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Comparative Binding Studies with Cholinergic Ligands and Histrionicotoxin at Muscarinic Receptors of Neural Cell Lines

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SUMMARY

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[^{3}H]Scopolamine and [^{3}H]quinuclidinyl-benzilate (QNB) have been used to study inhibitory acetylcholine receptors of neuroblastoma clone N1E-115 and excitatory acetylcholine receptors of NG108-15 neuroblastoma × glioma hybrid cells. Both [^{3}H]ligands bind with high affinity to muscarinic acetylcholine receptors of the cells. The apparent dissociation constants are 0.4 nM (N1E-115) and 0.5 nM (NG108-15) for [^{3}H]scopolamine, 0.06 nM (N1E-115) and 0.1 nM (NG108-15) for [^{3}H]QNB. The receptor concentration is 25 fmol/mg in N1E-115 and 40 fmol/mg in NG108-15. Binding and release of [^{3}H]-scopolamine are kinetically biphasic processes. [^{3}H]QNB has a similar rate of binding but a much slower rate of release from the receptor. Binding of both [^{3}H]ligands is competitively inhibited by compounds known to interact with muscarinic acetylcholine receptors. With 1 nM [^{3}H]ligand a 50% inhibition is caused by nanomolar concentrations of muscarinic antagonists, by 1 to 100 micromolar concentrations of muscarinic agonists, and by >100 micromolar concentrations of nicotinic cholinergic compounds. Slopes of approximately 1 were found for receptor antagonists and approximately 0.5 for receptor agonists in logit-log plots of competition data. Antagonist binding can therefore be described as interaction with a noncooperative class of receptors, whereas agonist binding exhibits negative cooperativity or heterogeneity in binding sites. The interaction of dihydroiso-histrionicotoxin (H_2-HTX) with muscarinic acetylcholine receptors of N1E-115 cells has also been studied by inhibition experiments. H_2-HTX inhibits [^{3}H]scopolamine binding in a noncompetitive manner causing a 50% inhibition at an applied concentration of 70 μM. The local anesthetic, tetracaine, shows almost identical characteristics. Dihydro-adaline, granatan-3β-ol and granatan-3α-ol are less strong inhibitors. Structural comparison of these compounds with H_2-HTX suggests that the two aliphatic side chains and the proximity of the hydroxyl and amino groups may be important features for the interaction of H_2-HTX with the muscarinic acetylcholine receptor.

INTRODUCTION

Cloned cell lines which possess exclusively one type of acetylcholine (ACh)[2] receptors have been developed in recent years. For instance, neuroblastoma clone N1E-115 has inhibitory muscarinic ACh re-

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[2] The following abbreviations are used: ACh, acetylcholine; QNB, quinuclidinyl benzilate; HTX, histrionicotoxin; H_2HTX, dihydroiso-histrionicotoxin; H_{12}-HTX perhydro-histrionicotoxin.

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ceptors. Upon stimulation with ACh, these cells respond with a membrane hyperpolarization and an increase in the level of cGMP (1). NG108-15 neuroblastoma × glioma hybrid cells, on the other hand, possess excitory muscarinic ACh receptors. They react to ACh-induced stimulation with a membrane depolarization and a decrease in the level of cAMP (22).

Cultured cells serve as valuable tools for investigating the ligand binding properties of individual types of ACh receptors and the possible interaction of new natural neurotoxins and synthetic compounds with these receptors. In the present work [^{3}H]-scopolamine has been introduced as a suitable radioactive ligand for monitoring muscarinic cholinergic binding. [^{3}H]Quinuclidinyl benzilate (QNB) has also been used in experiments described here. The binding properties of receptors in N1E-115 and NG108-15 cells toward known cholinergic agonists and antagonists have been investigated by measuring their ability to inhibit binding of the radioactive ligands. Labeled cholinergic ligands with high specific radioactivity previously have been used to demonstrate receptor-ligand binding in homogenates of brain and smooth muscle (3–5). Finally, the binding of dihydroiso-histrionicotoxin (H_2HTX, Fig. 1b) to muscarinic ACh receptors has been studied. The structurally related compounds, dihydro-adaline (Fig. 1c), granatan-3α-ol, granatan-3β-ol (Fig. 1d, e), and two local anesthetics have also been tested for their receptor affinity.

Histrionicotoxin (HTX, Fig. 1a) is an alkaloid isolated from the skin of the tropical frog, *Dendrobates histrionicus* (6). Albuquerque and coworkers have shown that HTX and its perhydro derivative (H_2-HTX) inhibit the ACh-elicited depolarization of mammalian and amphibian nerve-muscle preparations (7, 8). In the presence of the toxin, the depolarization produced by repeated application of ACh is progressively reduced. The blocking action of HTX was therefore explained in terms of an interaction with the cholinergic ionophore in its activated form. H_2-HTX also inhibits the depolarization produced by carbamylcholine at the monocellular electroplax of *Electrophorus electricus* (9) and it inhibits the ACh receptor-dependent Na^+ transport of cultured muscle cells (10). In the presence of H_2-HTX, the binding of agonists, such as ACh (9) and carbamylcholine (10), to the nicotinic ACh receptor is enhanced. Since the local anesthetics, prilocaine and dimethisoquin, also enhance ACh binding to the receptor, it was suggested that, at the receptor level, H_2-HTX acts as a powerful local anesthetic (9). From a recent study it was concluded that H_2-HTX inhibits the nicotinic ACh receptor by enhancing agonist-induced desensitization of the receptor (10).

MATERIALS AND METHODS

Compounds. [^{3}H](−)Scopolamine (11 Ci/mmol) was prepared in the following way (11). (−)Scopolamine hydrobromide (Sigma) was O-acetylated and N-demethylated (12, 13). After removal of the acetyl group, the *nor*-scopolamine was remethylated with [^{3}H]methyl iodide (New England Nuclear) and purified by thin layer chromatography on a Silica Gel F 254 plate, 0.25 mm thickness (EM Laboratories) in the solvent system chloroform-acetone-diethylamine (5:4:1). The product had a radiochemical purity of ≥95% as determined by thin layer chromatography in three solvent systems (hexane-toluene-diethylamine 4:1:1, ethanol-acetic acid-water 6:3:1, and the system used for purification) and scanning of the chromatograms (Autoscanner 880, Vanguard). As was shown by Schmidt *et al.* (12), the reactions used in this labeling procedure do not cause racemization at the asymmetric center of (−)scopolamine. The labeled alkaloid is therefore the pure pharmacologically active (−)enantiomer. [^{3}H](+/−)Quinuclidinylbenzilate (QNB) (8 Ci/mmol) was obtained from New England Nuclear. H_2-HTX was provided by Dr. J. Daly, Laboratory of Chemistry, NIAMDD, NIH, who isolated it from the Colombian frog, *Dendrobates histrionicus* (6). Dihydro-adaline was synthesized by Dr. E. Gössinger, Laboratory of Chemistry, NIAMDD, NIH. Granatan-3α-ol and granatan-3β-ol were gifts from Dr. G. Fodor, University of West Virginia, Morgantown, West Virginia. Other drugs were obtained from the following sources: ACh chloride, eserine sulfate, atropine sulfate,

