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Comparison of the Muscarinic Receptor Binding Activity of Some Tertiary Amines and Their Quaternary Ammonium Analogues

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SUMMARY

A series of tertiary amines and their *N*-methyl quaternary salts were examined for their ability to inhibit specific [^{3}H]3-quinuclidinyl benzilate binding to rat brain muscarinic receptors. The more flexible tertiary amines, like dimethylaminoethyl acetate, were less potent than their respective quaternary ammonium analogues, while rigid tertiary amines, like aceclidine, were more potent than their quaternary derivatives. The competition curves of most of the compounds were adequately described by a two-site binding equation. A good correlation between pharmacological activity and the high-affinity dissociation constant was observed. The influence of pH on the competitive inhibition of [^{3}H]3-quinuclidinyl benzilate binding by arecoline and scopolamine was also examined. The potency of these amines declined relative to that of their *N*-methyl derivatives as the pH increased from 8.0 to 9.0, suggesting that it is primarily the protonated form of arecoline and scopolamine which interacts with the muscarinic receptor.

INTRODUCTION

Since several cholinergic tertiary amines exist primarily in the protonated form at physiological pH, the difference between the muscarinic activity of most tertiary amines and their *N*-methyl quaternary ammonium derivatives cannot be explained on the basis of charge. The *N*-desmethyl tertiary amine derivatives of acetylcholine and its close structural analogues are much less biologically active than the parent quaternary ammonium compounds (1-3). In contrast, some semirigid tertiary amines, including aceclidine, arecoline and oxotremorine, are more potent than their *N*-methyl quaternary salts (4-6). Thus, for those compounds whose quaternary ammonium analogues are more potent than their tertiary derivatives, the basic nitrogen atom is contained on a flexible aliphatic chain, whereas it forms part of a relatively rigid ring in those tertiary amines which are more potent than their *N*-methyl salts.

In the present study, muscarinic receptor binding assays were used to investigate the affinity of a series of tertiary amines and their *N*-metho quaternary salts (see Fig. 1). The results of our binding experiments are in agreement with previous pharmacological data (4-6) and demonstrate that, although acetylcholine and similar analogues are more potent than the corresponding *N*-desmethyl tertiary amines, the converse is true for more rigid compounds. We have also investigated the influence of pH on muscarinic receptor binding and have obtained results consistent with the postulate that the charged forms of arecoline and scopolamine are the active pharmacological species.

METHODS

Rat striatum, hippocampus, and brain stem were used in muscarinic receptor binding assays. These brain regions were homogenized in 50 mM sodium-potassium phosphate buffer (81 mM Na^+, 9.5 mM K^+, 50 mM PO_4) at pH 7.4 with a Potter-Elvejhem glass homogenizer and Teflon pestle. The final homogenate concentrations for the brain stem, striatum, and hippocampus were 40, 5, and 5 mg of original wet tissue weight per milliliter of phosphate buffer. In experiments investigating the influence of pH on receptor binding, brain homogenates were made up in 50 mM sodium TAPS buffer ($pK_a = 8.2$).

[^{3}H]QNB[1] (29 Ci/mmole, New England Nuclear Corporation, Boston, Mass.) binding was measured according to the rapid filtration method of Yamamura and Snyder (7), with minor modifications. Routinely, brain homogenate (100 μl) was incubated with [^{3}H]QNB in a final volume of 2 ml containing 50 mM sodium-potassium phosphate buffer (pH 7.4). Incubations were carried out for 60 min at 37°, and binding in the presence of 10 μM atropine was considered nonspecific. For measurement of the competitive inhibition of [^{3}H]QNB binding by nonlabeled ligands, a total concentration of 0.8 nM [^{3}H]QNB was used. At this concentration, less than 5% of the total [^{3}H]QNB was bound. Physostigmine (10 μM) was included in binding assays when compounds susceptible to hydrolysis by cholinesterase were investigated. The addition of physostigmine to the assay did not markedly influence the competition curves of nonhydrolyzable agonists and antagonists. During experiments investigating the effect of pH on muscarinic receptor binding, TAPS buffer was used as the incubation medium. All of the cholinergic analogues used in this study were either synthesized as described previously (4, 5) or obtained from commercial sources and recrystallized.

