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Complex Allosteric Modulation of Cardiac Muscarinic Receptors by Protamine: Potential Model for Putative Endogenous Ligands

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SUMMARY

A large number of diverse pharmacological agents bind to a secondary domain on the muscarinic receptor, to influence allosterically the interaction of ligands at the primary binding site. Based on common structural features of these antagonists, we examined the interaction of protamine, an endogenous polycationic peptide, and of polyamines with muscarinic receptors in rat heart. Our results provide several lines of qualitative evidence that protamine allosterically modulates the conformation of muscarinic receptors, in a marked negatively cooperative manner. It decelerated the dissociation of *N*-[^{3}H]methylscopolamine ([^{3}H]NMS) initiated by atropine, in a concentration-dependent fashion. Inhibition by protamine of [^{3}H]NMS binding at equilibrium showed a distinct plateau, which increased in magnitude at higher ligand concentrations. Scatchard analysis of saturation isotherms of [^{3}H]NMS binding in the absence and presence of protamine indicated that protamine did not alter B_{max} in a statistically significant fashion, although there was a trend of a concentration-dependent increase in this parameter. On the other hand, it caused a marked concentration-dependent decrease in the affinity of [^{3}H]NMS, and this effect reached a ceiling limit. However, there were marked quantitative deviations of the interaction of protamine from a simple ternary allosteric model. Some of these discrepancies could be explained by the tendency of protamine to increase B_{max}. The allosteric actions of protamine demonstrated in kinetic and equilibrium experiments were selective for m1 and m2 muscarinic receptors, compared with m3, m4, and m5 receptors, as studied in Chinese hamster ovary cells transfected with the genes of the different muscarinic receptors. Arginine residues play an important role in the allosteric interaction of protamine, inasmuch as poly-L-arginine qualitatively mimicked the effects of protamine. In contrast, no effects of the polyamines spermine, spermidine, and putrescine were observed on [^{3}H]NMS binding. This is the first report on the allosteric modulation of muscarinic receptors by an endogenous peptide.

An allosteric modulator is defined as a compound that interacts with a binding site that is distinct from the primary binding site on a receptor, to modulate the binding of agonists and antagonists to the latter site. A large number of diverse pharmacological agents have been reported to be capable of modifying the conformation of muscarinic receptors allosterically, thereby preventing activation of the receptor. The first indication of an allosteric regulatory site on the muscarinic receptor was reported by Clark and Mitchelson (1). They demonstrated that muscarinic receptor-mediated negative inotropic effects in the heart were inhibited by gallamine in an allosteric fashion. Subsequently, the allosteric interactions of gallamine in heart and other tissues have been confirmed by using muscarinic receptor binding assays (2–7). In addition to gallamine, many other muscarinic antagonists have been found to interact with the receptor in an allosteric manner, e.g., AF-DX 116, methoctramine, himbacine, hexamethonium, quinidine, phencyclidine, and verapamil (8).

It has been noted that most allosteric muscarinic antagonists demonstrate selectivity for the M_2 receptor subtype (9). In fact, all selective M_2 muscarinic antagonists have been shown to influence the receptor conformation allosterically (8). Thus, allosteric interaction offers a unique opportunity to search for new selective muscarinic antagonists and to investigate their application in therapeutics. Furthermore, elucidation of the molecular mechanisms of the allosteric regulation of muscarinic receptors and the identification of some endogenous agents that interact allosterically with the receptors would be of great importance.

It is of interest that a common structural feature of the allosteric muscarinic receptor antagonists that have been reported so far is that they are either quaternary amines, or tertiary amines that would be protonated at physiological pH (8). Conversely, there are several conserved negatively charged

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ABBREVIATIONS: CHO, Chinese hamster ovary; NMS, *N*-methylscopolamine; QNB, quinuclidinyl benzilate; B_{max}, maximal ligand binding; K_d, equilibrium dissociation constant; IC_{50}, concentration of compound required to elicit 50% of its maximal inhibition of ligand binding or dissociation; DMEM, Dulbecco's modified Eagle's medium; $t_{1/2}$, half-life; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

aspartic acid residues in the muscarinic receptor sequences (10). Site-directed mutagenesis of the aspartate residue at position 71 of the m1 muscarinic receptor to asparagine results in a significant reduction of agonist-induced phosphoinositide hydrolysis (10) and also alters the magnitude of the allosteric interactions of gallamine with the receptor (11). Therefore, some of these multiple negatively charged aspartic acid residues might serve as a part of the allosteric binding site on muscarinic receptors.

Based on this hypothesis, we examined and identified prominent allosteric effects of the polycationic peptide protamine on cardiac muscarinic receptors, by using radioligand binding assays and different protocols designed to diagnose allosteric interactions (8, 12). However, there are several pieces of evidence suggesting a complex mode of allosteric modulation of the muscarinic receptor by protamine. The allosteric effects of protamine were also selective for the m1 and m2 muscarinic receptor subtypes, as compared in CHO cells transfected with the m1, m2, m3, m4, and m5 receptor genes. Basic arginine residues, which constitute approximately 90% of the sequence of protamine (13), play an important role in the regulation of muscarinic receptors by this peptide. The effects of other basic polypeptides and polyamines were also studied, to elucidate the structural features required for the allosteric interactions of protamine.

Experimental Procedures

Materials. [^{3}H]NMS (74 Ci/mmol) and [^{3}H]QNB (43 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Protamine, poly-L-arginine (M_r 11,600), poly-L-lysine (M_r 10,200), poly-D-lysine (M_r 13,000), poly-L-ornithine (M_r 11,700), spermine, spermidine, putrescine, choline chloride, and atropine were from Sigma (St. Louis, MO). Tissue culture supplies were obtained from GIBCO (Grand Island, NY).

Cell culture conditions. CHO cells stably transfected with restriction fragments containing the entire coding regions of the m1, m2, m3, m4, and m5 muscarinic receptor genes were provided by Drs. J. C. Venter, C. M. Fraser, and M. R. Brann, of the National Institutes of Health (10, 14). Cells were grown in tissue culture flasks (75 cm^2, 250 ml) in 20 ml of DMEM supplemented with 10% (v/v) bovine calf serum and 0.005% (w/v) geneticin. Cells were grown for 4–7 days at 37°, in an atmosphere consisting of 10% CO_2/90% humidified air. Subculture was achieved using D_1 solution containing 0.05% trypsin (15). After centrifugation of the cell suspension for 1 min at 300 × g, the supernatant was removed and the cells were resuspended in DMEM and distributed into flasks (1:6) on day 0. The culture medium was changed on day 4 after subculture, and every day thereafter, by the addition of 10 ml of fresh DMEM and removal of 10 ml of medium.

Membrane preparation. Frozen male Sprague-Dawley rat hearts were obtained from Hilltop Lab Animals, Inc. (Scottsdale, PA). Tissues were minced and homogenized in 50 mM NaCl/20 mM HEPES buffer (pH 7.4) for 30 sec, by Brinkmann Polytron (setting 7). Homogenates were centrifuged at 1000 × g for 10 min at 4°, and the resulting supernatant was centrifuged at 35,000 × g for 30 min. The pellet was suspended in 50 mM NaCl/20 mM HEPES buffer. Membranes from cultured cells were prepared in a similar fashion.

Radioligand binding assays. For the study of dissociation kinetics of radioligands, membranes were preincubated with a 1 nM concentration of either [^{3}H]NMS or [^{3}H]QNB for 90 min at 25°, in a final volume of 1 ml of 50 mM NaCl/20 mM HEPES buffer. Phosphate-based buffers were avoided, due to their co-precipitation with the polycationic peptides. Atropine (2 μM) was used to define the level of nonspecific binding. Dissociation of the radioligand-receptor complex was initiated by addition of a final concentration of 2 μM atropine to the assay tubes. The dissociation reaction was terminated at the desired time points by filtration under vacuum through Whatman GF/B glass fiber filters, using a Skafron cell harvester, followed by rapid washing of the filters two times with 5 ml of ice-cold 0.9% NaCl solution. Each filter was then extracted in a scintillation vial in 4 ml of scintillation fluid for at least 6 hr before the radioactivity was determined by liquid scintillation counting, with automatic correction for counting efficiency. In order to study the concentration dependence of the effects of protamine, poly-L-arginine, or other polypeptides on the dissociation rate, dissociation was initiated with 2 μM atropine, with or without increasing concentrations of the peptides, and the tissue was filtered at a time corresponding to approximately twice the half-life of the ligand-receptor complex in the absence of peptides.

