 pM. The mAchR subtypes in these plasma membranes were studied using competition experiments with selective antagonists. Pirenzepine displayed low competitive activity, having a pK_i of 6.91 ± 0.03, which was similar to that of AF-DX 116 (11[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6*H*-pyrido[2,3-b][1,4]benzodiazepine-6-one); (pK_i = 6.90 ± 0.04), whereas hexahydrodifenidol (HDD) and its *p*-fluoro-derivative (p-FHHSiD) showed higher affinities than pirenzepine, having pK_i values of 7.45 ± 0.05 and 7.17 ± 0.06, respectively. The antagonist 4-diphenylacetoxy-*N*-methylpiperidine methobromide (4-DAMP) showed a pK_i of 8.25 ± 0.03, which did not differ significantly from the affinity shown by methoctramine (pK_i = 8.00 ± 0.04). These data indicate that the mAchR associated with the plasma membrane fraction isolated from bovine airway smooth muscle can be classified as an M_2 subtype muscarinic receptor. NEM treatment altered the affinities of the mAchR towards specific antagonists, such as methoctramine (K_i increased 3 times), and the results indicated that the alkylated mAchR behaves as a chemically modified M_2 subtype. This suggests the presence of thiol groups controlling the antagonist binding activity of this muscarinic receptor subtype.

Key words: muscarinic receptors; airway smooth muscle; plasma membrane; methoctramine-sensitive SH groups; alkylating reagents on muscarinic receptors; SH groups at muscarinic receptors

Muscarinic acetylcholine receptors (mAchRs†) from tracheal smooth muscle have been classified as a mixed population of M_2 and M_3 subtypes [1–3]. This is in agreement with the finding that tracheal smooth muscle expresses mRNAs coding for both m2 and m3 receptors [4]. To understand the biochemistry and function of these mAchRs, we modified them chemically in a plasma membrane fraction isolated from bovine tracheal smooth muscle, using an alkylating agent, *N*-ethylmaleimide (NEM), and studied the L-[benzilic-4,4′-^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding activity in competition with several specific muscarinic antagonists.

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† Abbreviations: mAchR, muscarinic acetylcholine receptor; NEM, *N*-ethylmaleimide; AF-DX 116, 11[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-b][1,4]benzodiazepine-6-one; HDD, hexahydrodifenidol; p-FHHSiD, *p*-fluoro-hexahydrosiladifenidol; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methobromide; QNB, quinuclidinyl benzilate; and DTT, 1,4-dithiothreitol.

MATERIALS AND METHODS

The following compounds were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.): Trizma base, sucrose, 1,4-dithiothreitol (DTT), Sephadex G-50, atropine sulfate and NEM. Pirenzepine dihydrochloride and AF-DX 116 BS were obtained from Karl Thomae GmbH (Dr. H. Noll). 4-Diphenylacetoxy-*N*-methylpiperidine methobromide (4-DAMP) was a gift from Dr. R. B. Barlow (Bristol, U.K.) and J. L. Neumeyer (RBI, Natick, MA, U.S.A.). QNB and *p*-fluoro-hexahydrosiladifenidol (p-FHHSiD) were purchased from RBI. Methoctramine was donated by Dr. C. Melchiorre (Bologna, Italy). Hexahydrodifenidol (HDD) was a gift from Dr. G. Lambrecht (Frankfurt, F.R.G.). L-[^{3}H]QNB (45.5 Ci/mmol) was obtained from the Radiochemical Centre, Amersham (U.K.).

The plasma membrane fraction (P_1) was prepared as previously described [5, 6]. Before the alkylation experiments, aliquots of P_1 were diluted with 80 vol. of 20 mM Tris–HCl (pH 7.2)–0.5 mM DTT and centrifuged at 150,000 *g* for 30 min; the sediment was suspended in a small volume of incubation buffer (66 mM Tris–HCl, pH 7.8). Routinely, P_1 fraction (1 mg/mL of protein) was incubated for 30 min at 37° in the presence or absence of 2.5 mM NEM. Non-reactive NEM was removed by dilution (10×)