The binding parameters were determined from the experimental data by nonlinear least-squares regression analysis. The agonist/[^{3}H]QNB competitive inhibition data were fitted to the following two-site

[1] The abbreviation used is: QNB, (±)-3-quinuclidinyl benzilate.

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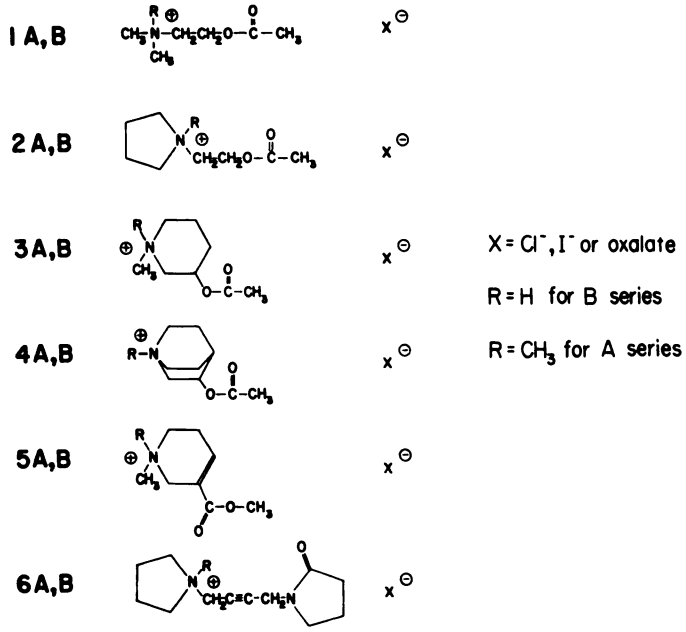

FIG. 1. *Structures of the various tertiary amines and their quaternary ammonium derivatives used in this study*

1A, Acetylcholine; *1B*, acetyldeanol; *2A*, *N*-(2-acetoxyethyl)-*N*-methylpyrrolidinium; *2B*, *N*-(2-acetoxyethyl)pyrrolidine; *3A*, *N,N*-dimethyl-3-piperidinyl acetate; *3B*, *N*-methyl-3-piperidinyl acetate; *4A*, *N*-methylaceclidine; *4B*, aceclidine; *5A*, *N*-methylarecoline; *5B*, arecoline; *6A*, *N*-methyloxotremorine, *6B*, oxotremorine.

competitive inhibition equation (8):

$$B = \frac{a}{1 + x/K_H'} + \frac{1 - a}{1 + x/K_L'} \quad (1)$$

where B is the proportion of [^{3}H]QNB bound, a is the proportion of high-affinity sites, K_H' and K_L' are the apparent dissociation constants of the high- and low-affinity sites, and x is the concentration of nonlabeled agonist. In some instances, the competition data were adequately described by the following one-site competitive inhibition equation:

$$B = \frac{1}{1 + x/K'} \quad (2)$$

where K' is the apparent dissociation constant of the nonlabeled ligand. The apparent dissociation constants (K') were corrected to give the true dissociation constants (K) using the following relationship:

$$K = K'/(1 + y/K_{QNB}) \quad (3)$$

In Eq. 3, y is the concentration of [^{3}H]QNB and K_{QNB} is the dissociation constant of [^{3}H]QNB, which was determined to be 0.14 nM. This value was calculated independently by nonlinear regression analysis of five-point [^{3}H]QNB binding isotherms. The concentration of nonlabeled inhibitor giving half-maximal receptor occupation (X_{50}) was calculated from the IC_{50} value (concentration of inhibitor that caused half-maximal inhibition of specific [^{3}H]QNB binding) according to the following relationship:

$$X_{50} = IC_{50}/(1 + y/K_{QNB}) \quad (4)$$

For the competition curves that were adequately described by the one-site equation (Eq.2), IC_{50} and X_{50} are essentially equivalent to K' and K, respectively.