For saturation experiments, membranes were incubated with [^{3}H]NMS (0.01–4 nM), in the absence or presence of increasing concentrations of protamine or poly-L-arginine, in a final volume of 1 ml of 50 mM NaCl/20 mM HEPES buffer, for 3 hr at 25°. Nonspecific binding was defined in the presence of 2 μM atropine but in the absence of peptides. The reactions were terminated by filtration under vacuum, and radioactivity was counted as described previously. Protein content was measured by the method of Lowry *et al.* (16). Whether the binding reaction was at equilibrium in the presence of high concentrations of protamine was tested by studying the time course of association of 0.3 nM [^{3}H]NMS in the presence of 100 μg/ml protamine at 25°. Under these conditions, the specific binding of the ligand reached equilibrium after 2 hr and remained stable until 4 hr (data not shown).

Data analysis. The $t_{1/2}$ of dissociation was calculated by linear regression, according to a first-order model. B_{max} and K_d values were calculated by Scatchard analysis (17). In Schild analysis (18), the ratios of the equilibrium dissociation constant of [^{3}H]NMS in the presence of increasing concentrations of protamine or poly-L-arginine (K'_d) to that in their absence (K_d) were applied to the following equation:

$$K'_d/K_d = (K_a + [A])/(K_a + [A]/\alpha)$$

where $[A]$, K_a, and α values are the concentration of an allosteric agent, its equilibrium dissociation constant at unoccupied receptors, and the cooperativity factor, respectively (12). These parameters were estimated using nonlinear regression analysis. The theoretical curve for a competitive interaction was obtained from the equation (18):

$$K'_d/K_d = 1 + [A]/[K_a]$$

Inhibition of ligand binding at equilibrium was fitted according to a four-parameter logistic sigmoid model, using the GraphPad program (ISI, Philadelphia, PA), to determine IC_{50} and maximal inhibition values obtained experimentally. Corresponding theoretical values that were expected according to a simple ternary allosteric model were calculated according to eq. 10 in Ref. 12, using the K_a and α values estimated as described above. Where appropriate, values are represented as means ± standard errors. Student's t test was used for statistical analysis of the data.

Results

Inhibition of [^{3}H]NMS binding at equilibrium by protamine in rat heart. Scatchard analysis of saturation isotherms of [^{3}H]NMS binding indicated that the affinity of [^{3}H]NMS for cardiac muscarinic receptors was significantly decreased in the presence of protamine (Table 1). However, there was a limit to this affinity change at higher concentrations of protamine (>100 μg/ml), indicating an allosteric mechanism of interaction. No statistically significant change in maximal [^{3}H]NMS binding was observed in the range of protamine concentrations used, although there was an obvious trend of a concentration-dependent increase in this parameter (Table 1). A statistically significant increase in B_{max} at protamine concentra-

TABLE 1

Effects of protamine on specific [^{3}H]NMS saturation binding

Heart homogenate was incubated with [^{3}H]NMS (0.01–3 nM), in the absence and presence of increasing concentrations of protamine, at 25° for 3 hr. K_d and B_{max} values were calculated by Scatchard analysis. Data are represented as means ± standard errors from 4–11 independent experiments.

Protamine	K_d	B_{max}
μg/ml	nM	fmol/mg of tissue weight
0	0.17 ± 0.01	3.6 ± 0.5
1	0.14 ± 0.02	3.1 ± 0.7
3	0.20 ± 0.03	3.6 ± 0.6
6	0.29 ± 0.04[a]	3.9 ± 0.4
10	0.54 ± 0.05[b]	4.3 ± 0.5
30	1.27 ± 0.15[b]	4.6 ± 0.6
60	1.44 ± 0.16[b]	4.7 ± 0.6
100	1.71 ± 0.11[b]	5.0 ± 0.6
300	1.66 ± 0.16[b]	4.8 ± 0.5
600	1.73 ± 0.16[b]	5.1 ± 0.8
1000	1.66 ± 0.08[b]	5.3 ± 0.7

[a] $p < 0.05$.
[b] $p < 0.01$, versus control.

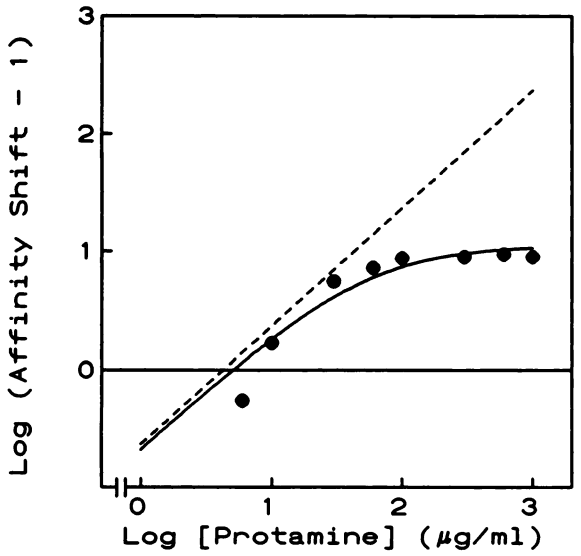

Fig. 1. Schild plot of the effects of protamine on [^{3}H]NMS saturation isotherms. Cardiac membranes were incubated with increasing concentrations of [^{3}H]NMS, in the absence or presence of protamine, for 3 hr at 25°. Affinity shift is the ratio between the ligand equilibrium dissociation constant in the presence and in the absence of protamine. – – –, Theoretical curve for a competitive interaction, with a slope of unity. The *data points* were fitted (——) according to the ternary model of allosteric interactions (12).

tions higher than 10 μg/ml ($p < 0.05$) was revealed when maximal binding in individual experiments was expressed as a ratio, relative to their own control.

Representation of these data by Schild regression (18) illustrates the allosteric nature of the interaction of protamine with cardiac muscarinic receptors. Unlike in competitive inhibition (theoretical line with a slope of 1), the shift of affinity of [^{3}H]NMS caused by protamine reached a plateau, with a cooperativity factor (α) of 12.2 ± 1.0 (Fig. 1). The calculated equilibrium dissociation constant of protamine for the unoccupied receptors (K_a) was 4.3 ± 0.7 μg/ml.

Fig. 2A shows that protamine inhibited the specific binding of 0.01 nM [^{3}H]NMS at steady state, in a concentration-dependent manner. However, maximal inhibition was achieved at 100–1000 μg/ml protamine, reaching a plateau at around 14% of binding in the absence of protamine. Increasing the concentration of [^{3}H]NMS resulted in a reduction in the magnitude of maximal inhibition of binding (Fig. 2). The potency of protamine in inhibiting [^{3}H]NMS binding decreased when the concentration of [^{3}H]NMS was increased, although the change did not reach statistical significance, except at 1.6 nM [^{3}H]NMS ($p < 0.05$) (Table 2). There was a good correlation between the ratio of increases in IC_{50} values obtained experimentally by elevating ligand concentration and the theoretical values calculated according to a simple ternary allosteric model of interaction (12) ($r = 0.9$, slope = 0.9) (Table 2). However, there was a consistent marked deviation of the absolute experimental IC_{50} values from the theoretical ones ($r = 0.9$, slope = 0.2) (Table 2). On the other hand, a good correlation existed between the experimentally determined and theoretically derived maximal inhibition of binding at different [^{3}H]NMS concentrations ($r = 1.0$, slope = 0.8) (Table 2), although deviations were still observed at higher ligand concentrations (Fig. 2). The latter deviations in maximal inhibition and IC_{50} values were reduced in magnitude when we accounted for the increase in B_{max} induced by protamine (Table 2).