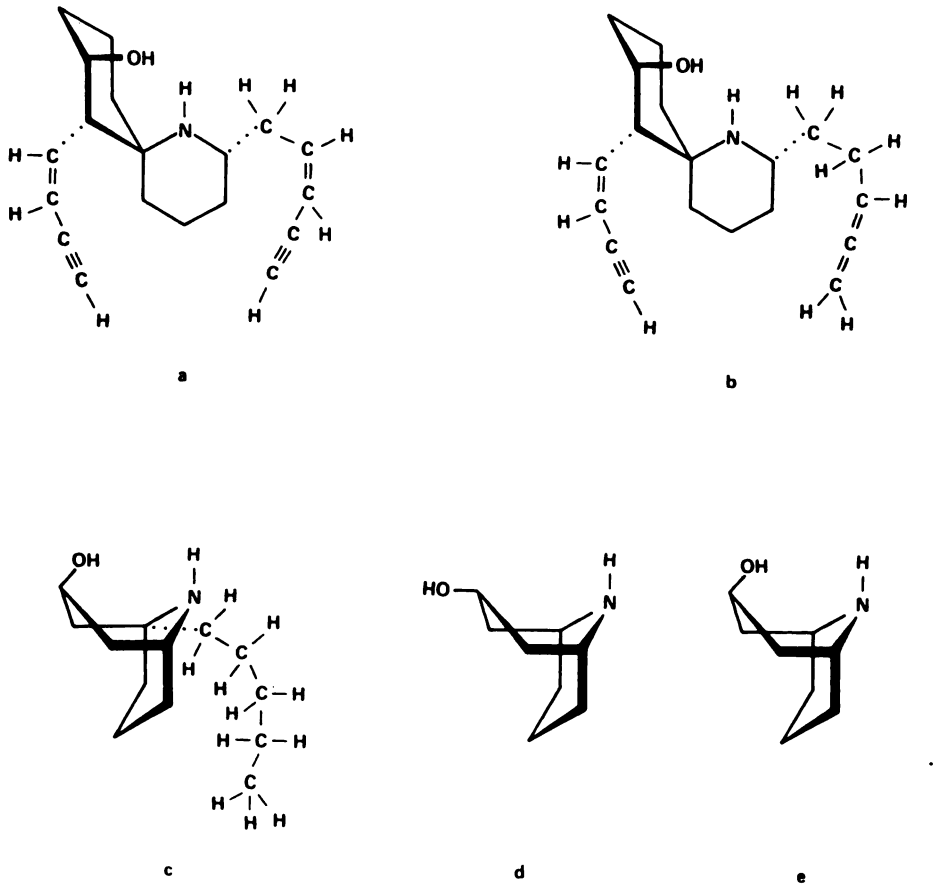

FIG. 1. *Molecular structures of histrionicotoxin (a), dihydroiso-histrionicotoxin (b), dihydro-adaline (c), granatan-3α-ol (d), and granatan-3β-ol (e)*

pilocarpine chloride, carbamylcholine chloride, (−)scopolamine hychochloride, d-tubocurarine chloride from Sigma, decamethonium bromide from K & K Laboratories, (+/−) QNB from Roche, oxotremorine from Aldrich.

Cell lines. Neuroblastoma cell line, N1E-115, was derived from the mouse C-1300 tumor and has been described previously (14). Neuroblastoma × glioma hybrid cell line, NG108-15, was obtained (2) by fusion of mouse neuroblastoma clone, N18TG-2 (15) and rat glioma clone, C6BU-1 (16), derived from C6 (17). Cells of subculture 5 to 20 were grown in plastic flasks of 75 cm^2 growth area (Falcon) as described (18).

[^{3}H]Ligand binding assay. The cells were collected for assays as follows. The attached cell layer was washed three times in the culture flask, each wash with 10 ml *buffer 1* (composition: 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 5.5 mM dextrose, 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes), adjusted to pH 7.4 with NaOH, and to 340 mosm/liter with NaCl). The cells were detached from the flask by addition of 2 times 3 ml *buffer 2* (composition: buffer 1 plus 2 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N′-tetraacetic acid (EGTA), readjusted to pH 7.4 with NaOH) and moderate shaking of the culture flask. Cells were counted in a hemacytometer to determine their concentration, and the cell suspension was diluted to about 10^6 cells/ml with buffer 2. Protein concentration was determined after the binding assay by precipitating a portion of the cell suspension with 5% trichloroacetic acid, centrifuging, dissolving in 1 N NaOH, and applying the

Lowry method with bovine serum albumin as a standard (19).

To assay [^{3}H]ligand total binding, 0.5 ml samples of the cell suspension were mixed with 0.5 ml buffer 2 containing the [^{3}H]ligand. After incubation at 24°C (60 min unless otherwise stated), 5 ml buffer 2 was added to the mixture, and the suspension was immediately filtered by vacuum through a glass fiber paper filter (GF/C, 2.4 cm diameter, Whatman). The filter was washed three times, each time with 5 ml buffer 2, placed in a counting vial, and dried for 3 hours at room temperature. Then 10 ml scintillation cocktail (Instagel, Packard) was added, and the vial was shaken. After an equilibration time of at least 3 hours, the radioactivity was measured by liquid scintillation spectrometry (Beckman LS 250) at a counting efficiency of 40%.

For determining nonspecific [^{3}H]ligand binding, the [^{3}H]scopolamine solutions used also contained unlabeled scopolamine (100 μM final concentration in the reaction mixture), whereas [^{3}H]QNB solutions contained oxotremorine (1 mM final concentration in the reaction mixture). Under these conditions, specific binding of the labeled ligands to receptors was completely inhibited, and their nonspecific binding to other membrane structures was not affected. This was shown by measuring the displacement of labeled ligands by increasing concentrations of up to 10 mM unlabeled scopolamine and oxotremorine. Specific [^{3}H]ligand binding is defined as total binding of [^{3}H]ligand minus nonspecific binding.

In order to test for competitive or noncompetitive inhibition, [^{3}H]ligand solutions were used containing increasing concentrations of [^{3}H]ligand and zero or a fixed concentration of the inhibiting unlabeled compound.

For determination of the association rate, equal volumes of the cell suspension and the [^{3}H]ligand solution were mixed at time zero. At indicated times, 1 ml samples of the mixture were diluted into 5 ml buffer 2 at 24°C, filtered, and immediately washed as described.

In order to determine the dissociation rate, equal volumes of cell suspension and [^{3}H]ligand solution were mixed and preincubated for 60 minutes. Then 100 μM unlabeled ligand was added (without changing the volume of the assay mixture by more than 1%) at time zero, and 1 ml samples were taken at indicated times and assayed as described. All experiments were carried out with duplicate or triplicate samples. The standard error in the replicates was usually 5–10% and, in some cases, 15%.

RESULTS

Dependence of [^{3}H]ligand binding on protein concentration and pH. When 1 nM [^{3}H]scopolamine is applied to cell suspensions, specific binding is proportional to protein concentration in the range 0–550 μg of protein (Fig. 2). The pH dependence of binding is shown in Fig. 3. Specific binding of [^{3}H]scopolamine to N1E-115 cells and [^{3}H]QNB to NG108-15 cells increases between pH 7 and 8. The maximum specific binding found within the range tested with the tritiated ligands was at pH 8.

Time course of association and dissociation. The rate of [^{3}H]scopolamine binding is shown in Fig. 4A. Half-maximum specific binding under the experimental conditions used is attained after 2.5 min. The rate of nonspecific binding decreases sharply at 3 min. Since the concentration of free [^{3}H]scopolamine remains virtually unchanged, the association can be treated as a pseudo first-order reversible reaction. The corresponding semilogarithmic plot is shown in Fig. 4B. It can be interpreted in terms of two intersecting lines, indicating two binding processes with different rate constants. From the slopes of the two lines, the association rate constants $k^I_{on} = 2.3 \times 10^8$ M^{-1} min^{-1}, and $k^{II}_{on} = 1.1 \times 10^8$ M^{-1} min^{-1} are obtained.

