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Influence of pH on the Binding of Scopolamine and *N*-Methylscopolamine to Muscarinic Receptors in the Corpus Striatum and Heart of Rats

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SUMMARY

The influence of pH on the binding of scopolamine and [^{3}H]*N*-methylscopolamine to muscarinic receptors in the heart and corpus striatum was investigated. The specific binding of [^{3}H]*N*-methylscopolamine in the heart and corpus striatum was relatively insensitive to pH over the range of 6 through 10 but decreased markedly below pH 6.0. This reduction in binding was attributed to a reversible decrease in the observed affinity without an effect on the binding capacity. The data are consistent with the postulate that [^{3}H]*N*-methylscopolamine competes with hydrogen ions for an acidic group on the muscarinic receptor that has a pK_A of approximately 5.5 in both the heart and corpus striatum. When measured by competitive inhibition of the binding of [^{3}H]*N*-methylscopolamine, the affinity of scopolamine decreased relative to that of [^{3}H]*N*-methylscopolamine as the pH increased from 6 to 10, confirming that it is primarily the protonated form of scopolamine that binds with muscarinic receptors.

Positive charge is perhaps the most essential structural feature of ligands that interact with muscarinic receptors. For example, most, if not all, muscarinic agonists are either partially charged tertiary amines or fully charged quaternary ammonium or sulfonium ions (see Ref. 1). In the few instances in which the question has been addressed, it has been shown that the agonist activity of muscarinic tertiary amines resides in the protonated species (2, 3). A similar picture emerges with regard to muscarinic antagonists, although it appears that the unprotonated form of some antagonists may contribute to binding affinity (4).

This cationic requirement of muscarinic ligands provides evidence for the existence of a complementary anionic site on the muscarinic receptor. If the anionic site is formed by the ionization of an acidic residue, then the binding of ligands to the muscarinic receptor should be affected by a change in pH in the range of the pK_A of the acidic group on the receptor. In a study in which this postulate was tested, it was noted that the binding of the muscarinic agonist [^{3}H]CD was unaffected by a change in pH from 8.0 to 6.0 but declined markedly as the pH decreased below 5.5 (5). These results led to the suggestion that a carboxylic acid group might be a likely candidate for the anionic site on the muscarinic receptor (5). Several investigators have now shown that the binding of both agonists and antagonists to muscarinic receptors is inhibited below pH 6.0 (6–8) and is prevented by agents that react with carboxyl groups (9).

Studies in which the aziridinium ion of [^{3}H]PrBCM was used to label muscarinic receptors covalently have also provided evidence for a carboxyl group at the anionic site of the receptor. It is likely that the anionic site participates in a covalent reaction with [^{3}H]PrBCM, because the reactive aziridinium ring of [^{3}H]PrBCM is structurally similar to the quaternary ammonium group of many muscarinic ligands. It has been shown that the covalent complex between solubilized muscarinic receptors and [^{3}H]PrBCM is cleaved by hydroxylamine (10), which would be expected for the ester linkage resulting from the covalent binding of [^{3}H]PrBCM to a carboxyl group on the receptor. On the basis of the latter expectation and the known primary structures of subtypes of the muscarinic receptor (11–15), Curtis and co-workers (16) have interpreted their peptide mapping studies on cerebral muscarinic receptors to indicate that [^{3}H]PrBCM labels a highly conserved aspartic acid in the putative third transmembrane helix of the receptor.