Deceleration of the dissociation rate of [^{3}H]NMS by protamine in rat heart. Dissociation of [^{3}H]NMS from muscarinic receptors in rat heart was initiated by addition of a receptor-saturating concentration of atropine (2 μM) after [^{3}H]NMS binding reached steady state. As shown in Fig. 3A, the dissociation of [^{3}H]NMS was rapid and monophasic, with a $t_{1/2}$ of 3 ± 0.02 min at 25°. Protamine at a concentration of 50 μg/ml markedly slowed the dissociation rate of [^{3}H]NMS, resulting in a $t_{1/2}$ value of 13 ± 1 min ($p < 0.01$), indicating cooperative interactions of protamine with the receptor.

In another type of experiment, ligand dissociation was allowed for 5 min (which is approximately twice the half-life of the [^{3}H]NMS-receptor complex), in the absence and presence of increasing concentrations of protamine. The inhibitory effects of protamine on the rate of [^{3}H]NMS dissociation were concentration dependent (Fig. 3B), with an estimated IC_{50} value of 43 ± 16 μg/ml. This is in good agreement with the potency of protamine in displacing [^{3}H]NMS binding at equilibrium (Table 2). It should be noted that protamine, at concentrations of >100 μg/ml, increased [^{3}H]NMS binding to a value higher than that when no ligand dissociation was induced (Fig. 3B). We have no explanation for this phenomenon, which has been reported recently for other allosteric muscarinic receptor antagonists (7).

In contrast, the effects of protamine in decelerating the rate of [^{3}H]QNB dissociation from cardiac muscarinic receptors were less pronounced. Thus, protamine maximally reversed the dissociation of this ligand after 10 hr (twice the half-life at 25°) from 20 ± 8% in its absence only to 38 ± 9% of nondissociated control binding in its presence, even at concentrations as high as 1 mg/ml (data not shown). Protamine decreased the specific binding of 0.04 nM [^{3}H]QNB at equilibrium with an IC_{50} of 13 ± 5 μg/ml and a maximal inhibition of 72 ± 0.3% (data not shown). The cooperativity factor of 3.6 calculated in this case (according to eq. 9 in Ref. 12) is smaller than that measured when [^{3}H]NMS was used as a ligand ($\alpha = 12.2$).

Antagonism by heparin and poly-L-aspartic acid of protamine-induced allosteric effects. Because protamine is a polycationic peptide, positively charged moieties might serve as important functional groups in eliciting its allosteric effects. Indeed, the polyanionic compounds heparin and poly-

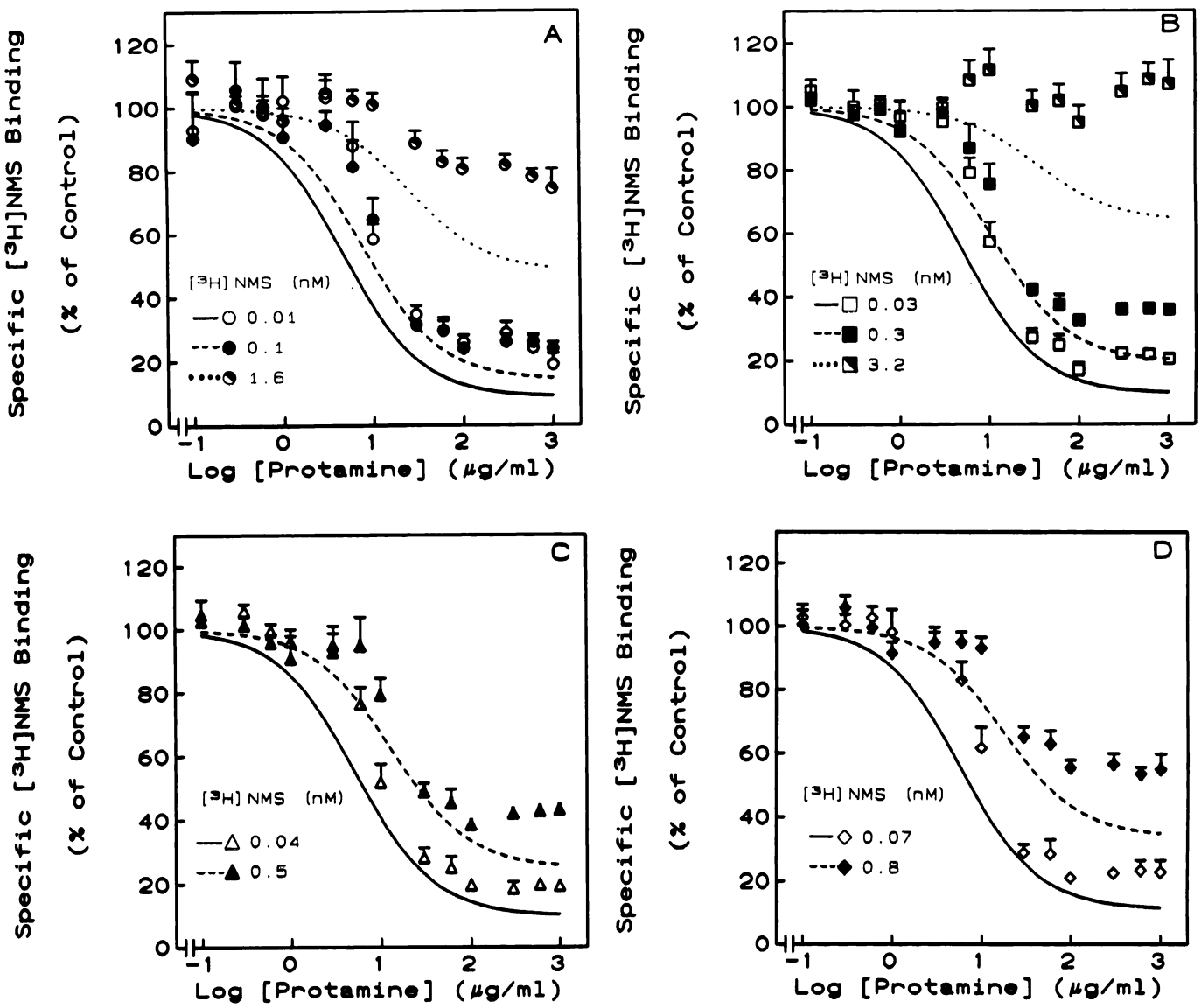

Fig. 2. Inhibition of [^{3}H]NMS binding at equilibrium by protamine in rat heart. Cardiac membranes were incubated with various concentrations of [^{3}H]NMS (A–D), in the absence or presence of increasing concentrations of protamine for 3 hr at 25°. The *curves* represent theoretical fits according to a simple allosteric model, as described by Ehlert (12) (α = 12.2, K_a = 4.3 μg/ml). Data shown are means ± standard errors from 4–11 independent experiments and are represented as percentage of control, which is the level of specific binding in the absence of protamine.

L-aspartic acid significantly inhibited the effects of protamine in altering [^{3}H]NMS dissociation or binding at equilibrium in the heart, in a concentration-dependent manner (Fig. 4). This suggests that neutralization of the dense positive charges on protamine could prevent its allosteric effects.

Role of arginine residues in the allosteric interactions of protamine. Because 90% of the amino acid residues in protamine sequences are arginines (13), we tested the effects of poly-L-arginine on [^{3}H]NMS binding in rat heart. Interestingly, poly-L-arginine (M_r 11,600) exhibited apparent allosteric behavior similar to that of protamine in its interaction with cardiac muscarinic receptors. Poly-L-arginine (0.5 μM) significantly increased the $t_{1/2}$ value of [^{3}H]NMS dissociation at cardiac muscarinic receptors from 3 ± 0.02 to 8 ± 1 min (Fig. 5A). This effect was concentration dependent, with an estimated IC_{50} value of 0.5 ± 0.1 μM (Fig. 5B).