The rate of dissociation of [^{3}H]scopolamine from the receptor was studied after adding a 10^5 fold excess of non-radioactive scopolamine (Fig. 5). A fast and a slow dissociation of ([^{3}H]scopolamine · ACh receptor) can be seen. The half-life of the fast and slow dissociating components which comprise approximately 25% and 75% of the specific binding are 3.8 and 11 minutes, respectively. The dissociation rate constants are $k^I_{off} = 1.8 \times 10^{-1}$ min^{-1} and k^{II}_{off}

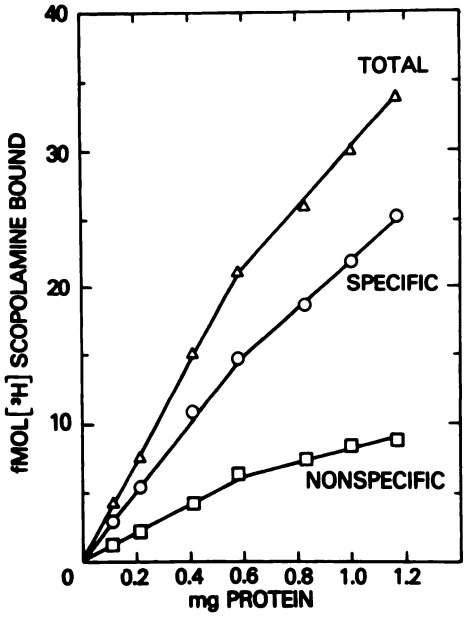

FIG. 2. *Binding of [^{3}H]scopolamine to N1E-115 cells as a function of cell protein concentration*

Cells were incubated for 60 min at 24° with 1 nM [^{3}H]scopolamine (for total binding) or with 1 nM [^{3}H]scopolamine plus 100 μM unlabeled scopolamine (for nonspecific binding).

= 6.3×10^{-2} min^{-1}. From the ratio of the respective rate constants, the following values of the dissociation equilibrium constant, K_D are obtained: k^I_{off}/k^I_{on} = 0.8 nM; k^{II}_{off}/k^{II}_{on} = 0.6 nM. [^{3}H]QNB binds to the receptor at a similar rate as [^{3}H]scopolamine. Its dissociation is slower, however, requiring more than 2 hours to be complete (experiments not shown here).

Saturability of specific binding. With increasing concentration of [^{3}H]scopolamine, specific binding reaches a saturation value. By contrast, nonspecific binding is proportional to the concentration of [^{3}H]ligand (Fig. 6A, B). Scatchard plots of [^{3}H]scopolamine specific binding obtained with N1E-115 and NG108-15 cells are shown in Fig. 6C. They reveal the presence of a high and a low affinity binding site in both cell lines. Values of the dissociation constants, K_D, and the concentration of binding sites, as evaluated from the Scatchard plots, are listed in Table 1.

With N1E-115 cells and [^{3}H]scopolamine, a value of K_D = 0.4 nM for the high affinity site is obtained from the Scatchard

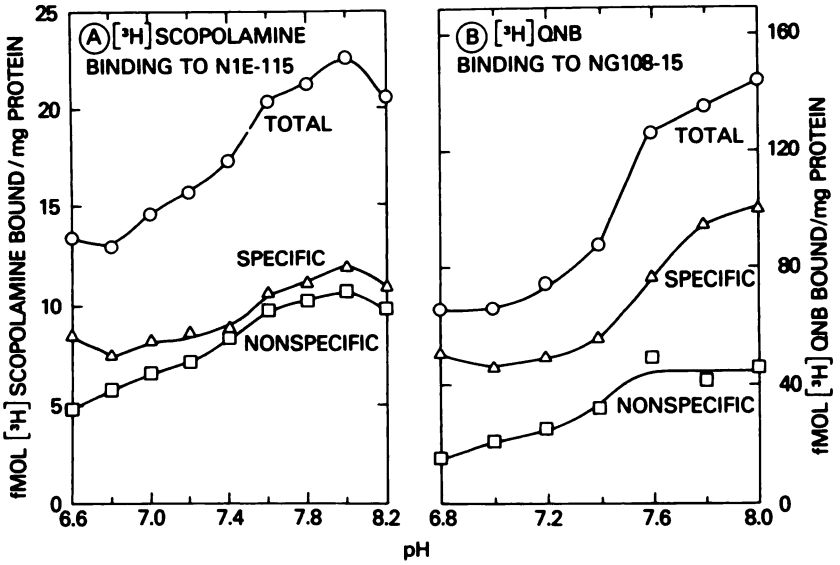

FIG. 3. *pH dependence of labeled ligand binding to cells*

Buffer solutions of the indicated pH values were prepared by varying the NaOH and NaCl concentrations of buffer 2 while maintaining an osmolarity of 340 mosm/liter. *A*, Samples containing 0.26 mg N1E-115 cells and 1 nM [^{3}H]scopolamine (for total binding) or 1 nM [^{3}H]scopolamine plus 100 μM unlabeled scopolamine (for nonspecific binding) were incubated for 60 min at 24°. *B*, Samples containing 0.44 mg NG108-15 cells and 1.2 nM [^{3}H]QNB (for total binding) or 1.2 nM [^{3}H]QNB plus 1 mM oxotremorine (for nonspecific binding) were incubated for 60 min at 24°.

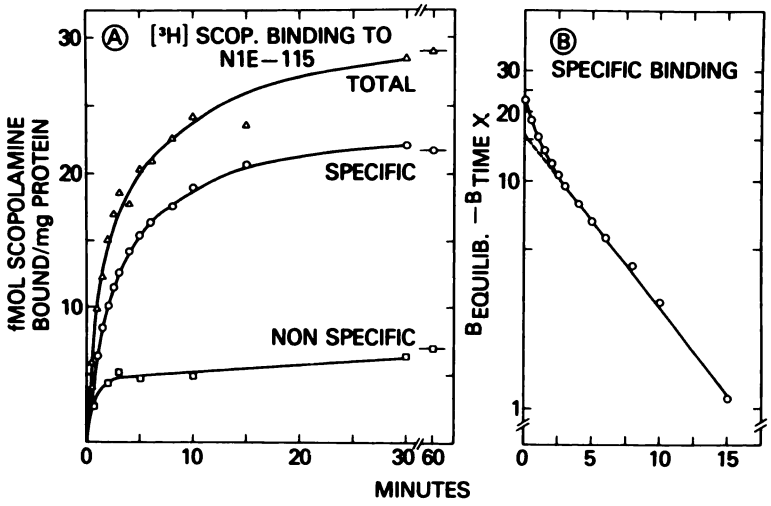

FIG. 4. *Rate of [³H]scopolamine binding to N1E-115 cells*

0.86 mg/ml cells were incubated at 24° with 1 nM [³H]scopolamine (for total binding) or 1 nM [³H]-scopolamine plus 100 μM unlabeled scopolamine (for nonspecific binding). At indicated times 1 ml samples were taken and assayed. *A*, Total, nonspecific and specific binding as a function of incubation time. *B*, The difference of specific binding at equilibrium and at the indicated times is plotted on a logarithmic scale as a function of incubation time. Values have been calculated for the data of part *A*.

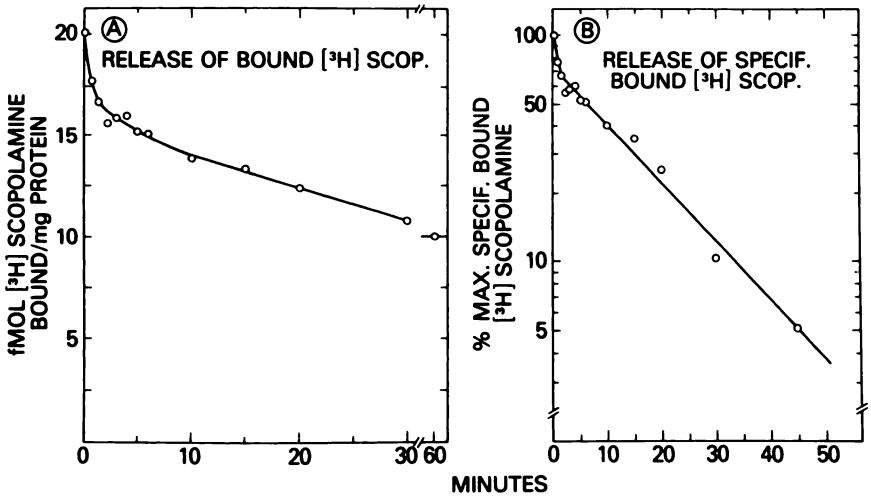

FIG. 5. *Rate of [³H]scopolamine release from N1E-115 cells*

0.84 mg/ml cells were incubated with 1 nM [³H]scopolamine for 60 min at 24°. Then 100 μM unlabeled scopolamine was added (= zero time) and 1 ml samples were taken at indicated times and assayed. *A*, Total binding as a function of time after addition of unlabeled scopolamine. *B*, Specific binding, on a logarithmic scale, is plotted as a function of time after addition of unlabeled scopolamine.