In this study, we have investigated the pH dependence of the

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ABBREVIATIONS: CD, (*cis*)-dioxolane; PrBCM, propylbenzilylcholine mustard; NMS, *N*-methylscopolamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TAPS, *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid; CHES, 2-[*N*-cyclohexylamino]ethanesulfonic acid; IC_{50}, 50% inhibitory concentration.

radioligand dissociation constant for muscarinic receptors in the corpus striatum and heart. The results of our studies are consistent with the postulate that the quaternary ammonium muscarinic antagonist [^{3}H]NMS competes with protons for an acidic residue on the muscarinic receptor that has a pK_A of approximately 5.5 in both the corpus striatum and heart. A recent study by Birdsall and co-workers (8) investigating the pH dependence of ligand binding to the heart is generally consistent with the results reported here. We have also investigated the influence of pH on the binding of tertiary amine scopolamine and have obtained results consistent with the postulate that the protonated base has about 70-fold higher affinity for muscarinic receptors than the free base.

Materials and Methods

Tissue preparation. The corpus striatum and heart were homogenized in approximately 20 volumes of 0.32 M sucrose, using a Polytron (setting 5; Brinkmann Instruments, Westbury, NY). The cardiac homogenate was filtered over four layers of cheese cloth. Tissue homogenates were centrifuged at 30,000 × *g* for 10 min and resuspended in 0.32 M sucrose to a concentration of approximately 1 mg/ml (corpus striatum) and 20 mg/ml (heart), based on the original wet weight of the tissue.

Radioligand binding assay. The binding of [^{3}H]NMS was measured using a centrifugation technique similar to that described by Birdsall *et al.* (17). Tissue homogenate (0.5 ml) was incubated in microfuge tubes at 25° for 15 min (heart) or 30 min (corpus striatum), in a final volume of 1.0 ml containing [^{3}H]NMS, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM dithiothreitol, 20 mM buffer (see below), and other nonlabeled scopolamine derivatives, as described in Results. All assays were run in triplicate, and nonspecific binding was estimated as the residual binding in the presence of 10 μM atropine. The incubations were stopped by centrifugation at approximately 30,000 × *g* for 10 min. The resultant pellets were washed superficially with two aliquots (1 ml each) of ice-cold saline. After the tubes were allowed to dry overnight, the pellets were dissolved in digestant liquid scintillation cocktail (Fluorosol; National Diagnostics, Somerville, NJ). The following buffers were used at the indicated pH values in the radioligand binding assays: Na citrate, pH 4.0–6.0; Na HEPES, pH 7.0; Na TAPS, pH 8.0–9.0; and Na CHES, pH 10.0. The concentration of the buffers was 20 mM with respect to the acid.

Calculations. In most experiments in which the influence of pH on the binding of NMS was investigated, the observed dissociation constant (K_{obs}) and binding capacity (B_{max}) of NMS were estimated by competitive inhibition of the binding of [^{3}H]NMS at a constant concentration by various concentrations of nonlabeled NMS and fitting of the data to the following equation by nonlinear regression analysis:

$$y = \frac{[[^3\text{H}]\text{NMS}]B_{max}}{[[^3\text{H}]\text{NMS}] + [\text{NMS}] + K_{obs}} \quad (1)$$

where y denotes specifically bound [^{3}H]NMS, and [[^{3}H]NMS] and [NMS] denote the molar concentrations of labeled and nonlabeled NMS, respectively. In a few experiments, the K_{obs} and B_{max} of [^{3}H]NMS were estimated by measurement of the binding of [^{3}H]NMS at various concentrations and fitting of the data to eq. 1 by nonlinear regression analysis, with the value of [NMS] set to zero. The concentration of scopolamine required for half-maximal displacement of [^{3}H]NMS binding (IC_{50}) was estimated by running scopolamine/[^{3}H]NMS competition experiments and fitting the data to the following equation by nonlinear regression analysis:

$$Y = \frac{1}{1 + [\text{I}]/IC_{50}} \quad (2)$$

where Y denotes the fractional binding of [^{3}H]NMS, and [I] denotes the concentration of nonlabeled competitor. The IC_{50} values of NMS were estimated in this manner as well.