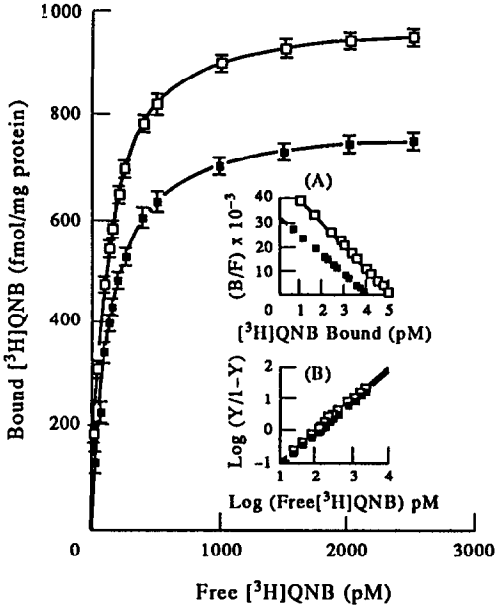

Fig. 1. [^{3}H]QNB saturation curves in bovine tracheal smooth muscle muscarinic receptors in control (□) and NEM-treated (■) membranes. Treatment of membranes and binding assays were performed using 2–3 μg of membrane protein, as described in Materials and Methods. In this typical curve, analysis indicated that QNB binding in native membranes had a $K_{D\text{app}}$ of 109 ± 15 pM and a receptor concentration (B_{max}) of 1007 ± 62 fmol/mg protein. NEM-treated membranes showed a $K_{D\text{app}}$ of 131 ± 13 pM and a B_{max} of 802 ± 31 fmol/mg protein. Data are the means ± SEM of three different membrane preparations, done in triplicate. Inset A: Scatchard plot of [^{3}H]QNB binding. Inset B: Hill plots of [^{3}H]QNB binding.

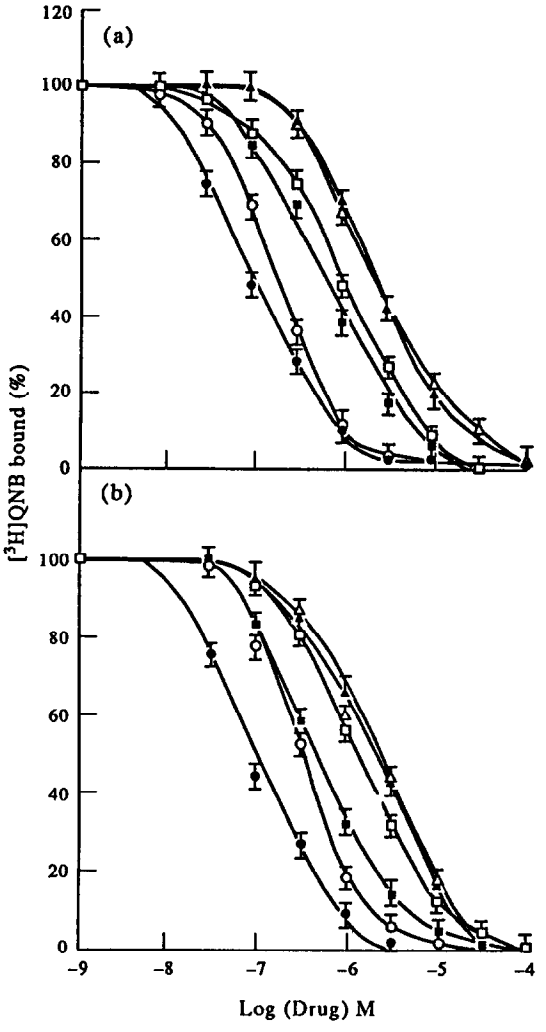

Fig. 2. Comparison of antagonist [^{3}H]QNB competition curves for bovine tracheal smooth muscle in control (a) and NEM-treated (b) membranes. Specific [^{3}H]QNB binding is expressed as a percentage of binding in the absence of antagonist compounds. Key: (●) 4-DAMP; (○) methoctramine; (■) HDD; (□) p-FHHSiD; (▲) pirenzepine; and (△) AF-DX 116 BS. The concentration of [^{3}H]QNB used for these assays was 625 pM, and each drug was incubated with 2–3 μg of membrane protein at 37°. Other details are given in Materials and Methods. Each point represents the mean ± SEM of four different membrane preparations, done in triplicate.