Poly-L-arginine decreased the affinity without significantly altering the B_{max} of [^{3}H]NMS. Similar to protamine, Schild regression of the effects of poly-L-arginine on saturation isotherms for [^{3}H]NMS binding also showed a ceiling effect on ligand affinity (Fig. 6), with a negative cooperativity value (α) of 12.6 ± 2.8 and a K_a value of 0.4 ± 0.04 μM. A different representation of the data showed that poly-L-arginine reduced the specific binding of a fixed concentration of [^{3}H]NMS to an apparent plateau at higher concentrations of the displacer. The magnitude of maximal inhibition decreased when the concentration of [^{3}H]NMS was increased (Fig. 7; Table 2). There was a reasonable correlation between the increases in the IC_{50} of poly-L-arginine due to increasing [^{3}H]NMS concentrations obtained experimentally and those calculated theoretically according to a simple ternary allosteric model (Fig. 7; Table 2) (r = 0.8 and slope = 2.3 for IC_{50} ratios relative to the values at 0.02 nM [^{3}H]NMS; r = 0.8 and slope = 1.2 for absolute values). An excellent correlation was observed for the magnitude of

TABLE 2

Relationship between [^{3}H]NMS concentration and potency of protamine and poly-L-arginine in inhibiting ligand binding at equilibrium

Experimental IC_{50} values and maximal inhibition were estimated according to a four-parameter logistic inhibition model, using the GraphPad program.

[^{3}H]NMS	Protamine potency		
	IC_{50}	Ratio of IC_{50} to that at lowest ligand concentration	Maximal inhibition
nM	μg/ml		%
0.01	20 ± 4 (5)[a] [13][b]	1.0	86 ± 3 (91) [92]
0.03	19 ± 5 (6) [10]	1.0 (1.1)	85 ± 1 (91) [91]
0.04	15 ± 5 (6) [9]	0.8 (1.1)	86 ± 1 (90) [91]
0.07	21 ± 6 (7) [11]	1.1 (1.3)	83 ± 1 (89) [89]
0.14	20 ± 6 (8) [10]	1.0 (1.7)	80 ± 1 (86) [87]
0.3	23 ± 7 (11) [13]	1.2 (2.3)	70 ± 1[c] (80) [80]
0.5	26 ± 9 (14) [13]	1.3 (2.9)	62 ± 1[c] (74) [74]
0.8	47 ± 20 (18) [13]	2.4 (3.9)	45 ± 4[c] (65) [63]
1.6	126 ± 45[c] (26) [22]	6.3 (5.5)	17 ± 5[c] (51) [45]
3.2	ND[d] (33) [31]		ND (36) [25]

[^{3}H]NMS	Poly-L-arginine potency		
	IC_{50}	Ratio of IC_{50} to that at lowest ligand concentration	Maximal inhibition
nM	μM		%
0.02	0.8 ± 0.3 (0.4)	1.0	100 ± 2 (91)
0.04	0.7 ± 0.2 (0.4)	0.9 (1.0)	95 ± 2 (91)
0.05	1.0 ± 0.5 (0.4)	1.2 (1.0)	93 ± 3 (90)
0.09	0.6 ± 0.1 (0.5)	0.8 (1.2)	91 ± 1[c] (89)
0.2	0.5 ± 0.2 (0.7)	0.6 (1.8)	85 ± 4[c] (86)
0.4	0.6 ± 0.2 (0.9)	0.8 (2.2)	81 ± 3[c] (80)
0.6	0.8 ± 0.3 (1.1)	1.0 (2.8)	75 ± 4[c] (76)
0.9	1.0 ± 0.2 (1.5)	1.2 (3.8)	62 ± 5[c] (67)
1.9	1.9 ± 0.4 (2.2)	2.4 (5.5)	33 ± 4[c] (52)
3.8	ND (2.9)		ND (37)

[a] Values in parentheses are the theoretical estimates calculated according to eq. 10 in Ref. 12, using k_d = 0.2 nM for [^{3}H]NMS, K_a = 4.3 μg/ml and α = 12.2 for protamine, and K_a = 0.4 μM and α = 12.6 for poly-L-arginine.

[b] The values in brackets were obtained by calculating specific [^{3}H]NMS binding as a fraction of B_{max} under each experimental condition. This method of normalization nullifies the changes in B_{max} in the presence of protamine.

[c] Significantly different from the value at the lowest concentration of [^{3}H]NMS, $p < 0.05$.

[d] ND, not determined, due to the lack of a significant effect.

maximal inhibition when experimental and theoretical values were compared (r = 1.0, slope = 0.8) (Table 2).

Allosteric interactions of other polycationic peptides, but not polyamines or choline chloride, with cardiac muscarinic receptors. In addition to poly-L-arginine, other positively charged polyamino acids, such as poly-L-lysine (M_r 10,200) and poly-L-ornithine (M_r 11,700), also exerted an allosteric effect on muscarinic receptors, which was demonstrated by the deceleration of the rate of [^{3}H]NMS dissociation induced by atropine and an incomplete inhibition of [^{3}H]NMS binding at equilibrium (Fig. 8). Table 3 presents the estimated parameters for the allosteric interactions of these polycationic peptides with cardiac muscarinic receptors. The higher α value of either protamine or poly-L-arginine, compared with that of poly-L-lysine and poly-L-ornithine, suggests that structure-related factors other than mere electrostatic effects might be implicated in the allosteric interactions. It is of interest that poly-D-lysine had a higher cooperativity factor (α = 4.3 ± 0.3) in inhibiting [^{3}H]NMS binding than did its L-isomer (α = 2.7 ± 0.4) ($p < 0.05$), showing a weak degree of stereoselectivity.

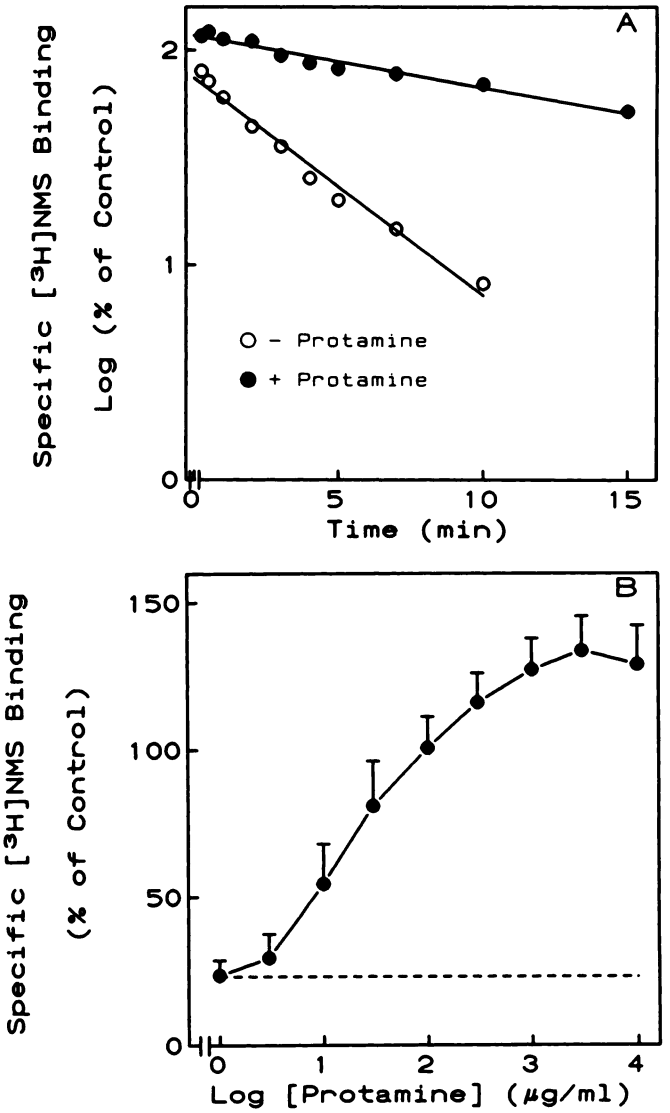

Fig. 3. Deceleration of the dissociation rate of [^{3}H]NMS by protamine in rat heart. A, Inhibition by protamine (50 μg/ml) of the dissociation rate of [^{3}H]NMS. Dissociation was initiated by 2 μM atropine, in the absence or presence of protamine, and allowed to proceed for the indicated time. B, Concentration-dependent inhibition by protamine of [^{3}H]NMS dissociation. Dissociation was initiated by 2 μM atropine, in the absence (- - -) or presence (●) of increasing concentrations of protamine for 5 min. Data shown are means ± standard errors from 4–11 independent experiments performed in duplicate, and the *points* were connected by *straight lines*. Control is the value of specific binding of [^{3}H]NMS before the initiation of dissociation.