plot. This is in good agreement with the kinetically determined K_D values. Specific [³H]QNB binding also saturates, and [³H]QNB has a higher affinity than scopolamine for the receptors of each cell line (data not shown). Experiments have not been done with very high [³H]QNB concentrations to test for binding to a low affinity site. The number of high affinity binding sites is the same for [³H]scopolamine and

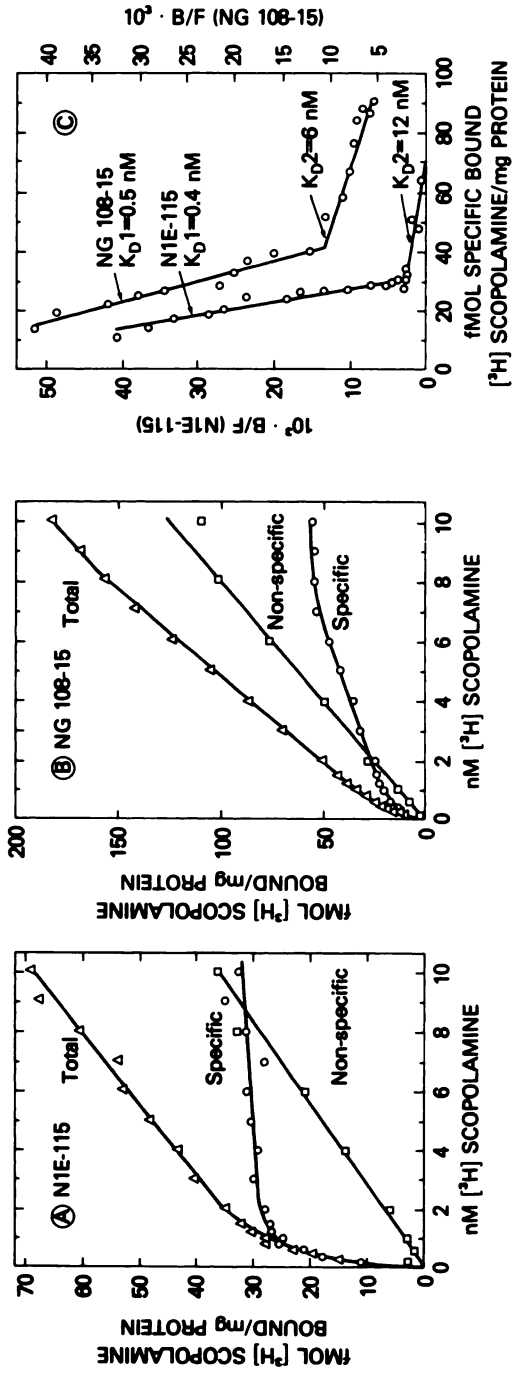

FIG. 6. *Binding of [³H]scopolamine to cells as a function of its concentration*

A, Samples containing 0.76 mg N1E-115 cells and increasing concentrations of [³H]scopolamine were incubated for 60 min at 24°. Nonspecific binding was determined in the presence of 100 μM unlabeled scopolamine. *B*, Samples containing 0.61 mg NG108-15 cells and increasing concentrations of [³H]scopolamine were assayed as described under A. *C*, Scatchard plots of the specific binding data, covering a wider range of [³H]scopolamine concentration (0.2–100 nM) than plots shown in parts A and B (0.2–10 nM).

TABLE 1

Binding data of [^{3}H]scopolamine and [^{3}H]QNB obtained from Scatchard plots

Cell line	[^{3}H]Ligand	High affinity binding site			Low affinity binding site		
		K_D	Concentration	Approximate Number of binding sites per cell (10^6 cells ≈1 mg protein ($\times 10^4$)	K_D	Concentration	Approximate Number of binding sites per cell (10^6 cells ≈1 mg protein ($\times 10^4$)
		nM	*fmol/mg protein*		*nM*	*fmol/mg protein*	
N1E-115	(−)Scopolamine	0.4	30	1.8	12	42	2.5
	(−)QNB	0.06	22	1.3	—	—	—
NG108-15	(−)Scopolamine	0.5	43	2.6	6	110	6.6
	(−)QNB	0.1	40	2.5	—	—	—

[^{3}H]QNB. A given ligand binds with similar affinity to the receptors of either cell line, as expressed by the similar K_D values.

Inhibition of [^{3}H]ligand binding by cholinergic ligands. In these experiments, increasing concentrations of cholinergic ligands were included in the assay mixture containing 1 nM [^{3}H]QNB or [^{3}H]scopolamine and 0.5–1.0 mg cell protein. Inhibition of [^{3}H]ligand specific binding as a function of the concentration of unlabeled ligands is shown for both cell lines in Fig. 7. In order to investigate the type of inhibition, binding of increasing concentrations of labeled ligand was determined in the absence and presence of a fixed concentration of oxotremorine. Double reciprocal plots of these data yield straight lines which intersect on the ordinate, indicating competitive inhibition of [^{3}H]scopolamine and oxotremorine or [^{3}H]QNB and oxotremorine (Fig. 8). The concentration curves of Fig. 7 are therefore interpreted as describing the concentration-dependent binding of the unlabeled ligands to the ACh receptor.

For a given cholinergic ligand, similar displacement curves of [^{3}H]ligand binding by unlabeled ligand were obtained with the two cell lines. The data demonstrate that muscarinic ACh receptor antagonists (QNB, scopolamine, atropine) have a higher affinity for the ACh receptor than the ACh receptor agonists (oxotremorine, pilocarpine, acetylcholine, carbamylcholine). Nicotinic ACh receptor ligands (*d*-tubocurarine, decamethonium) exhibit the lowest affinity.

The shape of the displacement curves shows that saturation of agonist binding extends over a broader concentration range than saturation of antagonist binding. This relationship is seen more clearly in Fig. 9, which shows logit-log plots of the competition data. Logit-log plots are analogous to Hill plots used in analyzing the binding of a single ligand. Although not identical with the Hill coefficient, the slope of a logit-log plot provides the same qualitative index of binding complexity.[3] With antagonists, slopes of approximately 1 (0.82 to 1.13) are obtained. On the other hand, agonists have slopes of about 0.5 (0.32 to 0.81). Individual values are listed in Table 2. A clearcut difference between antagonist binding and agonist binding thus emerges from these competition data.

Binding of antagonists can be described by the simple mass action equation:

$$R + L \underset{K_D}{\rightleftharpoons} RL \qquad (1)$$

where R, L, RL are symbols for the receptor, antagonist, and receptor-antagonist

[3] Personal comments by Dr. David Rodbard; *cf.* Rodbard, D. (1970) Acta Endocrinol. Suppl. *147*, 79–103; Rodbard, D. (1977) in *Health and Medical Physics* (Baarli, I., ed.), 204–219.

