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MYOCARDIAL MUSCARINIC ACETYLCHOLINE RECEPTOR: CHOLINE AND TRIS UNMASK HETEROGENEITY OF ANTAGONIST BINDING SITES

A. Sastre, K.M.M. Murphy and M.M. Rusher

Departments of Physiology and Neuroscience
The Johns Hopkins University School of Medicine
725 North Wolfe Street, Baltimore, Maryland 21205

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The binding properties of myocardial muscarinic acetylcholine receptors are altered in the presence of choline or Tris. The binding of the antagonist [^{3}H]quinuclidinyl benzilate is reduced in the presence of choline or Tris buffer, when compared to parallel determinations in a physiologic salt solution or phosphate buffer. Scatchard analysis indicates the reduced binding is due to a decrease in the apparent number of receptor sites. Experiments with other organic buffers exclude the possibility that the reduced binding in Tris is due to the absence of sodium ions. In the presence of choline or Tris up to 45% of the receptors are not accessible to [^{3}H]quinuclidinyl benzilate. The remaining sites maintain their high affinity for the antagonist. A heterogeneity of antagonist sites is evident.

INTRODUCTION

Studies with radiolabelled ligands have revealed a multiplicity of binding sites in the muscarinic acetylcholine receptor (mAChR). Two (1) or three (2) sites appear necessary to account for the complex binding isotherms obtained with agonists. In addition, mAChRs in homogenates from heart tissue exhibit agonist-specific interactions with Na^+ and GTP (3,4,5). In contrast, antagonists appear to bind to single, homogeneous class binding sites (6,7,8).

In this paper we report a surprising heterogeneity in the antagonist binding sites of cardiac mAChRs. This heterogeneity is unmasked by the cholinergic precursor and partial agonist choline (9,10) and the widely used amine buffer and cholinergic antagonist Tris. The effect of choline becomes evident at concentrations (0.1 - 1 mM) believed to be attained during synaptic transmission. The effect of Tris is evident at concentrations (10 - 100 mM) widely used in binding assays.

Abbreviations: mAChR: muscarinic acetylcholine receptor; QNB: quinuclidinyl benzylate; BES: (N,N-bis[2-hydroxyethyl]-2-aminoethane sulfonic acid); HEPES: (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid); MOPS: (morpholinopropane sulfonic acid); TES: (N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid); R_T: maximal binding capacity.

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MATERIALS AND METHODS

Hearts were excised from mongrel dogs anesthetized with 30 mg/kg i.v. Na^+-pentobarbital. Atria and atrial appendages were cleaned free of blood and connective tissue in ice-cold 0.9% NaCl. Subsequent steps were carried out in a cold room at 4°C. The tissue was blotted, weighed and homogenized in 19 volumes of 0.32 M sucrose with a PT-35K Polytron at maximum setting, using 4 15-sec bursts separated by 1-min cooling periods. Similar results were obtained when the binding assays were carried out with (i) whole homogenates; (ii) homogenates filtered through 6 layers of stainless steel wire mesh, or (iii) homogenates which were filtered, centrifuged at 40,000 x g for 15 min, and the pellet resuspended in the original volume of either 0.25 M sucrose or the assay buffers.

For the binding assays, 50 or 100 µl of tissue homogenate were added to tubes containing assay buffer (see below), [^{3}H]QNB (either [^{3}H](DL)QNB, 29 Ci/mmol, NEN or [^{3}H](L)QNB, 44 Ci/mmol, Amersham) and any unlabelled ligands in a total volume of 1 ml. Incubation was carried out for 60 min at 25°C in a temperature controlled shaking water bath. The reaction was terminated by chilling on ice and rapid filtration through Whatman GF/C filter disks. The assay tubes were rinsed with 3 ml of buffer, and the filters further rinsed with 10 ml of buffer (all at 1°C). Assays were carried out in duplicate, with duplicate determinations of non-specific binding (defined as the ^{3}H bound to the filters when the incubation medium contained 1 µM atropine). Specific binding was typically ≥ 90% of the total. Complete extraction of the filter-bound ^{3}H was obtained by incubation for 30 minutes with 100 µl NCS solubilizer (Amersham), followed by addition of 10 ml scintillation fluid and shaking overnight. Counting efficiency was determined using [^{3}H]toluene by the internal standard method.

Scatchard analysis of the binding isotherms for [^{3}H]QNB (40 pM to 2 nM) included a small correction for free ligand depletion at equilibrium. The concentration of mAChR was below 0.1 nM. Protein was determined by the method of Lowry et al (11).