RESULTS

Preliminary experiments showed that the ability of oxotremorine to compete with [^{3}H]QNB for muscarinic receptors varied in different regions of the brain (Fig. 2). The concentrations of oxotremorine resulting in half-maximal receptor occupation in the striatum, hippocampus, and brain stem were 1.03, 1.3, and 0.20 μM, respectively. The oxotremorine/[^{3}H]QNB competition curves deviated from the single-site equation, but nonlinear regression and analysis of variance showed that the data were adequately described by a two-site competitive inhibition model (Eq. 1). Regression analysis yielded similar values for the K_H and K_L of oxotremorine in the striatum (3.8 nM and 1.4 μM), hippocampus (4.4 nM and 1.6 μM), and brain stem (5.1 nM and 0.55 μM). In contrast, the percentage of high-affinity sites varied in the brain stem (30%), striatum (14%), and hippocampus (14%). Other investigators have found a qualitatively similar distribution of high- and low-affinity agonist sites within the brain (9, 10). Since the K_H and K_L of oxotremorine were similar in the three brain regions, and the brain stem contained the greatest proportion of high-affinity sites, this tissue was used in the remainder of the experiments. The greater contribution of high-affinity sites in the brain stem enabled a more accurate estimate to be made of the binding parameters of nonlabeled ligands.

The various tertiary amines and their *N*-metho quaternary derivatives shown in Fig. 1 were tested in muscarinic receptor binding assays, and a summary of the results of these experiments is shown in Fig. 3. The concentration of each analogue resulting in half-maximal receptor occupation (X_{50}) is given in Table 1 and was calculated from the IC_{50} for competitive inhibition of [^{3}H]QNB binding. Table 1 also shows the ratio of pharmacological activities of each pair as determined previously in the isolated guinea pig ileum (4, 5). These binding data on rat brain stem are consistent with the pharmacological activity of these ligands in the guinea pig ileum in the sense that the analogue which has the greater pharmacological activity also has greater potency in the [^{3}H]QNB binding assay.

Most of the competition curves shown in Fig. 3 are not

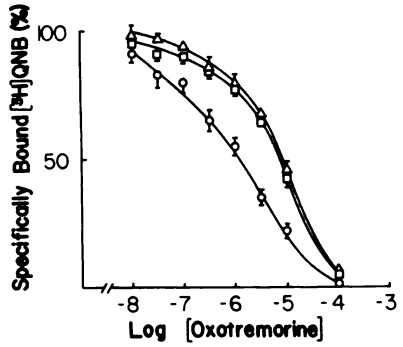

FIG. 2. *Competitive inhibition of [^{3}H]QNB binding by oxotremorine in the brain stem* (○), *hippocampus* (△), *and striatum* (□)

Mean binding values ± the standard error of the mean are shown. The theoretical curves are the least-squares fit to the data, assuming a two-site model.