Results

Influence of pH on [^{3}H]NMS binding. The effects of pH on the binding of [^{3}H]NMS to homogenates of the corpus striatum and heart are shown in Fig. 1. For these experiments, the specific binding of [^{3}H]NMS was measured at a concentration of 0.5 nM and in the presence of buffers ranging in pH from 4 to 10. It can be seen that the binding of [^{3}H]NMS to muscarinic receptors in both the heart and corpus striatum remained relatively constant over the pH range of 6 to 10 but declined markedly below pH 6.

To elucidate the mechanism by which pH affected the binding of [^{3}H]NMS, we examined the influence of pH on the observed dissociation constant and binding capacity of [^{3}H]NMS. For these experiments, the specific binding of [^{3}H]NMS was measured at various concentrations of radioligand in homogenates of the corpus striatum at pH 7.0 and pH 4.5. It can be seen in Fig. 2 that at pH 7.0 the binding of [^{3}H]NMS was consistent with a simple one-site model having an observed dissociation constant of 0.10 nM and a binding capacity of 0.10 pmol/mg of tissue. Reduction of the pH to 4.5 caused a 20-fold increase in the observed dissociation constant of [^{3}H]NMS. Nonlinear regression and analysis of variance showed no significant increase in residual error when the data were fitted simultaneously, sharing the control estimate of B_{max} between the data obtained at the two different pH values ($F_{1,8} = 0.4731$; $p = 0.543$). Prior incubation of striatal homogenate at pH 4.5 for 15 min at 25° had no significant effect on the binding properties of [^{3}H]NMS when measured subsequently at pH 7.0 (see Fig. 2). We conclude that reduction of the pH causes a reversible decrease in the observed affinity of [^{3}H]NMS for muscarinic receptors without influencing the binding capacity. These results are consistent with those of Anthony and Aronstam (7), who previously noted that hydrogen ions cause a reversible decrease in the affinity of [^{3}H]NMS for muscarinic receptors in the rat brainstem.

The simplest model to account for the effects of pH on the binding of [^{3}H]NMS is the following scheme, in which [^{3}H]NMS (D^+) competes with hydrogen ions (H^+) for an anionic

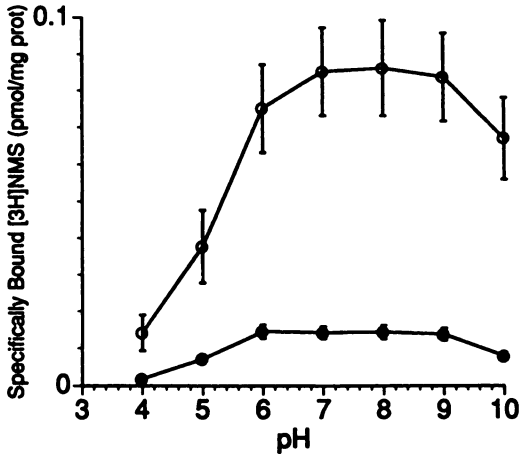

Fig. 1. Influence of pH on the binding of [^{3}H]NMS to the corpus striatum (○) and heart (●). Specific [^{3}H]NMS binding was measured at a radioligand concentration of 0.5 nM. Each point represents the mean binding value ± standard error of three experiments, each done in triplicate.