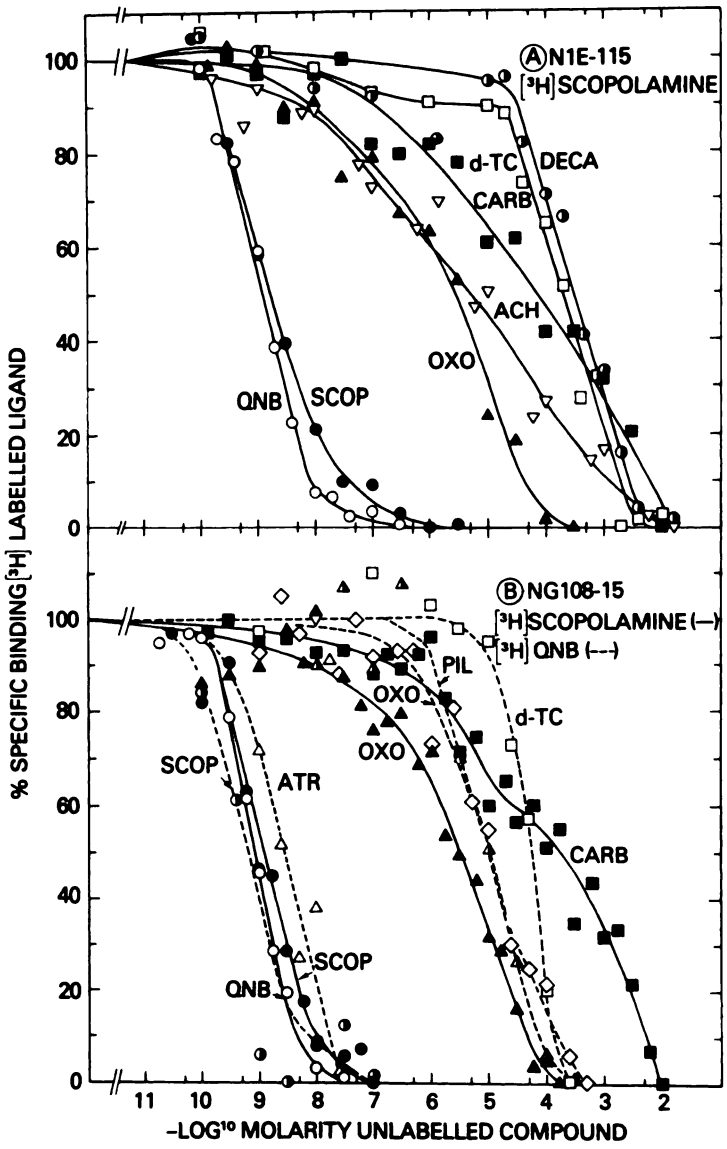

FIG. 7. *Inhibition of labeled ligand specific binding to cells by various cholinergic compounds*

Cells were incubated for 60 min at 24° with indicated concentrations of unlabeled cholinergic compounds plus 1 nM [^{3}H]scopolamine or 1.2 nM [^{3}H]QNB. Nonspecific binding has been subtracted from total binding and % maximum specific binding has been plotted. Maximum specific binding of N1E-115 cells varied from 16 to 33 fmol/mg and of NG108-15 from 30 to 54 fmol/mg. *A*, N1E-115 cells; inhibition of [^{3}H]scopolamine binding. Cell samples were as follows: 0.50 mg for scopolamine (—●—) and oxotremorine (—▲—), 1.0 mg for QNB (—○—), 1.10 mg for acetylcholine (—▽—), 0.60 mg for carbamylcholine (—■—). 0.75 mg for d-tubocurarine (—□—) and decamethonium (—◑—). *B*, NG108-15 cells; inhibition of [^{3}H]scopolamine (——) or [^{3}H]QNB (- - -) binding. The following cell samples were used: 0.50 mg for scopolamine (—●—), 0.50 mg of scopolamine (- -◑- -), 0.52 mg for QNB (—○—), 0.38 mg for atropine (- -△- -), 0.48 mg for oxotremorine (—▲—), 0.50 mg for oxotremorine (- -▲- -), 0.38 mg for carbamylcholine (—■—), 0.50 mg for pilocarpine (- -◇- -).

complex. The dissociation constant, K_D, for an unlabeled antagonist can be determined from inhibition of labeled antagonist binding. At equilibrium, binding of the unlabeled and the labeled antagonist can be described by the mass action equations

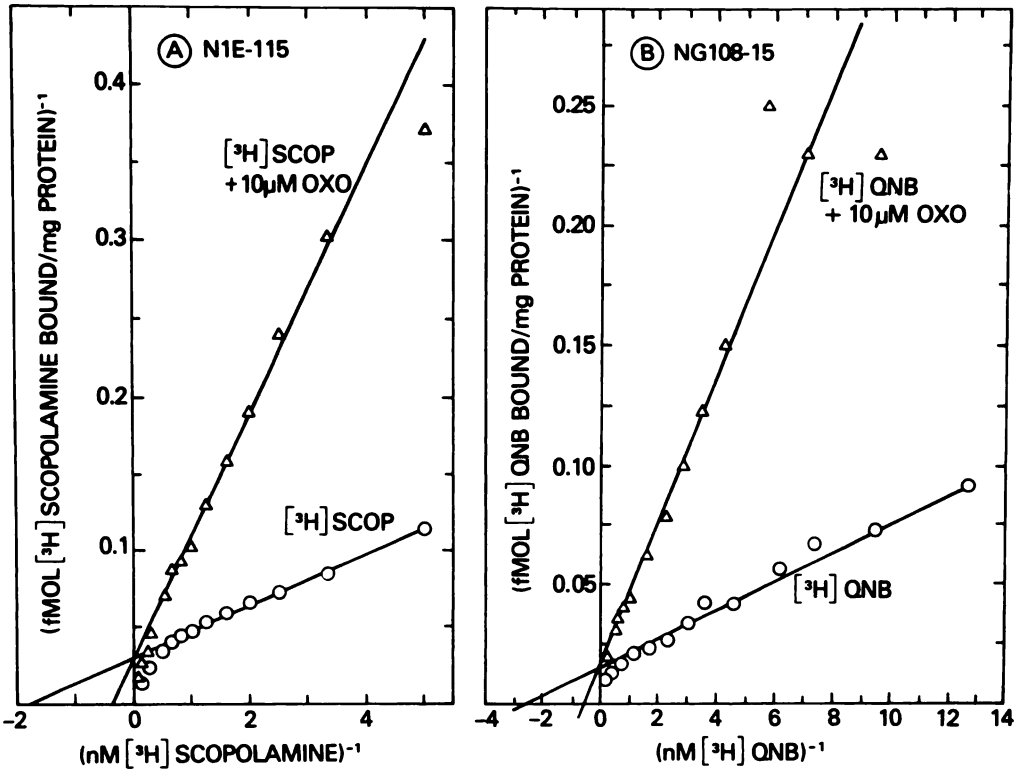

FIG. 8. *Inhibition pattern of oxotremorine*

Total binding of increasing concentrations of [³H]ligand was determined as described in legend of Fig. 6, either in absence of oxotremorine or in presence of 10 μM oxotremorine. Binding data are presented as double reciprocal plots. *A*, N1E-115 cells (0.50 mg); inhibition of [³H]scopolamine binding. *B*, NG108-15 cells (0.85 mg); inhibition of [³H]QNB binding.

$$K_D = \frac{[R][L]}{[RL]} \quad (1)$$

$$K_D^* = \frac{[R][L^*]}{[RL^*]} \quad (2)$$

(* refers to the labeled antagonist).

By combining equations (1) and (2), one obtains the expression

$$K_D = \frac{K_D^*\,[RL^*][L]}{[L^*][RL]} \quad (3)$$

Since K_D^* and $[RL^*]$ are known, K_D for the unlabeled antagonist can be calculated from equation (3) using the conservation substitutions:

$$[L^*] = [L_T^*] - [RL^*]$$

$$[RL] = [R_T] - K_D^* \frac{[RL^*]}{[L^*]} - [RL^*]$$

$$[L] = [L_T] - [RL]$$

(L_T^*, R_T, and L_T are the total concentrations added of radioactive ligand, receptor, and unlabeled antagonist, respectively.)