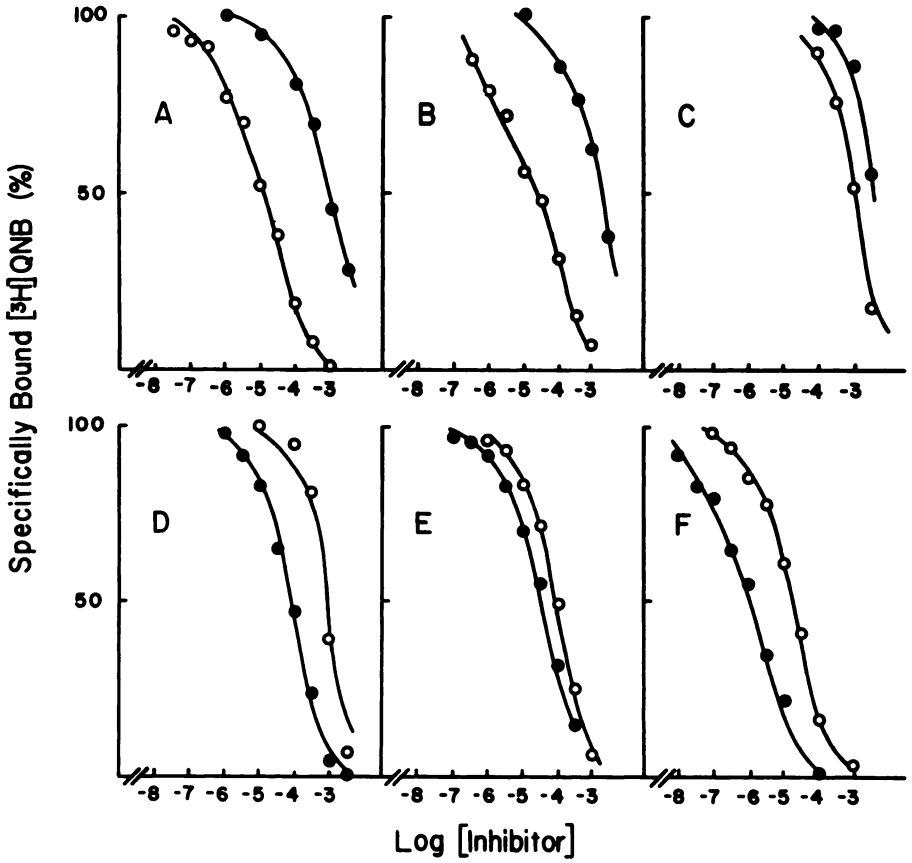

FIG. 3. *Competitive inhibition of [^{3}H]QNB binding to rat brain stem by a series of tertiary amines* (●) *and their quaternary derivatives* (○)
The following tertiary amines together with their *N*-methyl derivatives were investigated: A, acetyldeanol; B, *N*-(2-acetoxyethyl)pyrrolidine; C, *N*-methyl-3-piperidinyl acetate; D, aceclidine; E, arecoline; and F, oxotremorine. The data points represent the mean binding values of at least three experiments, each performed in triplicate. The theoretical curves are the least-squares fit to the data.

as steep as that expected for a simple one-site model. Preliminary regression analysis showed that the competition curves of *N,N*-dimethyl-3-piperidinyl acetate (*3A*), *N*-methyl-3-piperidinyl acetate (*3B*), and *N*-methylaceclidine (*4A*) were sufficiently described by the one-site model. For the other analogues, analysis of varience showed a significant reduction ($p < 0.05$) in residual error when the data were fitted to a two-site model as

TABLE 1
Binding parameters of tertiary amines and their quaternary derivatives

Compound	X_{50}[a]	K_H	K_L	K_L/K_H	$\frac{K_H \text{ (tertiary)}}{K_H \text{ (quaternary)}}$	Pharmacological activity[b] (tertiary/quaternary)
		μmoles/liter				
Acetylcholine	2.5	0.12	6.0	51	155	115
Acetyldeanol	130	18	320	18		
N-(2-Acetoxyethyl)-*N*-methylpyrrolidinium	3.5	0.097	13	139	390	398
N-(2-Acetoxyethyl) pyrrolidine	280	38	630	17		
N,N-Dimethyl-3-piperidinyl acetate	160	160		1	4.5	6.6
N-Methyl-3-piperidinyl acetate	700	700		1		
N-Methylaceclidine	120	120		1		
					0.020	0.0046
Aceclidine	12	2.3	24	10		
N-Methylarecoline	16	3.2	27	8.4	0.20	0.056
Arecoline	6.3	0.62	14	23		
N-Methyloxotremorine	2.8	0.35	6.3	18	0.020	0.10
Oxotremorine	0.20	0.0070	0.63	90		

[a] The X_{50} values are the geometric means of estimates from at least three experiments.
[b] The pharmacological activity is the ED_{50} for stimulation of contractions of the guinea pig ileum, taken from refs. 4 and 5.