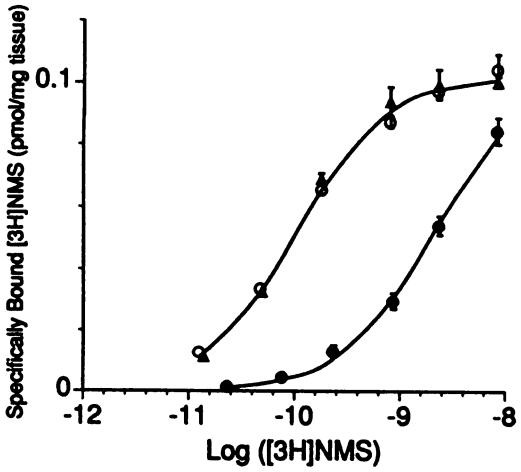

Fig. 2. Effect of low pH on the binding of [^{3}H]NMS to the corpus striatum. The specific binding of [^{3}H]NMS was measured at various concentrations of radioligand at pH 7.0 (○, ▲) and pH 4.5 (●) in the corpus striatum. For these experiments, the striatal homogenate was first preincubated at pH 7.0 (○, ●) and pH 4.5 (▲) for 15 min. The homogenates were centrifuged at 30,000 × *g* for 10 min and resuspended in 0.32 M sucrose. Specific [^{3}H]NMS binding was then measured in these homogenates at the indicated pH values, as described in Materials and Methods. Each point represents the mean binding value ± standard error of three experiments, each done in triplicate. The mean binding values are plotted against the logarithm of the free concentration of [^{3}H]NMS. The two theoretical curves represent the least squares fit to a simple one-site model, sharing the same estimate of B_{max} between the two sets of data.

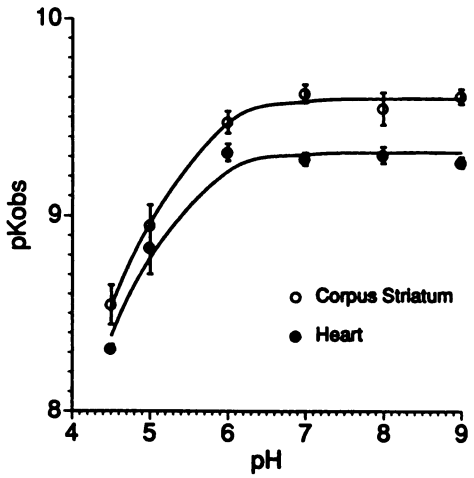

Fig. 3. Influence of pH on the observed affinity of [^{3}H]NMS in the corpus striatum and heart. The negative logarithm of the observed dissociation constant (pK_{obs}) of [^{3}H]NMS is plotted against the pH. Each point represents the estimate of pK_{obs} obtained from the results of three experiments, each done in triplicate. The theoretical curves represent the least squares fit to the logarithmic form of eq. 3.

site on the muscarinic receptor (R^-):

$$\begin{array}{ccc} & H^+ & \\ & + & \\ D^+ + R^- & \rightleftharpoons^{K_D} & DR \\ & \downarrow\uparrow K_A & \\ & HR & \end{array}$$

In this scheme, K_A denotes the dissociation constant of the acidic group on the receptor, and K_D denotes the dissociation constant of the radioligand NMS. It can be shown that the observed dissociation constant of NMS (K_{obs}) is related to the hydrogen ion concentration and the K_A of the acidic group by the following equation:

$$K_{obs} = K_D(1 + [H^+]/K_A) \quad (3)$$

To determine the suitability of the model, we measured the K_{obs} of NMS over a range of pH values and fitted the data to the logarithmic form of eq. 3 by nonlinear regression analysis. For these studies, the K_{obs} of NMS was estimated by running NMS/[^{3}H]NMS competition experiments in the corpus striatum and heart, as described in Materials and Methods. It can be seen in Fig. 3 that the K_{obs} of NMS remained relatively constant over the pH range of 6–9 but decreased markedly below pH 6.0. The data were consistent with the model described above, as shown by the good agreement between the data points and the logarithmic form of the regression eq. 3 (Fig. 3, *solid curves*). Regression analysis yielded similar estimates of 5.52 ± 0.21 and 5.38 ± 0.04 for the pK_A values of the acidic group on the muscarinic receptor in the corpus striatum and heart, respectively. The estimates of the true dissociation constant of NMS in the absence of any hydrogen ions were 0.26 and 0.48 nM in the corpus striatum and heart, respectively. These values are also listed in Table 1. We have no adequate explanation for the discrepancy between the estimates of the dissociation constants for NMS in the corpus striatum in Figs. 2 and 3 but presume it is related to experimental error.