On the other hand, no effects of spermine, spermidine, putrescine, or choline chloride on either dissociation kinetics or equilibrium binding of [^{3}H]NMS were observed (Fig. 8).

Selectivity of protamine-induced allosteric effects among various muscarinic receptor subtypes. Fig. 9A demonstrates the influence of increasing concentrations of protamine on atropine-induced dissociation of [^{3}H]NMS from m1, m2, m3, m4, and m5 muscarinic receptor subtypes in membranes prepared from CHO cells. There was no difference in the potency of protamine in inducing deceleration of ligand dissociation at the m1 and m2 receptors, with respective estimated IC_{50} values of 24 ± 6 μg/ml and 15 ± 3 μg/ml ($p > 0.05$). Interestingly, however, protamine was markedly less potent in altering ligand dissociation at the m3, m4, and m5 receptor

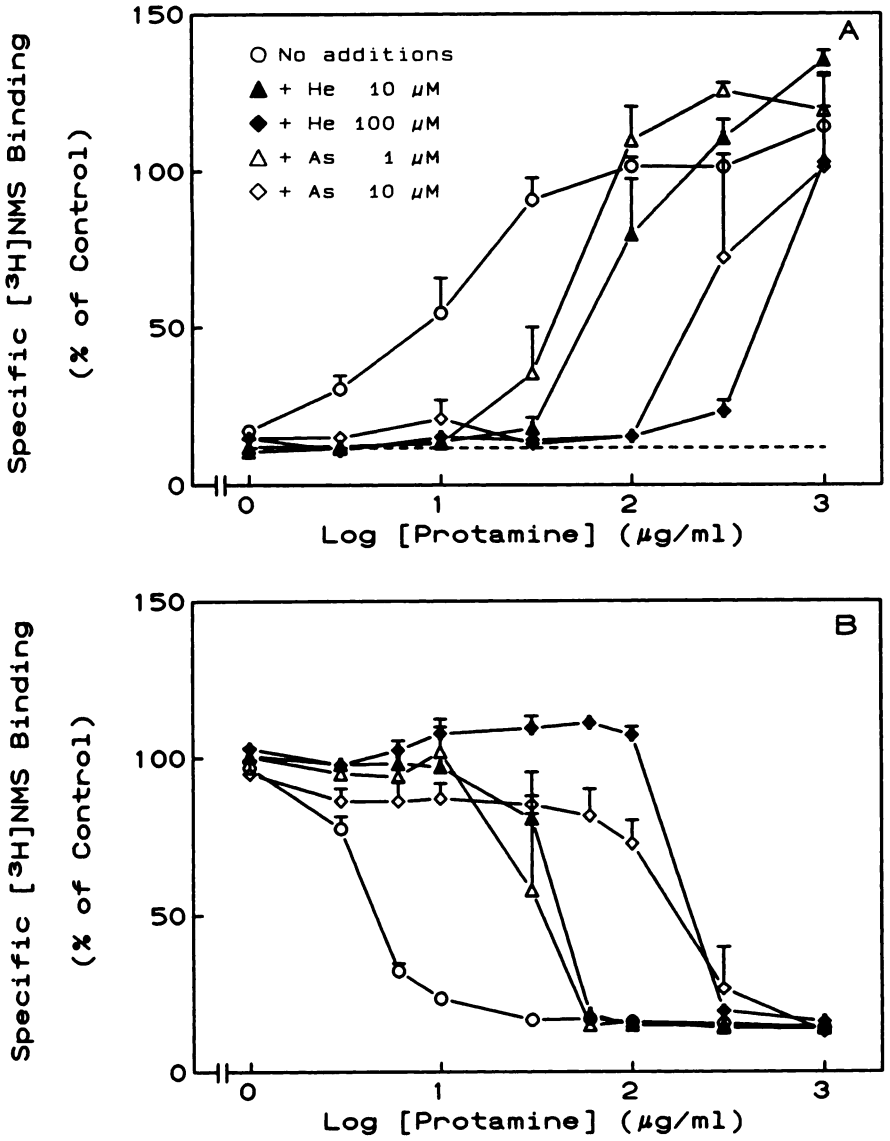

Fig. 4. Influence of heparin and poly-L-aspartic acid on protamine-induced allosteric interactions with cardiac muscarinic receptors. A, Effects on protamine-induced modification of [^{3}H]NMS dissociation. - - -, Extent of dissociation at 5 min at 25° in the presence of 2 μM atropine alone. Control is specific [^{3}H]NMS binding before the initiation of dissociation. B, Effects on protamine-induced inhibition of [^{3}H]NMS binding at equilibrium. Control is [^{3}H]NMS binding in the absence of protamine. Data shown are means ± standard errors from three independent experiments performed in duplicate, and the *data points* were connected by *straight lines*. *He*, heparin; *As*, poly-L-aspartic acid. The same symbols were used in A and B.

subtypes, with IC_{50} values of 104 ± 22 μg/ml in m3, 95 ± 18 μg/ml in m4, and 220 ± 60 μg/ml in m5 cells, which are significantly higher than the IC_{50} values at either m1 or m2 receptors ($p < 0.01$). Protamine was also selective regarding its maximal effect on [^{3}H]NMS dissociation (m1 > m2 > m3 > m4 > m5) (Fig. 9A). In equilibrium displacement binding experiments, protamine exhibited the following rank order of potency: m2 ≥ m1 > m4 ≈ m3 ≈ m5 (Fig. 9B). The estimated IC_{50} of protamine at m1 and m2 receptors averaged 176 ± 57 and 85 ± 39 μg/ml, respectively. It should be noted, however, that the potency of protamine in interacting with cardiac M_2 receptors was higher than that obtained in CHO cells expressing the same receptor subtype. One possible explanation is that membrane constituents in addition to the specific sequence of receptor protein influence the interaction of protamine with the receptor. Protamine was significantly less potent at the other three receptor subtypes, with observed inhibition of ligand binding at the highest concentration used (1000 μg/ml) of 30 ± 6%, 44 ± 9%, and 52 ± 8% at m3, m4, and m5 receptors, respectively (Fig. 9B).

Discussion

The allosteric nature of the interaction of protamine with cardiac muscarinic receptors has been demonstrated in radioligand binding assays by using several qualitative diagnostic experimental protocols. On the other hand, the observed profile of protamine binding to muscarinic receptors deviated quantitatively from a simple ternary model (12), suggesting a more complex mode of interaction. Some of these deviations could be accounted for by the effects of protamine on the B_{max} of [^{3}H]NMS binding. Arginine residues in the sequence of protamine appear to play an important role in this allosteric interaction, and these effects of protamine could be neutralized by polyanions. Furthermore, the interaction of protamine with muscarinic receptors is selective for the m1 and m2 muscarinic receptor subtypes, compared with the m3, m4, and m5 subtypes.

The molecular mechanisms underlying allosteric interactions of a variety of compounds with muscarinic receptors remain unclear. It has been proposed that one type of allosteric interaction can take place upon binding of an agent to an ion channel