compared with a one-site model. The estimate of the proportion of high-affinity sites varied among the compounds and had a mean and standard deviation of 24 ± 9%. Since errors in the estimate of the affinity and proportion of the high-affinity site are correlated, we reasoned that it would be informative to reanalyze the data assuming a fixed proportion of high-affinity sites. This constraint seemed valid since previous experiments on muscarinic receptors in various regions of the rat brain have suggested that the proportion of high-affinity sites within a given region is constant and independent of the ligand (10). To establish an accurate value for the proportion of high-affinity sites, the average estimate for the high-affinity component of the competition curves of those compounds [acetylcholine (*1A*), *N*-(2-acetoxyethyl)-*N*-methylpyrrolidinium (*2A*), and oxotremorine (*6B*)] having a large ratio of K_L/K_H was determined. This mean estimate (34%) was used as a constant in the two-site competitive inhibition equation (Eq. 1) for nonlinear regression analysis. Table 1 shows the results of regression analysis of the competition data assuming a constant proportion (34%) of high-affinity sites. Analysis of variance showed no significant increase in residual error when the proportion of high-affinity sites was held constant ($F = 1.391$; $p = 0.23$).

In a study investigating the influence of pH on the muscarinic activity of arecoline, oxotremorine, and pilocarpine, it was demonstrated that the hydronium ions of these amines were responsible for their muscarinic activity (4). Thus, it was of interest to determine whether the charged forms of tertiary amines were pharmacologically active in the muscarinic receptor binding assay. For these experiments, we investigated the influence of pH on the competitive inhibition of [^{3}H]QNB binding by arecoline ($pK_a = 7.72$; ref. 4) and scopolamine ($pK_a = 7.53$; ref. 11) and their quaternary derivatives. These compounds were used since a comparison of an agonist and an antagonist could be made and since the pK_a values of the tertiary bases are relatively close to physiological pH. Changing the pH did not significantly affect the IC_{50} values of the quaternary derivatives. Figure 4 shows that the IC_{50} of arecoline and scopolamine increases relative to that of their quaternary derivatives as the pH increases from 8.0 to 9.0. These data are consistent with the hypothesis that the binding activity of these tertiary amines is proportional to the fraction of drug ionized.

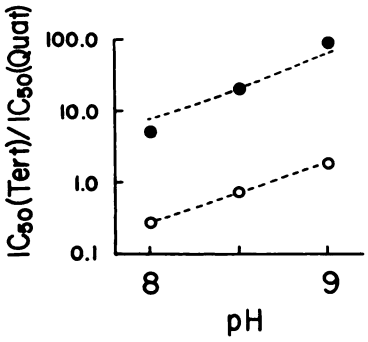

FIG. 4. *Influence of pH on the potency of arecoline* (○) *and scopolamine* (●) *relative to their N-methiodides, expressed as ratio of their* IC_{50} *values for displacement of specific [*^{3}*H]QNB binding*

Each point represents the geometric mean of values determined from three experiments. The *dotted lines* represent the predicted ratio of IC_{50} values assuming that only the protonated form of the tertiary amine displaces specific [^{3}H]QNB binding.

DISCUSSION

In studies of structure-activity relationships, it is useful to consider the contribution of both affinity and efficacy to the observed pharmacological activity of a drug. By comparing the ED_{50} for contraction of the guinea pig ileum with the muscarinic receptor binding affinity, it should be possible to assess the extent to which structural modifications alter the affinity and efficacy components of cholinergic analogues. However, this approach is complicated since the binding of cholinergic agonists can be resolved into two major, high- and low-affinity receptor populations in addition to a minor population of super-high-affinity sites (10). In the present study, we have calculated only the binding parameters of the high- and low-affinity sites since it is difficult to fit the agonist/[^{3}H]QNB competition data to a three-site model by nonlinear regression analysis. Birdsall and co-workers (10, 12, 13) have postulated that the various agonist binding sites are the same macromolecule and that the differences in affinity are the result of various environmental or coupling states of the receptor. Ultimately, this model predicts an agreement between K_L and the pharmacologically determined dissociation constant, a parameter which takes into account spare receptors. This model also predicts a good correlation between efficacy and K_L/K_H and an agreement between K_H and the ED_{50} for contraction of the guinea pig ileum (13). As shown in Fig. 5A, we observed good correlation ($r = 0.92$) between the ED_{50} for stimulation of contractions of the guinea pig ileum and the K_H values determined in binding experiments on the rat brain stem. Since the pharmacological experiments and binding experiments were carried out in different tissues under different ionic conditions, a better agreement between binding constants and pharmacological activity would be expected if the ratio of pharmacological activities of each quaternary-tertiary pair is compared with the corresponding ratio of K_H values. In this way, the change in the pharmacological activity caused by quaternization of the compound is compared with the change in binding activity at the high-affinity site, thereby controlling against differences in experimental conditions. Figure 5B shows that there is better correlation ($r = 0.97$) between the ratio of ED_{50} values and the ratio of K_H values of each pair of quaternary and tertiary amines, with the data points tending to fall on the line of equivalence ($Y = X$). There was also a good correlation ($r = 0.89$) between the ED_{50} for stimulation of contraction of the guinea pig ileum and the K_L value of each compound; however, the slope of the linear regression line was significantly less than 1 (0.67), so that the K_L value consistently underestimated the pharmacological activity of each compound. This finding is consistent with the results of Birdsall *et al.* (10, 12, 13), who have found good agreement between K_L and the pharmacologically determined dissociation con-