with the incubation buffer and centrifugation at 150,000 *g* for 30 min. The sediment was washed and suspended with the same buffer. The [^{3}H]QNB binding assay [5, 6] was started by adding membrane protein (1.0 to 3.0 μg) in 66 mM Tris–HCl (pH 7.8) and L-[^{3}H]QNB (0.1 to 2.5 nM) for saturation experiments to a final volume of 240 μL; in the case of drug displacement studies, the tubes contained the same buffer and increasing concentrations of unlabeled ligands plus L-[^{3}H]QNB (0.625 nM) to a final vol. of 120 μL. After 30 min of incubation at 37°, the incubation mixture was placed onto a pre-centrifuged Sephadex G-50 column (3 mL) equilibrated with 0.25 M sucrose–5 mM Tris–HCl (pH 7.8) and immediately centrifuged at 700 *g* for 1.5 min to remove free [^{3}H]QNB [5, 6]. The column effluent containing the bound [^{3}H]QNB (protein recovery was 95–98%) was transferred to vials containing 5 mL of Aquasol®. The radioactivity was measured in a RackBeta liquid scintillation counter (LKB, Wallac 1214/1219), and all samples were counted with approximately the same efficiency. Specific binding was calculated by subtracting the non-specific binding (which was less than 1% of total binding measured with 1 μM atropine sulfate) from the total binding [5, 7]. In all binding experiments, no more than 5% of the fixed radioligand concentration was bound to the membranes. Similar amounts of active receptors of control and NEM-treated membranes were employed in the antagonist binding displacement experiments. Protein was determined using bovine serum albumin as standard, as described elsewhere [8]. The program LIGAND [9] was used to analyze saturation isotherms, which allowed the quantification of the receptor site concentration (B_{max}) and the apparent dissociation constants ($K_{D\text{app}}$). The K_D (true values) were

Table 1. pK_i values and pseudo-Hill coefficients (n_H) of muscarinic receptor antagonist binding in native and NEM-modified plasma membranes

	Native membranes		NEM-treated membranes		Ratio
	pK_i	n_H	pK_i	n_H	(K_i)
4-DAMP	8.25 ± 0.03	0.96 ± 0.06	8.08 ± 0.03	0.99 ± 0.04	1.47
Methoctramine	8.00 ± 0.04	1.18 ± 0.04	7.52 ± 0.03*†	1.22 ± 0.04	3.00
HDD	7.45 ± 0.05	1.02 ± 0.03	7.42 ± 0.07	0.91 ± 0.05	1.09
p-FHHSiD	7.17 ± 0.06	0.95 ± 0.15	6.94 ± 0.05	1.03 ± 0.15	1.69
AF-DX 116	6.90 ± 0.04	1.02 ± 0.01	6.75 ± 0.02	0.98 ± 0.08	1.35
Pirenzepine	6.91 ± 0.03	0.92 ± 0.03	6.75 ± 0.02	1.03 ± 0.06	1.42
Atropine	9.27 ± 0.05	0.96 ± 0.07	9.33 ± 0.05	1.01 ± 0.07	1.00

The K_i ratio was calculated using K_i values from NEM-treated and native membranes. Results are the means ± SEM of four experiments performed in triplicate.

* Significantly different ($P < 0.001$) from the pK_i value for methoctramine in native membranes (Student's t-test).

† Significantly different ($P < 0.001$) from the pK_i value for 4-DAMP in NEM-treated membranes (Student's t-test).

determined graphically by plotting experimental $K_{D\text{app}}$ versus receptor concentration and extrapolating to zero receptor concentration, as suggested by Fields *et al.* [7]. To calculate IC_{50}, the data between 10 and 90% of the total binding displacement curve were linearized by computer-assisted linear regression allowing the calculation of IC_{50} and the Hill pseudo-coefficients (n_H) [10]. The K_i values were calculated following the method described by Cheng and Prusoff [11] with estimated IC_{50} by using the K_D values as previously calculated.