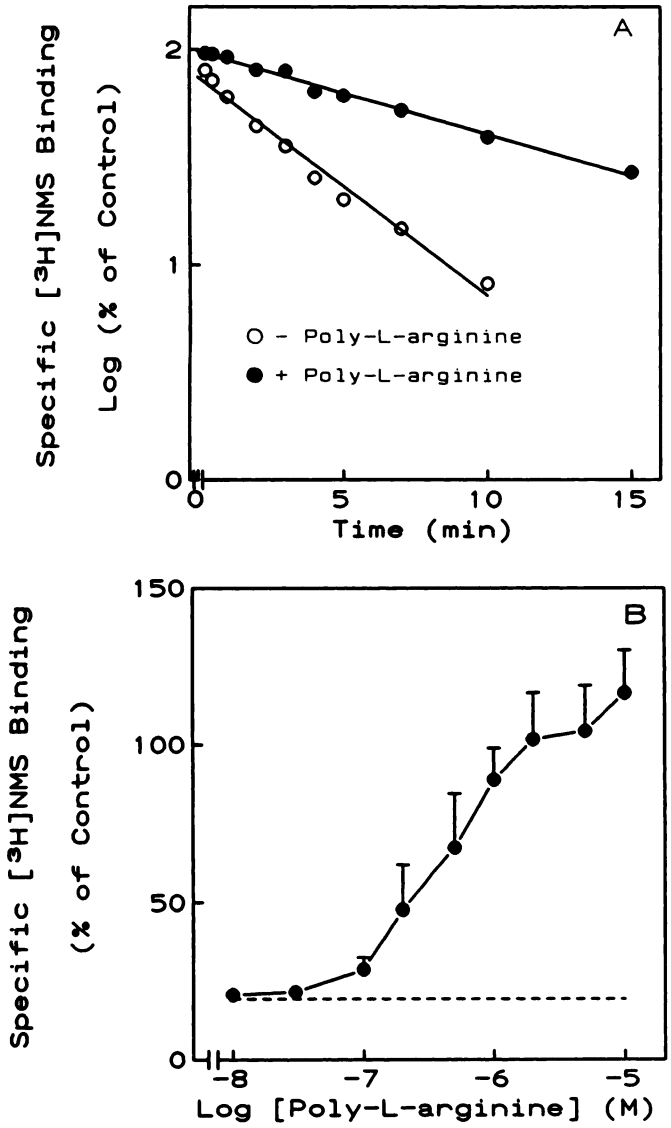

Fig. 5. Deceleration of the dissociation rate of [^{3}H]NMS by poly-L-arginine in rat heart. A, Inhibition by poly-L-arginine (0.5 μM) of the dissociation rate of [^{3}H]NMS. Dissociation was initiated by the addition of 2 μM atropine, in the absence or presence of poly-L-arginine, and allowed to proceed for the indicated times at 25°. Control is the specific binding of [^{3}H]NMS before addition of atropine or atropine plus poly-L-arginine. *Data points* were fitted by linear regression. B, Concentration-dependent inhibition by poly-L-arginine of [^{3}H]NMS dissociation. Dissociation was induced in the absence (- - -) or presence (●) of increasing concentrations of poly-L-arginine for 5 min. *Data points* were connected by *straight lines*. Control is the binding of [^{3}H]NMS before initiation of dissociation. Data shown are the means ± standard errors from four independent experiments performed in duplicate.

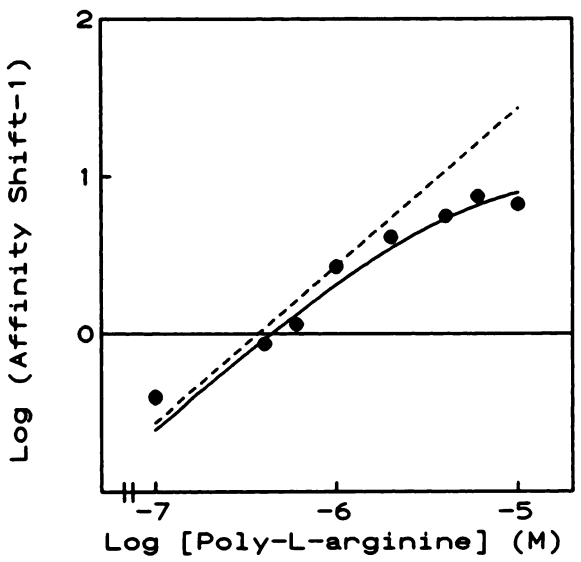

Fig. 6. Schild plot of the effects of poly-L-arginine on [^{3}H]NMS saturation isotherms in rat heart. Cardiac membranes were incubated with a series of [^{3}H]NMS concentrations, in the absence and presence of increasing concentrations of poly-L-arginine. Affinity shift is the ratio between the ligand equilibrium dissociation constant in the presence and in the absence of poly-L-arginine. - - -, Theoretical curve for competitive interaction, with a slope of unity. The *data points* were fitted as shown (——) according to the ternary model of allosteric interactions (12). Data shown are means ± standard errors from four independent experiments performed in duplicate.

that is coupled to muscarinic receptors through a GTP-binding protein (19, 20). In a more generalized model of receptor allosterism, it has been suggested that both an allosteric antagonist and a competitive ligand can bind simultaneously to distinct domains on the receptor, to form a ternary complex, where there is a reciprocal effect of the two agents on their affinity of binding to the receptor (3, 12). The latter model adequately explains the allosteric interactions of gallamine with muscarinic receptors (3). These effects of gallamine persist upon receptor solubilization (3) and are not altered upon uncoupling of the receptor from its associated ion channels in the heart (9).

Protamine decelerated the rate of dissociation of [^{3}H]NMS, induced by a high concentration of atropine, from cardiac muscarinic receptors. Such an effect is a hallmark of an allosteric cooperative interaction (8). This effect was concentration dependent. Protamine also incompletely inhibited ligand binding at equilibrium, and its maximal inhibition was inversely proportional to ligand concentration. It also decreased the affinity of [^{3}H]NMS for cardiac muscarinic receptors, in a concentration-dependent fashion, and this effect demonstrated a marked ceiling value. This was reflected as a curvilinearity of the Schild regression, which clearly deviated from what is expected for a competitive interaction. The latter two experimental protocols strongly suggest that protamine influences muscarinic receptor conformation in a negatively cooperative manner (12, 21, 22). The magnitude of this cooperative interaction (α = 12.2) is comparable to that of gallamine in the heart using [^{3}H]NMS as a ligand (α = 13) (3). Gallamine is a prototype for muscarinic receptor allosteric antagonists (2, 4). In contrast, protamine exhibited minimal effects on the rate of dissociation of [^{3}H]QNB in the heart and inhibited its binding at equilibrium with a smaller α value of 3.6. These results support the notion that the apparent nature of cooperative interactions of a compound with muscarinic receptors depends on the ligand used (4).

There is also some evidence, however, that the binding of protamine to cardiac muscarinic receptors deviates from a simple ternary allosteric mechanism (12). First, this is supported by the departure of the data points in the Schild plot and inhibition curves from the theoretical values calculated according to this specific model of drug-receptor interaction (Figs. 1 and 2; Table 2). Second, there was a noticeable trend of a concentration-dependent increase in the B_{max} of [^{3}H]NMS in the presence of protamine. In fact, this effect offered a partial explanation for the apparent discrepancy in the mag-