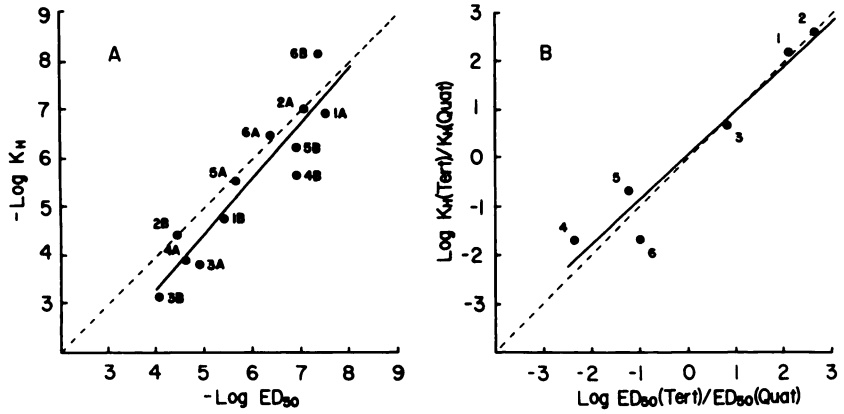

FIG. 5. *Correlation between the high-affinity dissociation constant and pharmacological activity of tertiary amines and their quaternary derivatives*

A. The negative logarithm of the K_H value of the various compounds is plotted against the negative logarithm of the ED_{50} for stimulating contractions of the guinea pig ileum. The pharmacological data are from refs. 4, 5, and 14. The *solid line* represents the least-squares fit to the data (slope = 1.15, Y intercept = −1.31).

B. The logarithm of the ratio of K_H values of each tertiary amine and its quaternary derivative is plotted against the logarithm of the ratio of their respective ED_{50} values for stimulating contractions of the guinea pig ileum. The *solid line* represents the least-squares fit to the data (slope = 0.93, Y intercept = 0.08). In A and B, the *dotted line* is the line of equivalence ($Y = X$).

stant. In view of the correlations noted previously between efficacy and K_L/K_H and between affinity and K_L, the present data suggest that quaternization of acetyldeanol and *N*-(2-acetoxyethyl)-pyrrolidine causes an increase in both affinity and efficacy whereas quaternization of aceclidine, arecoline, and oxotremorine leads to a decrease in affinity and efficacy.

The results of our binding experiments with racemic aceclidine and its methiodide generally confirm a previous study of the binding of the enantiomers of aceclidine and its methiodide (14). In that study, both enantiomers of aceclidine were more potent than their methiodides, with the (+)-enantiomers showing the largest ratio of binding activity.