Assay buffers included: Na^+-PO_4, K^+-PO_4, Tris, BES, HEPES, MOPS, TES; and PBS (Dulbecco's phosphate buffered saline, containing in mM: NaCl, 138; KCl, 2.6; $CaCl_2$, 1.0; $MgSO_4$, 0.5; Na_2HPO_4, 8; and KH_2PO_4, 1.5). All were adjusted to pH 7.4 at 25°C, the phosphate buffers by the proper ratio of monobasic and dibasic salts; Tris with either the fully neutralized HCl salt or by concentrated HCl; BES, HEPES, MOPS and TES with 1 N NaOH. The organic buffers, atropine-SO_4 and choline Cl were obtained from Sigma; all other reagents were of the highest purity commercially available.

RESULTS AND DISCUSSION

The initial observation is illustrated in Figure 1. Assay of atrial homogenates in Tris reduces the amount of [^{3}H]QNB bound at all concentrations, with respect to the binding observed in Na^+-PO_4 buffer in a parallel determination. The maximal binding capacity (R_T) measured in the physiological PBS was the same as in Na^+-PO_4, thus the latter was used as a convenient "reference" buffer. A Scatchard transform of the binding isotherms reveals the reduced binding in Tris to be due to a surprising reduction in the apparent R_T. This effect on R_T depends on the concentration of Tris up to 50 mM (which decreases R_T by 45%); no further reduction is seen at 100 mM Tris with respect to a

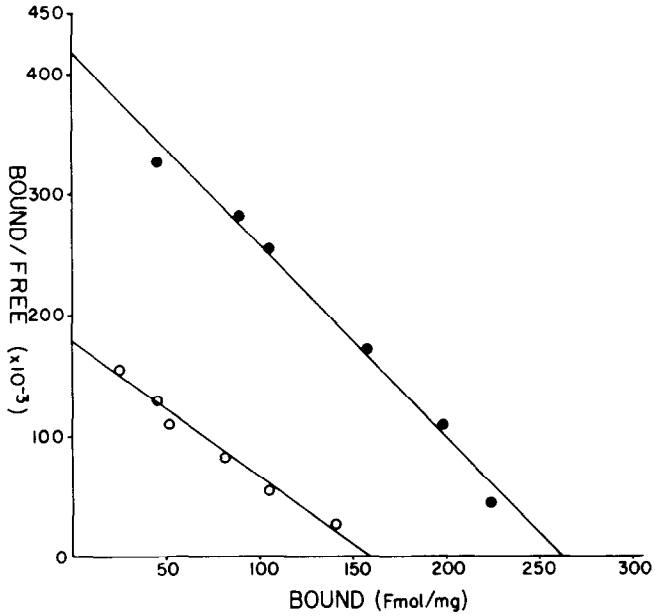

Figure 1: Scatchard plot of [^{3}H]QNB binding to canine atrial homogenates in 50 mM Na^+-PO_4 buffer (●) or 50 mM Tris-HCl buffer (○), both pH = 7.4 at 25°C.

Na^+-PO_4 control of comparable ionic strength (Table 1). The K_D for [^{3}H]QNB increases in both buffers with ionic strength (12), however, the difference in K_D as measured in Na^+-PO_4 and Tris is reduced as the concentration of the buffer (and the ionic strength) is increased (Table 1). It is evident that Tris can "mask" up to 45% of the QNB binding sites while exhibiting a negligible affinity for the remaining sites; a heterogeneity of antagonist sites is evident.

A similar situation exists with respect to choline (Figure 2). In the range of 1 μM to 1 mM, statistically significant reductions in R_T of 12% at 0.1 mM ($P < 0.05$, N=4) and 32% at 1 mM ($P < 0.001$, N=8) are seen. In contrast to Tris, choline does exhibit significant affinity for the remaining QNB sites, as inferred from the increased apparent K_D for QNB (from 0.10 nM to 0.19 nM in 50 mM Na^+-PO_4 plus 1 mM choline). The weak affinity of choline for the QNB sites is expected from and consistent with previous displacement studies (13,14,15); the effect on R_T is surprising.

These results raise the question of whether the observed reduction in R_T results from the presence of Tris (or choline) or the absence of Na^+ in Tris buffer. Two classes of experiments exclude the second alternative. In the

TABLE 1. Effects of Tris and NaCl on the affinity and maximal binding capacity of atrial homogenates for [^{3}H]QNB

	K_D ($\times 10^{-10}$M)	% Change in R_T	
10 mM Na^+-PO_4	0.53 ± 0.07	-	n=5
10 mM Na^+-PO_4 + 100 mM NaCl	0.62 ± 0.11	+7 (*)	n=4
10 mM Tris	1.60 ± 0.38	-29 ± 4 (**)	n=5
10 mM Tris + 100 mM NaCl	0.87 ± 0.20	+2 (*)	n=4
50 mM Na^+-PO_4	1.01 ± 0.60	-	n=12
50 mM Tris	1.55 ± 0.38	-45 ± 5 (***)	n=8
100 mM Na^+-PO_4	2.43 ± 1.29	-	n=4
100 mM Tris	2.76 ± 0.42	-44 ± 5 (**)	n=4