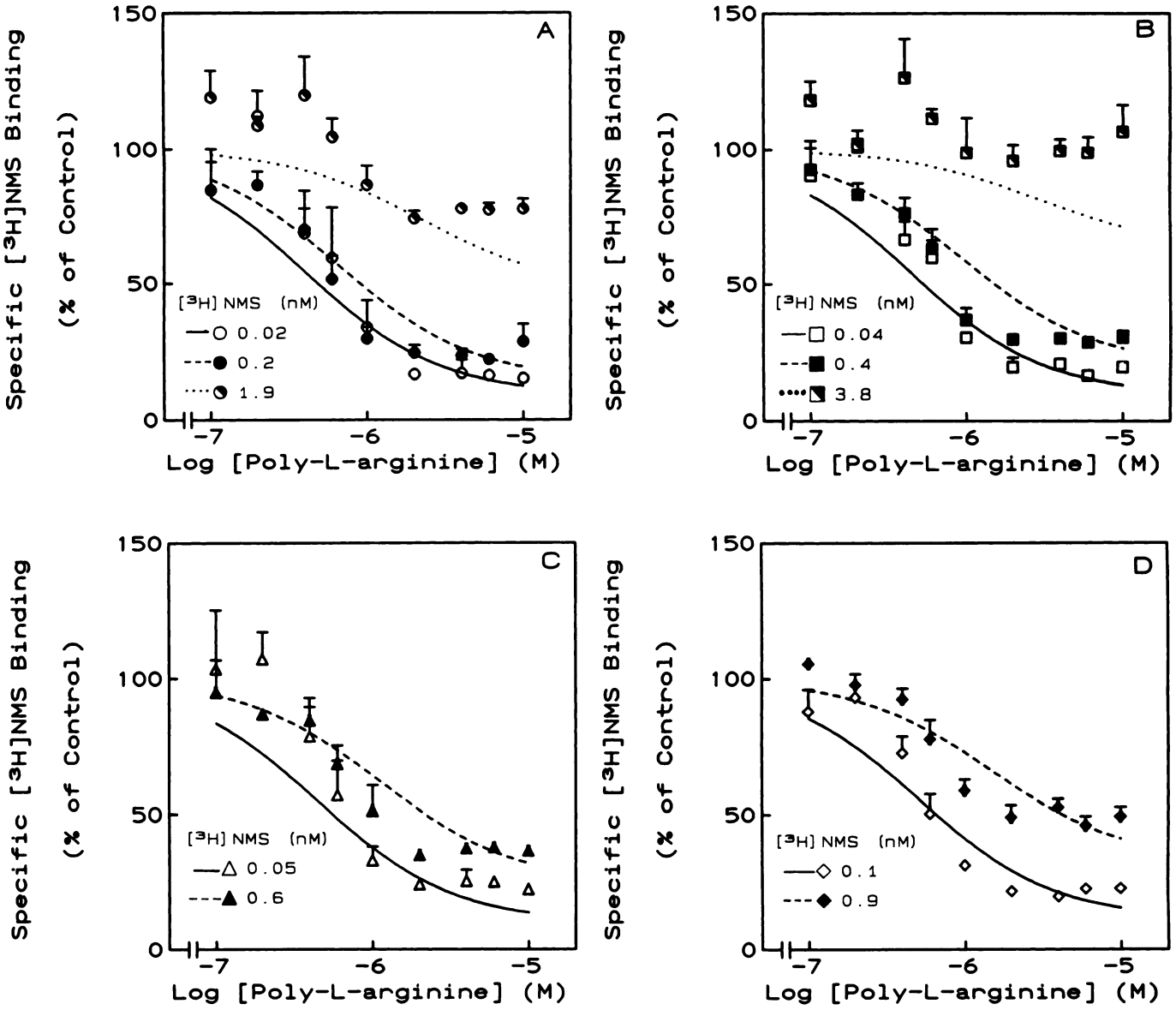

Fig. 7. Inhibition of [^{3}H]NMS binding at equilibrium by poly-L-arginine in rat heart. Cardiac membranes were incubated with different concentrations of [^{3}H]NMS (A–D), in the presence of increasing concentrations of poly-L-arginine, at 25° for 3 hr. The *curves* are theoretical fits according to a simple allosteric model, as described by Ehlert (12) (α = 12.6, K_a = 0.4 μM). Data shown are the means ± standard errors from four independent experiments and are represented as a percentage of control, which is the specific binding of [^{3}H]NMS in the absence of poly-L-arginine.

nitude of the maximal inhibition of binding of different [^{3}H] NMS concentrations by protamine and its IC_{50}, in relation to the values expected for a simple allosteric modulator (Table 2). Schild regressions were not equally affected by this increase in B_{max} produced by protamine, because ligand K_d values were used for these calculations. Thus, this method of computation, in essence, has the same effect in nullifying the influence of changes in B_{max} as does normalization of saturation isotherms in the absence and presence of protamine to an equal maximal binding capacity. Third, protamine was almost equipotent in equilibrium and dissociation kinetics experimental protocols in the heart. In fact, it was even more potent in decreasing the ligand dissociation rate than at inhibiting steady state binding in m1- or m2-expressing CHO cells. In general, allosteric antagonists exhibit a higher apparent potency in inhibiting equilibrium binding than in affecting the rate of ligand dissociation, although there are exceptions to the rule (9). This apparent differential potency is likely due to preoccupation of receptors with the radioligand in the latter type of experimental design. However, no constant quantitative relationship of the potency ratios under these two conditions should necessarily be expected for a given allosteric antagonist, because the outcome is dependent on the magnitude of cooperative interactions, the on-rate of the allosteric compound, and the off-rate of the radioligand.

The amino acid sequence of muscarinic receptors contains several highly conserved negatively charged aspartic acid residues, which have been shown to be important for binding of agonists and antagonists or for agonist-induced changes in receptor conformation (10). It has been suggested that some of these conserved groups might also be involved in the interaction of muscarinic receptor allosteric antagonists (23, 24), most of which are positively charged at physiological pH (8). In fact, we recently demonstrated that mutagenesis of certain aspartic

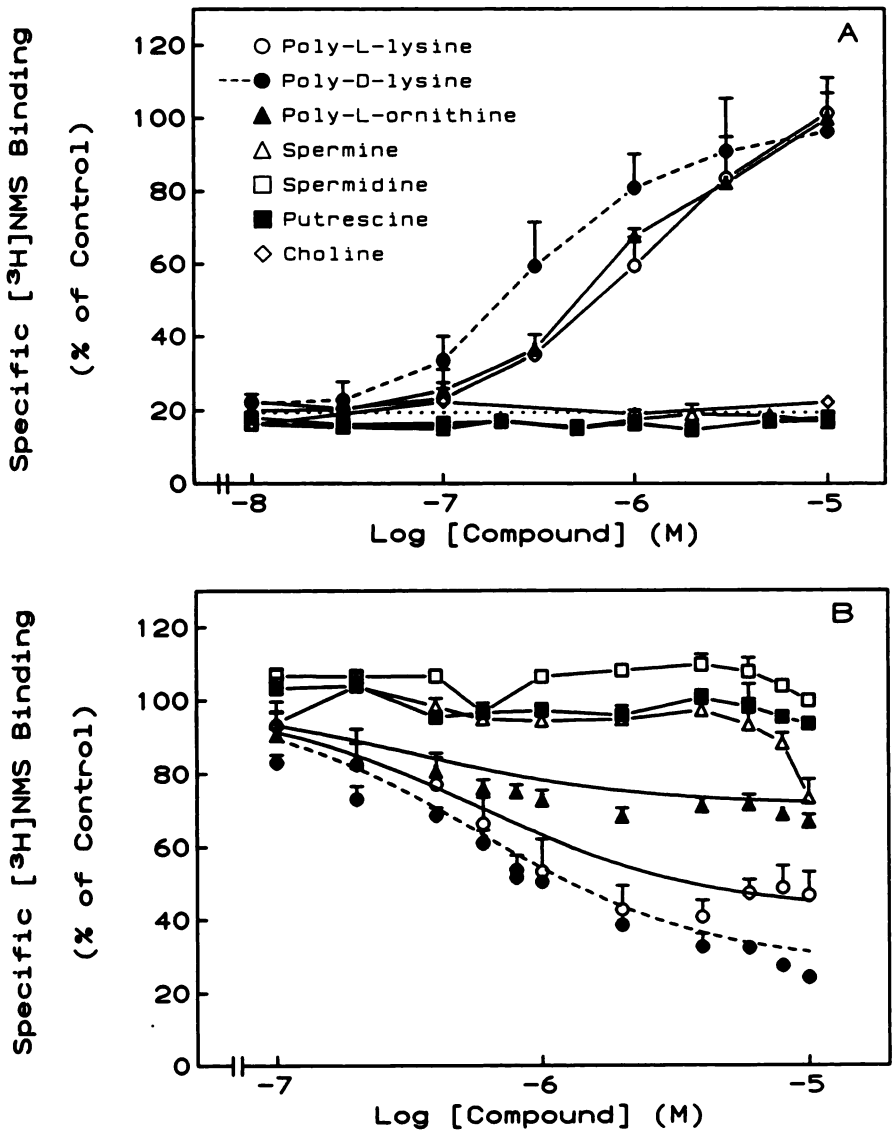

Fig. 8. Allosteric interactions of other polycationic amino acids, but not polyamines or choline chloride, with cardiac muscarinic receptors. A, Concentration-dependent effects on [^{3}H]NMS dissociation. Dissociation was allowed to proceed in the presence of 2 μM atropine, without (· · ·) or with (*symbols*) increasing concentrations of the test substances, for 5 min at 25°. Control is specific binding of [^{3}H]NMS before dissociation. *Data points* were connected by *straight lines*. B, Effects on specific binding of 0.04 nM [^{3}H]NMS at equilibrium. Curves for poly-L-ornithine and the two isomers of poly-lysine were fitted by computer according to a simple allosteric model (12), using the estimates of α and K_a listed in Table 3. Other *data points* were connected by *straight lines*. Control is the specific binding of [^{3}H]NMS in the absence of inhibitors. Data shown are means ± standard errors from three to six independent experiments performed in duplicate. The same symbols were used in A and B.