TABLE 1

Dissociation constants of scopolamine derivatives for muscarinic receptors in the heart and corpus striatum

The dissociation constants for NMS were estimated by nonlinear regression analysis of the data in Fig. 3 according to the logarithmic form of eq. 3. The dissociation constants for scopolamine were estimated by nonlinear regression analysis of the data in Fig. 4 according to the logarithmic form of eq. 5. The values represent the negative logarithm of the dissociation constant ± the standard error.

Compound	− log dissociation constant	
	Corpus striatum	Heart
NMS	9.59 ± 0.19	9.32 ± 0.05
Protonated scopolamine	9.10 ± 0.20	8.64 ± 0.05
Unprotonated scopolamine	7.26 ± 0.21	6.82 ± 0.07

Influence of pH on scopolamine binding. If coulombic forces are involved in the binding of NMS to muscarinic receptors, then it might be expected that the protonated form of the tertiary amine scopolamine binds to the muscarinic receptor with much higher affinity than the unprotonated base. To test this postulate, we ran scopolamine/[^{3}H]NMS competition experiments at various pH values to investigate the influence of pH on the affinity of scopolamine for muscarinic receptors in the corpus striatum and heart. An advantage of this type of experiment is that the affinity of scopolamine can be measured relative to a drug of constant charge (i.e., NMS) to control for the competitive effect of hydrogen ions and for the potential effects of pH on the conformation of the receptor. This strategy has been used previously in studies of the influence of pH on the pharmacological activity of tertiary bases at muscarinic receptors in the isolated guinea pig ileum (2–4).

The results in Fig. 4 show the ratio of the IC_{50} value of NMS divided by that for scopolamine plotted against pH. It can be seen that the potency of scopolamine declines relative to that of NMS as the pH increases in both the heart and corpus striatum. It is apparent that the relative potency of scopolamine reaches a maximum plateau at low pH, when the base is fully protonated.

Although it is clear that the data in Fig. 4 are generally

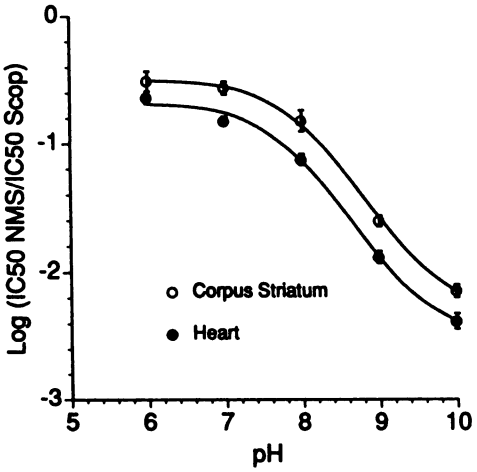

Fig. 4. Influence of pH on the potency of scopolamine relative to NMS, expressed as the ratio of their IC_{50} values for competitive inhibition of the binding of [^{3}H]NMS in the corpus striatum and heart. Each point represents the estimate obtained from the results of three experiments, each done in triplicate. The theoretical curves represent the least squares fit to the logarithmic form of eq. 6 (corpus striatum: $pA_0 = 0.49 \pm 0.02$, $p\alpha = -1.83 \pm 0.06$, $pK_A = 7.87 \pm 0.02$; heart: $pA_0 = 0.68 \pm 0.02$, $p\alpha = -1.83 \pm 0.06$, $pK_A = -7.70 \pm 0.02$).