Binding of agonists appears more complex than binding of antagonists, as indicated by logit-log plots with slopes of less than one. In order to describe the data empirically, a modified form of Eq. (1) has been proposed (20):

$$K = \frac{[R][L]^n}{[RL]} \quad (1a)$$

This empirical equation, which is independent of molecular mechanisms, allows one to approximate agonist-receptor affinity. At a concentration of ligand giving 50% saturation of receptors (L_{50}), Eq. (1a) yields the expression

$$K = [L_{50}]^n$$

The constant K, and hence L_{50}, can be

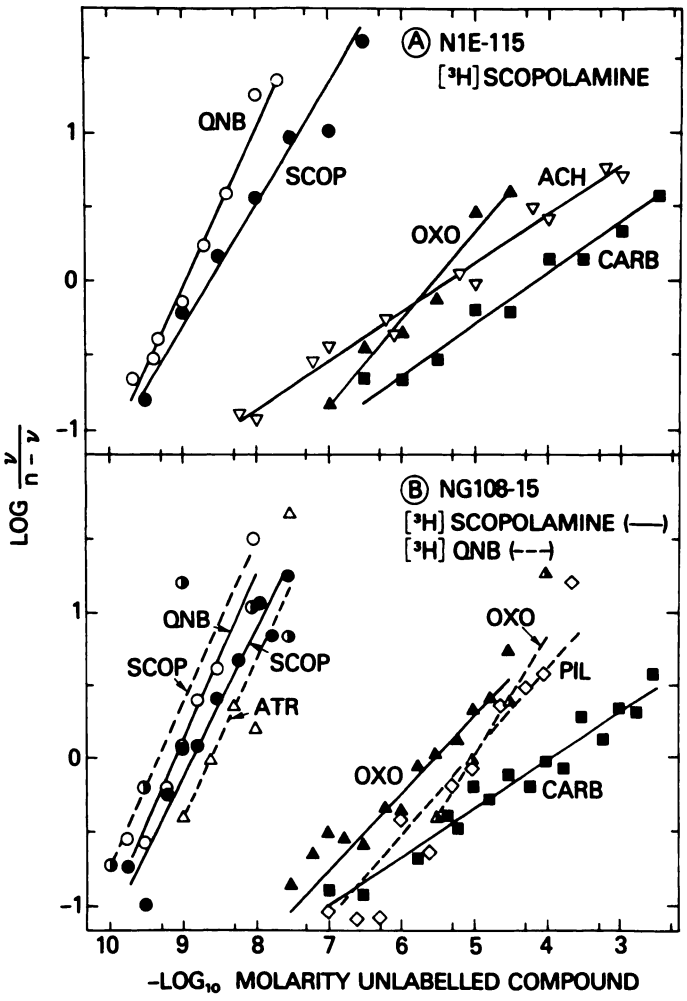

FIG. 9. *Logit-log plots of inhibition of [^{3}H]ligand binding by cholinergic ligands*

Data of Fig. 7 were used for these plots. In the ordinate, the symbol n represents the maximum amount of [^{3}H]ligand displaced at saturating concentrations of unlabeled cholinergic compound; v is the amount of [^{3}H]-ligand displaced at nonsaturating concentrations of cholinergic compound. *A*, N1E-115 cells; inhibition of [^{3}H]-scopolamine binding of QNB (—○—), scopolamine (—●—), oxotremorine (—▲—), acetylcholine (—▽—), carbamylcholine (—■—). *B*, NG108-15 cells; inhibition of [^{3}H]scopolamine (——) or [^{3}H]QNB (- - - -) binding by QNB (—○—), scopolamine (—●— and - -◑- -), atropine (- -△- -), oxotremorine (—▲— and - -▲- -), pilocarpine (- -◇- -), carbamylcholine (—■—).

calculated in a manner analogous to the procedure described above for obtaining K_D values by substituting Eq. (1a) for Eq. (1). The calculation provides a measure of the concentration of the unlabeled agonist necessary to occupy one-half of the receptor sites in the absence of the competing labeled ligand.

In Table 2, K_D values for the antagonists and L_{50} values for the agonists have been calculated from the competition data. IC_{50} values (the ligand concentration necessary to inhibit binding of a given concentration of labeled ligand by 50%) and "n" values (slopes of logit-log plots) also are presented. Receptors found on the two cell lines have an affinity for muscarinic ligands nearly identical to the affinity of brain muscarinic receptors previously investigated using [^{3}H]QNB (4, 5). Antagonists bind especially

TABLE 2

Hill coefficients and binding affinity data of cholinergic compounds

Compound	Cell line	[^{3}H]Ligand used for determination	Hill coefficient	IC_{50}[a]	K_D[b]	$[L_{50}]$[c]
			n	*μM*	*nM*	*μM*
(±)QNB	NG108-15	Scopolamine	1.13	0.0008	0.25	
	N1E-115	"	1.07	0.0011	0.31	
Scopolamine	NG108-15	Scopolamine	0.99	0.0013	0.45	
	N1E-115	"	0.82	0.0024	0.68	
(±)Atropine	NG108-15	QNB	1.06	0.0025	0.23	
Oxotremorine	NG108-15	QNB	0.81	10.0		0.52
	"	Scopolamine	0.52	2.9		0.34
	N1E-115	"	0.59	2.7		0.33
Pilocarpine	NG108-15	QNB	0.56	9.0		0.12
ACh	N1E-115	Scopolamine	0.33	4.3		0.10
Carbamylcholine	NG108-15	Scopolamine	0.32	115		4.2
	N1E-115	"	0.34	67		1.3

[a] IC_{50} is the concentration of cholinergic compound which inhibits binding of 1 nM [^{3}H]scopolamine or 1.2 nM [^{3}H]QNB to receptor by 50%.

[b] K_D is the apparent dissociation constant of the cholinergic compound-receptor complex, calculated from competition data as described in text.

[c] $[L_{50}]$ is the equilibrium concentration of free cholinergic compound at 50% saturation of its binding to receptor, calculated from competition data as described in text.

well, with calculated dissociation constants for the active stereoisomers ranging from 0.1 to 0.7 nM. Calculated values for the concentrations of agonists required to half-occupy receptor sites ranged from 0.1 μM for acetylcholine, the most potent agonist tested, to 4.2 μM for carbamylcholine, the least potent agonist tested.

Inhibition of [^{3}H]scopolamine binding by H_2-HTX and related compounds. Displacement experiments with H_2-HTX and structurally related compounds were performed as described before, with [^{3}H]scopolamine as the labeled ligand (Fig. 10). Fifty percent inhibition of [^{3}H]scopolamine binding was obtained by either 70 μM H_2-HTX, 250 μM dihydro-adaline, 4 mM granatan-3β-ol or >10 mM granatan-3α-ol.

With regard to the postulated analogies between the action of H_2-HTX and local anesthetics (9), inhibition experiments also were carried out with tetracaine and cocaine. The inhibition curve obtained with tetracaine is almost superimposable on the curve obtained with H_2-HTX, indicating a very similar influence of both substances on receptor properties. Cocaine shows a weaker effect, producing 50% inhibition at 1 mM.

It should be noted that in Fig. 10, the ordinate represents total binding of [^{3}H]-scopolamine. H_2-HTX, dihydro-adaline, and the local anesthetics inhibit not only specific but also nonspecific binding of [^{3}H]scopolamine. The cholinergic ligands, on the other hand, inhibit only specific binding of the [^{3}H]ligand.

Figure 11 shows double reciprocal plots of [^{3}H]scopolamine specific binding in the absence and presence of fixed concentrations of either H_2-HTX or tetracaine. In both cases, straight lines are obtained which do not intersect on the ordinate, indicating that H_2-HTX and tetracaine are noncompetitive inhibitors of [^{3}H]scopolamine binding.