TABLE 3

Allosteric effects of polycationic peptides on cardiac muscarinic receptors

Data were obtained from dissociation kinetic and equilibrium [^{3}H]NMS binding studies, as described in Experimental Procedures. IC_{50} values for dissociation were estimated graphically. IC_{50} values at equilibrium were estimated by computer fitting according to a ternary allosteric model, using the estimated α values for each compound (12). These values were calculated by the equation $\alpha \approx 1/Y'$, which is the special case (at very low concentrations of radioligand) of the equation $\alpha = (K_d + [D](1 - Y'))/K_d \cdot Y'$ (12). $[D]$ is the concentration of [^{3}H]NMS (0.04 nM) and Y' is the fraction of [^{3}H]NMS binding remaining at the plateau. In dissociation experiments, the concentration of the polycationic amino acids was 0.5 μM.

	Deceleration of dissociation rate		Inhibition of [^{3}H]NMS binding at equilibrium	
	$t_{1/2}$	IC_{50}	α	IC_{50}
	min	*μM*		*μM*
Atropine alone	3.0 ± 0.02			
+Poly-L-lysine	6.6 ± 0.1[a]	0.4 ± 0.1	2.7 ± 0.4	0.5 ± 0.2
+Poly-D-lysine		0.3 ± 0.1	4.3 ± 0.3	0.5 ± 0.1
+Poly-L-ornithine	8.5 ± 0.4[a]	0.6 ± 0.1	1.5 ± 0.1	0.3 ± 0.1

[a] $p < 0.01$, versus atropine.

acid residues of the m1 muscarinic receptor, to asparagine, resulted in a significant modification of the allosteric interactions of gallamine with the receptor (11). It is highly plausible that the allosteric effects of protamine on cardiac muscarinic receptors might entail similar electrostatic interactions, for the following reasons. First, poly-L-arginine elicits allosteric effects on cardiac muscarinic receptors that are qualitatively similar to those of protamine. Protamine is mostly composed of arginine residues (13). These effects of poly-L-arginine showed less marked deviations from a simple allosteric model than did those of protamine. Second, the effects of protamine are antagonized by polyanions such as heparin and poly-L-aspartic acid. Third, mutagenesis of certain aspartate residues in the m1 receptor sequence, particularly residue 122, alters the allosteric effects of protamine.[2] Fourth, other polycationic peptides, such as poly-L-lysine and poly-L-ornithine, mimic the effects of protamine on cardiac muscarinic receptors, albeit with varying magnitudes of cooperativity. Thus, structural features in addition to mere electrostatic interactions may influence allosteric

[2] J. Hu and E. E. El-Fakahany, unpublished observations.

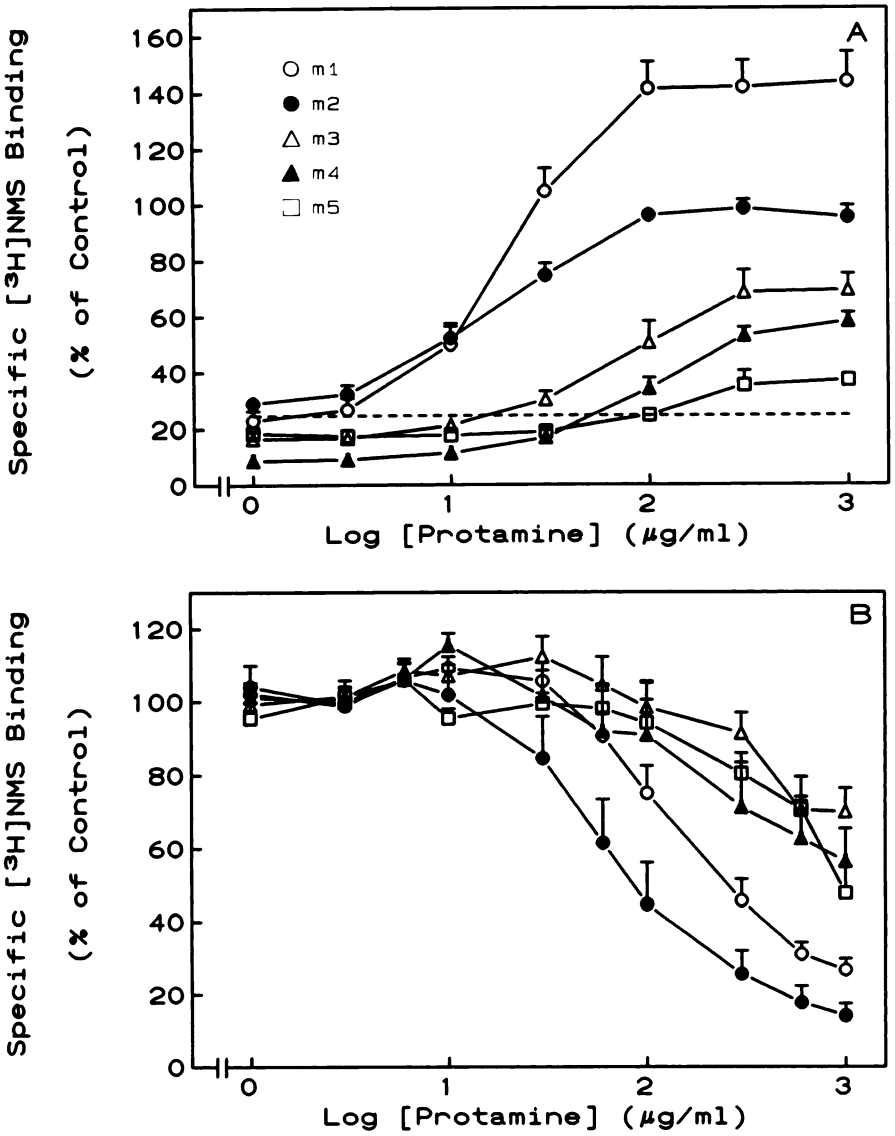

Fig. 9. Allosteric interactions of protamine with the different muscarinic receptor subtypes in membranes of CHO cells. A, Concentration-dependent inhibition of [^{3}H]NMS dissociation by protamine. Dissociation was initiated by 2 μM atropine and was allowed to proceed at 25°, in the absence (– – –) or presence (*symbols*) of increasing concentrations of protamine, for 20 min (m1), 5 min (m2), 80 min (m3), 120 min (m4), or 140 min (m5), in CHO cells. Control is specific binding before dissociation. B, Inhibition by protamine of binding of 0.04 nM [^{3}H]NMS at equilibrium. Control is binding in the absence of protamine. Data shown are means ± standard errors from four to seven independent experiments performed in duplicate, and the *data points* were connected by *straight lines*. The same symbols were used in A and B.

binding. This is also supported by the absence of effects of choline and the polyamines spermine, spermidine, and putrescine on either the dissociation rate of [^{3}H]NMS or its binding at equilibrium. The allosteric effects of polycationic peptides, e.g., poly-lysine, exhibited some stereoselectivity. This stereoselectivity is comparable in magnitude to and is in the same direction as that of the allosteric effects of the isomers of verapamil (25).

The allosteric effects of protamine were selective for the m1 and m2 subtypes of muscarinic receptors, compared with the m3, m4, and m5 subtypes. This was demonstrated in both kinetic and equilibrium studies. This observation agrees with previous findings that all known agents that are selective for m2 versus m3 receptors interact with muscarinic receptors in an allosteric fashion (8, 9, 26–30).

In summary, our data provide a clear qualitative indication of an allosteric mode of interaction of protamine with muscarinic receptors. However, there are marked quantitative differences between the observed effects of protamine and those expected in the case of a simple allosteric receptor antagonist. These deviations signify a more complex mechanism of protamine-receptor interactions. Nevertheless, our data suggest that protamine-like peptides might function as putative endogenous allosteric modulators of the conformation of muscarinic receptors. In fact, preliminary experiments from this laboratory have shown that other peptides enriched in arginine or lysine, such as dynorphin A-(1–13), histones, and myelin basic protein, also interact with muscarinic receptors in a negatively cooperative manner.[2] The search for such endogenous substances that interact with muscarinic receptors is of utmost importance, due to their potential modulatory role in the fine tuning of cholinergic neurotransmission in a variety of physiological and pathological conditions.

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