Values expressed as mean ± SD. K_D and R_T determined from Scatchard transforms of binding isotherms. Changes in R_T in Tris buffer (with or without added NaCl) are calculated using the respective Na^+-PO_4 concentration as baseline. (*) not significant; (**) $p<0.01$; (***) $p < 0.001$.

first set of experiments, binding isotherms obtained in the organic buffers BES, HEPES, MOPS and TES at 50 mM were compared to 50 mM Na^+-PO_4. Changes in R_T of +1% to -12% are seen; none are statistically significant. Furthermore, there is no correlation between the effect and the amount of Na^+ (introduced as NaOH when neutralizing the free acid); these range from +1 to -6% in BES & TES (2 mM Na^+) to -12% in HEPES (22 mM Na^+).

In the second set of experiments, isotherms were obtained in Na^+-PO_4 (or K^+-PO_4) and Tris, with or without added NaCl (or KCl). No significant increase in R_T occurs in phosphate buffer when Na^+ is increased from 10 to 110 mM (Table 1). In contrast, the presence of NaCl "restores" some of the sites "masked" by Tris; 30 - 50 mM Na^+ is required for a detectable effect, and 80 -100 mM Na^+ is needed to restore full binding in 10 mM Tris. Furthermore, Na^+ can be readily replaced by K^+. The type of principal anion appears to be immaterial, with comparable results obtained using Na_2SO_4 and K_2SO_4 (data not shown). Exogenous divalent cations do not appear essential, since comparable R_T values are obtained in PBS (which contains Ca^{++} and Mg^{++}) and the other

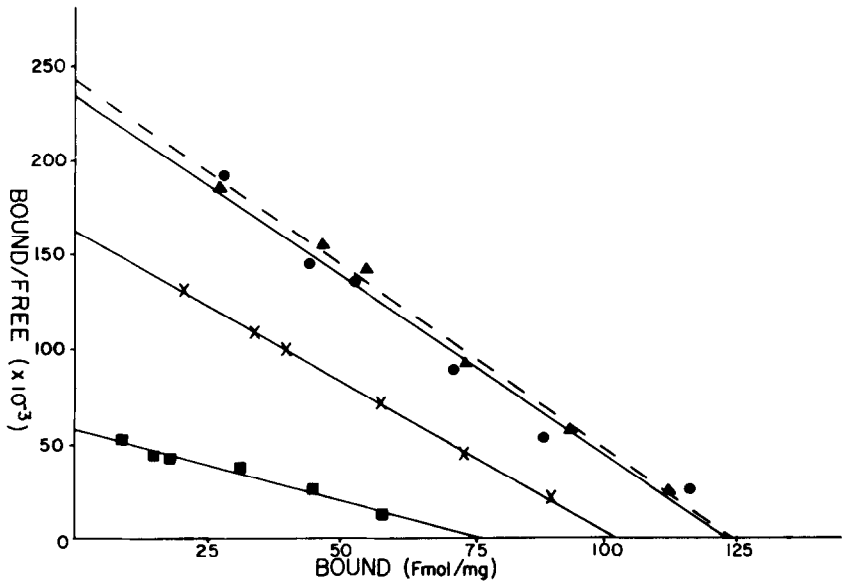

Figure 2: Scatchard plot of [^{3}H]QNB binding to canine atrial homogenates in 50 mM Na^+-PO_4 buffer: (●) control; (▲) plus 10^{-5} M choline Cl (dotted line); (X) plus 10^{-4} M choline Cl; (■) plus 10^{-3} choline Cl. Results from a different homogenate than that illustrated in Figure 1.

metal-free buffers. We conclude that it is the presence of Tris and not the absence of Na^+ that produces these effects.

In conclusion, choline and Tris bind to a site in the mAChR that unmasks an unexpected heterogeneity in antagonist binding sites. In the presence of Tris or choline, up to 45% of the antagonist sites are not accessible to concentrations of [^{3}H]QNB up to 2 nM. The remaining sites maintain their high affinity for this antagonist. This choline/Tris site (i) is distinct from the previously described agonist and antagonist binding sites; (ii) exhibits a unique structure-activity profile; and (iii) exhibits complicated interactions with several monovalent ions (Sastre, manuscripts in preparation). This site would be expected to be occupied by choline during synaptic transmission (16), and preliminary experiments on isolated cardiocytes in vitro suggest a physiologic role for this site in the modulation of chronotropic responses to acetylcholine (Sastre and Rusher, unpublished observations).

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