H_2-HTX has been shown to increase the affinity of the nicotinic ACh receptor for agonists (9, 10). In order to test whether H_2-HTX has an analogous effect at the

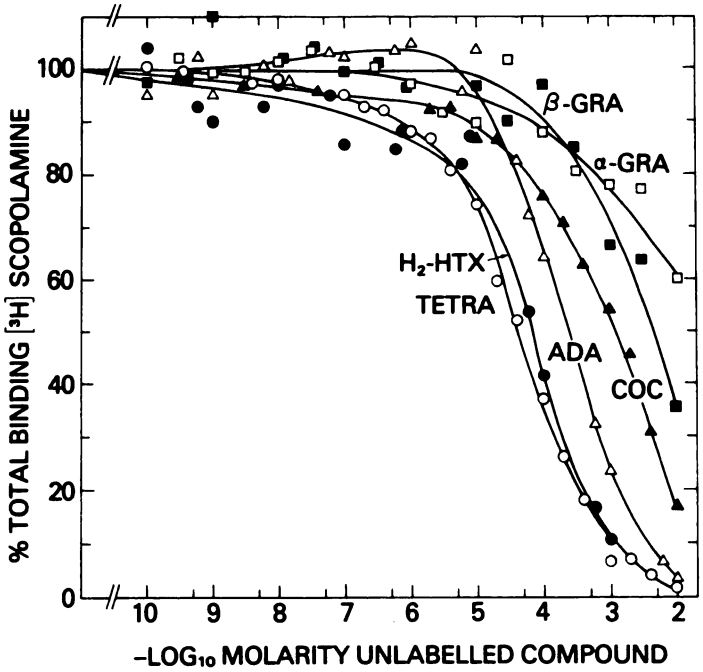

FIG. 10. *Inhibition of [³H]scopolamine binding to N1E-115 cells by H_2-HTX and various other compounds.* Cells were incubated for 60 min at 24° with increasing concentrations of the inhibiting compounds and 1 nM [³H]scopolamine. The following cell samples were used; 0.69 mg of tetracaine (—○—), 1.10 mg for H_2-HTX (—●—), 1.18 mg for dihydro-adaline (—△—), 0.68 mg for cocaine (—▲—), 1.2 mg for granatan-3α-ol (—□—), and granatan-3β-ol (—■—).

muscarinic ACh receptor of N1E-115 cells, the experiment represented in Table 3 was carried out. 10 μM H_2-HTX or 200 nM ACh were applied separately and inhibited the binding of 1 nM [³H]scopolamine by 32% and 43%, respectively. Both compounds combined, at the same concentrations as above, produced a 38% inhibition. These results indicate that H_2-HTX does not cause an increase of affinity of the muscarinic receptor for agonists.

DISCUSSION

The results have shown that [³H]scopolamine can be used as a convenient labeled ligand for studying muscarinic ACh receptors. The alkaloid binds to receptors of two cloned cell lines, one with inhibitory (N1E-115), the other with excitatory ACh receptors (NG108-15): (i) with high affinity, (ii) in a saturable and reversible manner, and (iii) with kinetics which are compatible with a filtration assay.

The dissociation constants of [³H]scopolamine at two cell lines (K_D = 0.4 and 0.5 nM, respectively) are somewhat higher than those of the synthetic muscarinic antagonist, [³H]QNB (K_D = 0.06 and 0.1 nM, respectively). Binding site concentrations of both cell lines determined with either [³H]scopolamine or [³H]QNB, are found in good agreement (Table 1). N1E-115 cells and NG108-15 cells have 25 and 40 fmol muscarinic binding sites per mg protein, respectively. As reported by Yamamura and Snyder, the concentration of specific [³H]QNB binding sites ranges from 35 to 478 fmol/mg protein in different regions of rat brain (4) and from 28 to 208 fmol/mg protein in various peripheral tissues (lung, spleen, heart, and ileum) of guinea pig (5). According to Hulme *et al.* (21), a synaptosome preparation from rat cerebral cortex contains 1.7 pmol muscarinic binding sites per mg protein. Thus, the two cell lines used in this study have a comparatively low content of muscarinic receptors.

Binding of both labeled ligands occurs at

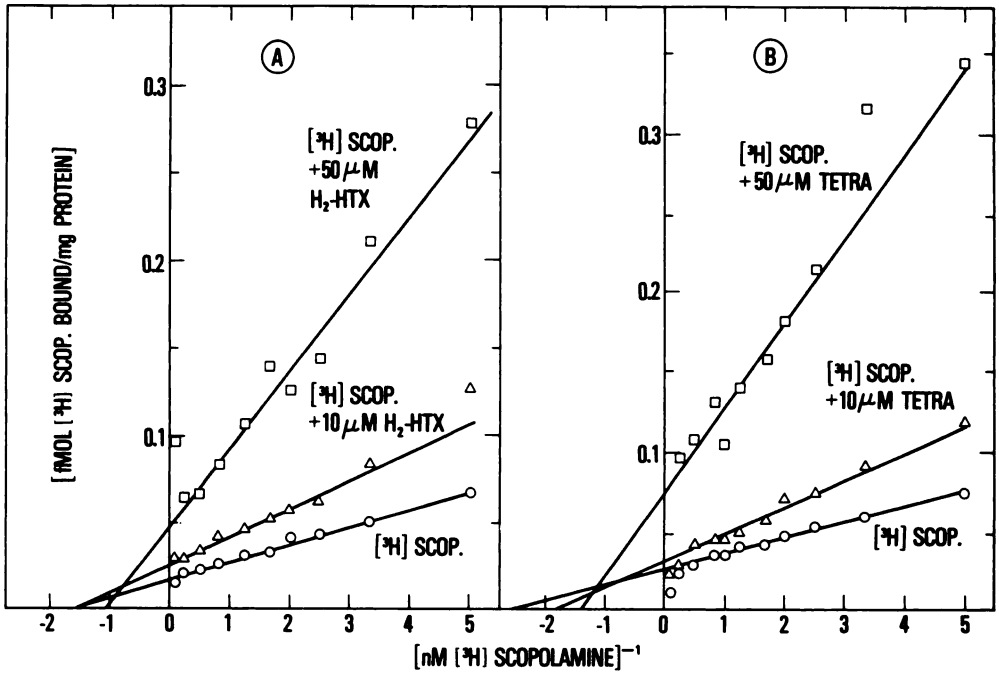

FIG. 11. *Inhibition pattern of H_2-HTX (A) and tetracaine (B)*

N1E-115 cells (A: 1.04 mg; B: 0.53 mg) were incubated for 60 min at 24° with increasing concentration of [^{3}H]scopolamine in the absence and presence of fixed concentrations of DHTX or tetracaine, respectively. Nonspecific binding of [^{3}H]scopolamine was determined in presence of 100 μM unlabeled scopolamine and has been subtracted. Specific binding of [^{3}H]scopolamine is presented in double reciprocal plots.

TABLE 3

Test for cooperativity of H_2-HTX and ACh binding to N1E-115 cells

Applied concentrations of unlabeled compounds	[^{3}H]Scopolamine specific binding[a]	% Inhibition of [^{3}H]scopolamine specific binding
	fmol/mg protein	
None	16.2	0
2 nM ACh	17.7	0
200 nM ACh	9.2	43
10 μM H_2-HTX	11.0	32
2 nM ACh plus 10 μM H_2-HTX	10.0	38
200 nM ACh plus 10 μM H_2-HTX	10.0	38

[a] Average of triplicate samples.

similar rates, reaching saturation within 30 to 60 min (Fig. 4). Dissociation from the receptors is, however, considerably faster with [^{3}H]scopolamine (~60 min) (Fig. 5) than with [^{3}H]QNB (>120 min). Binding as well as dissociation of [^{3}H]scopolamine are kinetically biphasic processes suggesting the existence of at least two states of the excitatory and inhibitory muscarinic ACh receptors.

As was mentioned in the INTRODUCTION, N1E-115 cells and NG108-15 cells produce opposite responses upon stimulation of their muscarinic ACh receptors. Ligand binding properties of both cell lines, however, are similar. As shown by the inhibition of labeled ligand binding with various cholinergic compounds (Fig. 7), the receptors clearly exhibit muscarinic specificity. Antagonists are bound with higher affinity (IC_{50} in the nanomolar range) than agonists (IC_{50} in the range of 1 to 100 micromolar). The receptor affinity for nicotinic cholinergic compounds is low (IC_{50} in the range of 100 micromolar) (Table 2).