The results of our experiments investigating the influence of pH on the binding of arecoline and scopolamine are consistent with the postulate that the binding activity of these amines resides in the charged species. Since the tertiary amines examined in the present study exist mainly in the protonated form at physiological pH, the change in pharmacological activity caused by *N*-methylation cannot be explained on the basis of charge. If we consider agonist analogues only, the difference in pharmacological activity between quaternary ammonium compounds and their *N*-desmethyl derivatives appears to be related to structural rigidity. For those compounds whose quaternary ammonium analogues are more potent than their tertiary derivatives, the basic nitrogen is contained on a flexible aliphatic chain. In contrast, when the basic nitrogen forms part of a relatively rigid ring, the difference in pharmacological activity between the tertiary amine and its quanternary salt is not as great or else the tertiary amine is more active. An explanation for these differences in potency has been given elsewhere (4, 5). The data presented here are consistent with previous pharmacological data and illustrate a good agreement between pharmacological activity and binding activity.

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REFERENCES

1. Stehle, R. L., K. I. Melville, and F. K. Oldham. Choline as a factor in the elaboration of adrenaline. *J. Pharmacol. Exp. Ther.* **56**:473–481 (1936).
2. Brimblecome, R. W., and D. G. Rowsell. A comparison of the pharmacological activities of tertiary bases and their quaternary ammonium derivatives. *Int. J. Neuropharmacol.* **8**:131–141 (1969).
3. Van Rossum, J. M. Pharmacodynamics of parasympathetic drugs: mechanism of action and structure-action relationship of tertiary ammonium salts. *Arch. Int. Pharmacodyn.* **140**:592–605 (1962).
4. Hanin, I., D. J. Jenden, and A. K. Cho. The influence of pH on the muscarinic action of oxotremorine, arecoline, pilocarpine, and their quaternary ammonium analogues. *Mol. Pharmacol.* **2**:352–359 (1966).
5. Cho, A. K., D. J. Jenden, and S. I. Lamb. Rates of alkaline hydrolysis and muscarinic activity of some aminoacetates and their quaternary ammonium analogues. *J. Med. Chem.* **15**:391–394 (1972).
6. Mashkovsky, M. D., and L. N. Yakhontov. Relationships between chemical structure and pharmacological activity in a series of synthetic quinuclidine derivatives. *Prog. Drug. Res.* **13**:293–339 (1969).
7. Yamamura, H. I., and S. H. Snyder. Muscarinic cholinergic binding in rat brain. *Proc. Natl. Acad. Sci. U. S. A.* **71**:1725–1729, (1974).
8. Birdsall, N. J. M., A. S. V. Burgen, and E. C. Hulme. Muscarinic receptors: biochemical and binding studies, in *Recent Advances in Receptor Chemistry* (F. Gualtieri, M. Giannella, and C. Melchiorre, eds.). Elsevier/North-Holland Biomedical Press, 71–96 (1979).
9. Aronstam, R. S., L. G. Abood, and W. Hoss. Influence of sulfhydryl reagents and heavy metals on the functional state of the muscarinic acetylcholine receptor in rat brain. *Mol. Pharmacol.* **14**:575–586 (1978).
10. Birdsall, N. J. M., E. C. Hulme, and A. S. V. Burgen. The character of the muscarinic receptors in different regions of the rat brain. *Proc. R. Soc. Lond. B Biol. Sci.* **207**:1–12 (1980).
11. Barlow, R. B., and E. A. Winter. Affinities of the protonated and non-protonated forms of hyoscine and hyoscine N-oxide for muscarinic receptors of the guinea-pig ileum and a comparison of their size in solution with that of atropine. *Br. J. Pharmacol.* **72**:657–664 (1981).
12. Birdsall, N. J. M., A. S. V. Burgen, and E. C. Hulme. The binding of agonists to brain muscarinic receptors *Mol. Pharmacol.* **14**:723–736 (1978).
13. Birdsall, N. J. M., A. S. V. Burgen, and E. C. Hulme. Correlation between the binding properties and pharmacological responses of muscarinic receptors, in *Cholinergic Mechanisms and Psychopharmacology* (D. J. Jenden, ed.). Plenum Press, New York, 25–33 (1977).
14. Ringdahl, B., F. J. Ehlert, and D. J. Jenden. Muscarinic activity and receptor binding of the enatiomers of aceclidine and its methiodide. *Mol. Pharmacol.* **12**:594–599 (1982).

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