consistent with the hypothesis that the binding activity of scopolamine resides mainly in its protonated form, it is possible to obtain a more unbiased quantitative assessment of this hypothesis using a procedure similar to that described by Hanin *et al.* (3). The scheme describing the binding of protonated (BH^+) and unprotonated (B) scopolamine to the muscarinic receptor (R^-) at neutral to high pH is:

$$\begin{array}{ccc} & R^- & R^- \\ & + \quad K_A & + \\ H^+ + & B \rightleftharpoons & HB^+ \\ \alpha K_D & \downarrow\uparrow & \downarrow\uparrow \; K_D \\ & BR^- & HBR \end{array}$$

in which K_D and αK_D denote the dissociation constants of the protonated (HBR) and unprotonated (BR^-) ligand receptor complexes, respectively, and K_A denotes the dissociation constant for scopolamine (i.e., $K_A = [H^+][B]/[HB^+]$). It can be shown that the observed dissociation constant (K_{obs}) of scopolamine for the receptor complex is given by:

$$K_{obs} = \frac{K_D}{(1 - 1/\alpha)\dfrac{[H^+]}{[H^+] + K_A} + 1/\alpha} \qquad (4)$$

It follows that the ratio of the ligand-receptor dissociation constant for NMS divided by that for scopolamine is given by:

$$A = A_0\left((1 - 1/\alpha)\frac{[H^+]}{K_A + [H^+]} + 1/\alpha\right) \qquad (5)$$

in which A denotes the ligand-receptor dissociation constant for NMS divided by that for scopolamine, A_0 denotes the ligand-receptor dissociation constant for NMS divided by that for protonated scopolamine, and α denotes the ratio of ligand-receptor dissociation constants for unprotonated and protonated scopolamine. It can be assumed that the ratio of ligand-receptor dissociation constants A is also equivalent to the ratio of IC_{50} values for NMS and scopolamine, because the concentration of [^{3}H]NMS was the same in both the NMS/[^{3}H]NMS and scopolamine/[^{3}H]NMS competition experiments. To assess the model, we fitted the data in Fig. 4 to the logarithmic form of eq. 5. Regression analysis of the data in the corpus striatum and heart yielded similar estimates of 7.87 ± 0.02 and 7.70 ± 0.02, respectively, for the pK_A values of scopolamine. Protonated scopolamine was estimated to be 68- and 67-fold more potent than unprotonated scopolamine at muscarinic receptors in the corpus striatum and heart, respectively. To determine whether the data could be explained assuming that only protonated scopolamine binds with the muscarinic receptor, we fitted the data to the logarithmic form of eq. 6, setting $1/\alpha = 0$. However, analysis of variance showed a significant increase in residual error ($F_{1,2} = 37.2$; $p = 0.0170$), indicating that the free base probably contributes to binding at high pH. The estimates of the dissociation constants for NMS and the ionized and unionized forms of scopolamine in the corpus striatum and heart are given in Table 1.

Discussion

Our observations regarding the pH dependency of the dissociation constant of [^{3}H]NMS for cardiac muscarinic receptors are generally consistent with those recently published by Birdsall *et al.* (8), who concluded that [^{3}H]NMS competes with protons for an acidic residue on the muscarinic receptor. Our estimate of the pK_A (5.4) of the acidic group on cardiac muscarinic receptors is in general agreement with that (5.9) estimated by Birdsall and co-workers in studies of the influence of pH on the binding of [^{3}H]NMS. The reversibility of the effect of pH on [^{3}H]NMS binding is consistent with this postulate and makes it unlikely that the decrease in binding noted at low pH was due to damage to the receptor. Our estimate of the pK_A of the acidic residue on striatal muscarinic receptors (5.5) is approximately the same as that measured on cardiac muscarinic receptors (5.4), and both estimates are in the upper range of that expected for a carboxyl group. This moderately high pK_A could be attributed to an inductive effect of neighboring residues on the receptor. As mentioned above, molecular biological and biochemical studies on the muscarinic receptor indicate that a highly conserved aspartate carboxyl group may be the anionic site for ligands on the muscarinic receptor. When rationalized from this viewpoint, our studies indicate that the pK_A of the relevant aspartic acid is approximately 5.5.