The K_D values determined for the antagonists, QNB, scopolamine, and atropine agree well with reported data on the binding of these compounds to muscarinic sites of other biological materials. These include

rat brain homogenate (4), subcellular fractions of rat cerebral cortex (2), and homogenate of longitudinal muscle of guinea pig ileum (5). The IC_{50} values determined for the agonists, oxotremorine, pilocarpine, ACh, and carbamylcholine, are dependent on the concentration of the radioactive ligand used in the displacement experiments. If this is corrected for, they are in good agreement with IC_{50} values reported for these compounds with the tissues mentioned above (4, 5). Thus, muscarinic receptors have very similar ligand binding properties whether they occur in these cultured cell lines or in various tissues.

As shown by double-reciprocal plots, the agonist oxotremorine and the antagonists [^{3}H]scopolamine and [^{3}H]QNB compete for the same binding site (Fig. 8). An important difference between antagonist and agonist binding is revealed, however, by logit-log plots of the competition data obtained with both cell lines (Fig. 9). Antagonists bind with "n" values (slope of logit-log plot equals "n") of about 1, while agonists bind with "n" values of about 0.5 (Table 2). Birdsall and Hulme have reported similar findings describing [^{3}H]ligand binding to receptors of brain homogenate (3). An "n" value of 1 can be explained by binding of antagonists to a noninteracting homogeneous class of receptor sites. However, several mechanisms could account for the "n" values of 0.32 to 0.81 as observed with agonists (22): (i) molecular heterogeneity of agonist binding sites, (ii) negative cooperativity between agonist binding sites, (iii) agonist-induced change of receptor affinity (i.e., receptor desensitization), (iv) change of affinity of the receptor for agonists upon coupling to an effector. The present data do not distinguish between these mechanisms. As reported by Hulme *et al.* (21, 23), negative cooperativity and receptor desensitization can be ruled out as possible reasons for the low Hill coefficients they observe for muscarinic agonist binding to brain subcellular fractions. According to these authors, agonist binding data can be interpreted in terms of saturation of a high affinity and a low affinity binding site. By comparison of the respective binding constants with pharmacological activities of the agonists, they postulate that the high affinity binding site represents the uncoupled receptor, and the low affinity binding site represents the receptor coupled to an effector. A detailed model of agonist and antagonist interaction with muscarinic acetylcholine receptors of NG108-15 will be presented elsewhere.[4] Further experiments, however, are required to specifically prove or disprove the various possible mechanisms of heterogeneous agonist binding mentioned before.

Another aspect of the present work concerns the influence of DHTX and related compounds on ligand binding to muscarinic acetylcholine receptors. Detailed studies with H_2-HTX so far have employed only the N1E-115 cell line. It is of interest for future studies to examine interaction of H_2-HTX with the depolarizing muscarinic receptors of NG108-15. Half-maximal inhibition of [^{3}H]scopolamine binding to N1E-115 cells is produced by 70 μM H_2-HTX. Thus H_2-HTX is as potent as carbamylcholine and only slightly less potent than other muscarinic agonists in blocking scopolamine binding to muscarinic receptors (Table 2 and Fig. 10). H_2-HTX is more potent as a muscarinic blocker, with respect to binding, than the nicotinic ligands tested. Without further evidence, however, we cannot as yet say whether or not H_2-HTX is acting directly at the scopolamine binding site. The noncompetitive nature of the inhibition (shown in Fig. 11) suggests the possibility that H_2-HTX may be acting at a site distinct from the receptor binding site, and H_2-HTX may be some way be masking binding sites indirectly.

Interaction of H_2-HTX with nicotinic acetylcholine receptor systems has been studied elsewhere in some detail. H_2-HTX has about the same potency in inhibiting *alpha*-bungarotoxin binding to nicotinic receptors as we have observed for inhibition of scopolamine binding to muscarinic receptors (24). On the other hand, histrionicotoxins interact at much lower concentrations with other binding sites. H_{12}-HTX binds with a K_d of 0.4 μM to a protein fraction from *Torpedo ocellata* electric or-

[4] Klein, W. L. & Nirenberg, M., manuscript in preparation.

gan which could be separated from the ACh binding protein (25). H_2-HTX inhibits the depolarization produced by carbamylcholine on isolated electroplax of *Electrophorus electricus* with a K_i of 0.8 μM (9) and inhibits ACh receptor-dependent Na+ transport of cultured muscle cells with a K_i of 0.2 μM (10). It seems quite likely that, in the nicotinic system, the ion conductance block caused by histrionicotoxins probably results from binding to a site different from the ACh recognition site (7). If H_2-HTX action on the muscarinic system is analogous to its action on the nicotinic system, we might predict physiological inhibition of muscarinic activation at even lower doses than observed for inhibition of scopolamine binding. Inhibition by H_2-HTX of muscarinic receptor activation has not yet been tested, however.

Interaction of H_2-HTX with nicotinic and muscarinic receptors is dissimilar in at least one respect. H_2-HTX (1 μM) causes an increase of carbamylcholine binding to the nicotinic receptor of cultured muscle cells (10). It was suggested that H_2-HTX increases the affinity of the desensitized form of the receptor for agonists and thereby stabilizes the desensitized state of the nicotinic receptor. By contrast, the binding of ACh to the muscarinic receptor is not increased even in the presence of 10 μM H_2-HTX, as indicated by the data of Table 3.

There is a striking similarity between the interaction of H_2-HTX and tetracaine with the muscarinic ACh receptor. Very similar concentrations of both compounds inhibit specific as well as nonspecific [^{3}H]scopolamine binding (Fig. 10). As shown by double reciprocal plots (Fig. 11), both compounds act as noncompetitive inhibitors of [^{3}H]scopolamine binding. At the nicotinic ACh receptor of cultured muscle cells, however, tetracaine does not, in analogy to H_2-HTX, cause an increase of affinity for agonists (unpublished results).

A decrease of affinity to the receptor is observed in the series H_2-DHTX dihydroadaline, granatan-3β-ol, granatan-3α-ol (Fig. 10). These compounds have similar bicyclic structural elements with a secondary amino and a secondary alcohol function. While in DHTX two aliphatic side chains are attached to the ring system, dihydro-adaline has only one aliphatic side chain, and the granatanols have none. Thus, the observed order of receptor affinity suggests that lipophilic side chains contribute to the receptor binding of these compounds.

As can be seen from molecular models, the amino and hydroxyl groups of H_2-HTX and dihydro-adaline are in very similar and close conformational arrangements, permitting a hydrogen bond interaction. The hydroxyl group of granatan-3β-ol has the same conformation as in dihydro-adaline whereas, in granatan-3α-ol it points in the opposite direction and cannot interact with the amino group. Since granatan-3α-ol has a lower affinity to the receptor than granatan-3β-ol, a certain orientation of the hydroxyl group, probably in relation to the amino group, appears to be important for causing receptor blockade.

Note added in proof: In this report we have filled a need described in a recent review [J. Patrick, S. Heinemann and D. Schubert, *Ann. Rev. of Neuroscience*, **1**, 417–443 (1978)] by introducing the use of cloned cell lines for studies of ligand interaction with muscarinic acetylcholine receptors. The usefulness of such studies in cloned cell lines of neuronal origin is underscored by recent behavioral studies implicating muscarinic receptors in human learning [Sitaram, N., Weingartner, H. and Gillin, J. C. (1978) *Science*, **201**, 274–276]. Our equilibrium data, obtained with cloned cells, reveal receptor properties that are identical with the equilibrium characteristics described in the extensive studies of A. S. V. Burgen *et al.* [*Molec. Pharmacol.* **14**, (1978)] with brain homogenate, while our kinetic studies have uncovered new properties of muscarinic receptors.

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