RESULTS

We have demonstrated previously the presence of mAchRs in bovine tracheal smooth muscle plasma membranes [5, 6]. In this study, these mAchRs present in the plasma membrane subfraction (P_1) were further characterized pharmacologically. In addition, an evaluation of the [^{3}H]QNB binding parameters of NEM-treated mAchRs was performed. It was found that 2.5 mM NEM significantly reduced the [^{3}H]QNB binding activity as compared with control membranes. This inhibition was not increased by higher concentrations of NEM. A typical saturation binding curve of [^{3}H]QNB to bovine tracheal smooth muscle membranes is shown in Fig. 1. The curve was hyperbolic, reaching saturation at 1 nM [^{3}H]QNB using different receptor concentrations as protein concentrations (1.5, 2.0, 2.5 and 3.0 μg of membrane protein). From all these binding isotherms, we calculated the specific binding parameters for the mAchRs present in these native membranes as having a maximum binding capacity (B_{max}) of 1116 ± 142 fmol/mg protein (N = 9) and a dissociation constant (K_D) of 36 pM. It is important to emphasize that these K_D values were calculated by plotting experimental $K_{D\text{app}}$ vs receptor concentration and extrapolating to zero receptor concentration as described in Materials and Methods. As others have reported [7], a close correlation between $K_{D\text{app}}$ and receptor concentration was found, which is difficult to explain. When this plasma membrane subfraction was treated with 2.5 mM NEM, a reduction of the B_{max} ($P < 0.05$) was observed, decreasing it to 853 ± 59 fmol/mg protein (N = 9), and the K_D values increased to 61 pM. This change in K_D values was additionally supported in saturation experiments using the same amounts of active receptors of both native or alkylated membrane preparations, and we found a 2-fold ratio in the $K_{D\text{app}}$ between NEM membranes and control membranes.

The analysis of the saturation isotherms using Scatchard and Hill plots (Fig. 1, insets A and B), rendered binding parameters for [^{3}H]QNB as n_H close to unity for both experimental conditions, which were not different from the data calculated with the LIGAND program [9]. The data showed that the radioligand binds to a single class of saturable high-affinity binding sites in both control and NEM-treated membranes. The pharmacological profile of [^{3}H]QNB binding was established using selective antagonists, and the specific binding of [^{3}H]QNB to plasma membrane fractions was inhibited in a concentration-dependent fashion by the following antagonists: atropine, 4-DAMP, methoctramine, HDD, p-FHHSiD, AF-DX 116 and pirenzepine (Fig. 2a, Table 1). In each case, competition experiments generated displacement curves with Hill pseudo-coefficients of nearly 1.0. Moreover, for the native plasma membrane fraction, the order of potency of selective antagonists studied was atropine > 4-DAMP = methoctramine > HDD > p-FHHSiD > AF-DX116 = pirenzepine. This indicates a pharmacological profile characteristic of a M_2 muscarinic receptor subtype, as suggested by McIntosh and Blazynski [12]. NEM induced changes in these displacement curves, as shown in Fig. 2b. Thus, the curves of some of the antagonists in NEM-treated membranes were shifted to the right; this change was significant for methoctramine ($P < 0.001$). This pK_i change induced by NEM also altered the behavior of methoctramine with relation to 4-DAMP ($P < 0.001$). In addition, some observable changes were found for p-FHHSiD, AF-DX 116, pirenzepine and 4-DAMP, with no changes being determined for atropine and HDD. Thus, the

relative potency of the antagonists in the NEM-treated membranes was as follows: 4-DAMP > methoctramine ≥ HDD > p-FHHSiD > AF-DX 116 = pirenzepine (Fig. 2b, Table 1).