To date, five subtypes of the muscarinic receptor have been cloned (11–15) and designated m1 to m5 by Bonner and coworkers (13, 15). The mammalian myocardium appears to contain a nearly uniform population of M2 muscarinic binding sites (18) and m2 mRNA (19), whereas the corpus striatum contains mainly M1 and non-M1 non-M2 binding sites (18) and m1 and m4 mRNA (20). The similarity in the pK_A values of the acidic residue on muscarinic receptors in these two tissues provides no evidence for differences in the pK_A values of subtypes of the muscarinic receptor.

The results of our studies investigating the effects of pH on the binding of [^{3}H]NMS are generally consistent with a previous report on the influence of pH on the binding of the quaternary ammonium muscarinic agonist [^{3}H]CD (5). Although no quantitative estimate of the pK_A of the muscarinic receptor was made in that prior study, the effects of pH on the binding of [^{3}H]CD at a single concentration were similar to those observed in this study for [^{3}H]NMS (see Fig. 1). The similarity of these data suggests that quantitative analysis of

the effects of pH on [^{3}H]CD binding would yield an estimate of the pK_A of the acidic residue on the recognition site of the muscarinic receptor similar to that reported here. Collectively, these results are consistent with the model that [^{3}H]CD and [^{3}H]NMS bind with the charged form of the receptor only and that the influence of pH on the binding of these two radioligands can be attributed to a change in the ionization of the same acidic residue at the ligand recognition site. However, this conclusion may not apply to all ligands that interact with the muscarinic receptor. In a recent study investigating the influence of pH on the binding of several ligands to cardiac muscarinic receptors, Birdsall and co-workers (8) provided evidence for two acidic groups modulating cardiac muscarinic receptors, having pK_A values of approximately 5.4 and 6.8. These investigators suggested that ligands may interact with one or the other of these residues or that ligands bind only to the residue with the pK_A of 5.4 and that the conformation of the receptor is altered by the ionization of a more distant residue having a pK_A of 6.8.

It has been reported previously that the IC_{50} value of scopolamine increases approximately 10-fold relative to that for NMS as the pH increases from 8 to 9 (21). The results of the present study are consistent with these prior observations but somewhat different from those of Asselin *et al.* (6), who only noted a 2–3-fold change in the potency of scopolamine over the same range in pH. By measuring the relative binding affinity of scopolamine over a wider range of pH (pH 6–10), it was possible to estimate the pK_A of scopolamine from the binding data as well as to determine whether both the protonated and unprotonated forms of the base bind with the muscarinic receptor. The estimate of the pK_A of scopolamine was practically the same when measured in binding studies on both the heart (7.70) and corpus striatum (7.87) and similar to that (7.53) measured by chemical titration (4). In a prior study of the influence of pH on the potency with which scopolamine and NMS competitively antagonized contractions of the guinea pig ileum, results consistent with the postulate that the unprotonated form of scopolamine is only 10-fold less potent than the protonated form were obtained (4). In contrast, we estimate nearly a 70-fold difference between the potencies of the charged and uncharged forms of scopolamine. Although we have no satisfactory explanation for this discrepancy, it is conceivable that this difference is related to differences in tissue preparations. It should be noted that the present study was carried out over a wider range of pH (pH 6–10) than the former study (4), and we observed a 44-fold decrease in binding affinity of scopolamine relative to NMS as the pH increased from 6 to 10. This decrease in potency cannot be explained by a mere 10-fold difference between the binding affinities of the charged and uncharged forms of scopolamine.

Our results indicate that NMS binds mainly, if not entirely, to the charged form of an acidic residue on the recognition site of the muscarinic receptor having a pK_A of approximately 5.5. It is conceivable that relatively high concentrations of NMS might bind to the uncharged form of the muscarinic receptor. Nevertheless, the agreement of our data with a simple competitive model for NMS and hydrogen ions indicates that hydronium ions are perhaps the simplest molecules that exhibit moderately potent competitive binding characteristics (i.e., pK_i = 5.5) at muscarinic receptors.

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