DISCUSSION

Plasma membrane fractions (P_1-fraction) from bovine tracheal smooth muscle having highly enriched (7 times) mAchR activity, measured as [^{3}H]QNB binding, were obtained using a procedure described previously [5, 6]. In previous studies, two plasma membrane fractions (P_1 and P_2) that were isolated showed a high amount of muscarinic receptor activity, with the P_1 fraction being the most abundant fraction and showing the highest specific activity for [^{3}H]QNB binding. In this study, we demonstrated that this (P_1) plasma membrane fraction contained mAchR subtypes sensitive to NEM (2.5 mM). There is a controversy in the literature concerning the classification of the muscarinic subtypes in tracheal smooth muscle. Thus, the affinity exhibited by pirenzepine correlates well with the absence of the M_1-subtype in this plasma membrane fraction [2, 3]. However, AFDX116 and PZ showed similar antagonist binding behavior, which resembles the pattern displayed in functional studies in intact tracheal smooth muscle described by Garssen *et al.* [13]. The high binding affinity showed by methoctramine suggests the presence of the M_2 subtype. Similarly, based on the potency exhibited by the antagonists, and according to the scheme proposed elsewhere [12, 14, 15], this native plasma membrane fraction from tracheal smooth muscle displays an M_2 mAchR subtype. This fact is supported by a comparison of the pharmacologic profile obtained in these membrane fractions with the antagonist binding profile of m2 mAchRs expressed in CHO-K1 cells transfected with different mAchR subtypes described by Buckley *et al.* [16] and Dorje *et al.* [17]. In addition, these results correlate with those of other studies using methoctramine, which report the presence of the M_2-subtype in the tracheal smooth muscle particulate fractions [3, 18, 19]. However, the presence of M_2 and M_3 mAchRs in particulate preparations from tracheal smooth muscles has been suggested [2, 3]. In our preparation, the only evidence for the existence of the M_3 subtype was the high affinity shown by 4-DAMP relative to the other specific antagonists used, as suggested elsewhere [15].

To understand the molecular and structural basis of drug–receptor interactions, it is necessary not only to compare the affinity constants measured under one condition but also to evaluate the effect of a variety of perturbations such as the chemical or genetic modification of the receptor. For this reason we used a chemical (NEM) treatment (alkylation) of the receptor. The [^{3}H]QNB binding saturation isotherm of NEM-treated tracheal smooth muscle plasma membranes showed a significant reduction of the mAchR binding sites (B_{max}) and a nearly 2-fold increase in the K_D values. It is well known that alkylating and sulfhydryl reagents affect the topology of the macromolecular environment of the mAchR and alter its responses to ligand binding [20]. NEM-treated smooth muscle plasma membranes showed similar behavior, suggesting that the thiol-disulfide state of mAchRs may be involved in the antagonist binding activity, as suggested elsewhere [21]. This thiol-disulfide state of the receptor seems to have a physiological relevance in some tissues. Moreover, it has been reported that NEM discriminates between the chronotropic and inotropic responses to mAchR stimulation in rat atrium [22], and NEM sensitivity may be associated with the receptor molecule as well as G-proteins. It is well known that G-proteins are inactivated by NEM [23], and muscarinic receptors are coupled to G-proteins [15]. The reduction in B_{max} caused by NEM alkylation may be due to a direct effect on the receptor molecule, and it is not likely to be the result of alkylation of receptor-associated G-proteins, which would lead to uncoupling of the receptor–G-protein interactions but not to the reduction in B_{max}. It is well known that mAchRs have some cysteine residues as free SH groups [15] and others involved in disulfide bonds [24, 25]. Consequently, tracheal smooth muscle muscarinic receptor population may also be modified by NEM acting on the receptor molecule itself. Thus, the NEM effect suggested the presence of free SH groups on the surface of the native receptor. In this respect, the possible site of action of NEM could be two cysteine residues found at the transmembrane helix (VII) of the native receptor molecule, which may regulate antagonist binding sites, a possibility recently postulated, using techniques of molecular biology, for m1 mAchRs [26]. Although NEM (2.5 mM) treatment of the tracheal smooth muscle plasma membranes produced differential changes in the muscarinic ligand displacement curves, the relative potency of the antagonists remained close to the control profile. However, the behavior exhibited by methoctramine is probably due to the divalent ligand nature of this compound, and it has been proposed that this polymethylene tetramine binds to two similar, if not identical, vicinal receptor sites [27]. In summary, these data suggest that multiple classes of NEM-reactive thiol groups are present in the mAChRs associated with these plasma membrane smooth muscle preparations. Reaction of some of these decreases the B_{max} and the affinity for [^{3}H]QNB, whereas reaction of others alters antagonist binding, specifically methoctramine affinity. These observations support the hypothesis that methoctramine interacts with receptor domains that are additional to those of the other antagonists studied here.

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