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OBLIGATORY ROLE OF A TRIS/CHOLINE ALLOSTERIC SITE IN GUANINE NUCLEOTIDE REGULATION OF [^{3}H]-L-QNB BINDING TO MUSCARINIC ACETYLCHOLINE RECEPTORS

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Tris and choline reduce the maximal binding capacity (R_T) of the muscarinic cholinergic antagonist [^{3}H]-L-quinuclidinyl benzilate ([^{3}H]-L-QNB) to atrial membranes, when compared to control values in physiological salt solution (PBS) or NaPi buffer. Addition of guanine nucleotides (GN) to incubations containing choline or Tris reverses the effect of choline and Tris on R_T and restores it to levels determined in NaP_i or PBS alone. GN addition fails to alter R_T or K_D values determined in NaP_i or PBS in the absence of choline and Tris. This GN effect follows a nucleotide specificity similar to that of the GN regulatory proteins coupled to adenylate cyclase. Tris or choline are required for the expression of GN regulation of [^{3}H]-L-QNB binding to muscarinic acetylcholine receptors (mAChR). An allosteric site recognizing choline and Tris appears involved in the interaction between the guanine nucleotide regulatory protein and antagonist binding to mAChR.

Guanine nucleotides (GN) and Na^+ have well documented effects (1,2,3) on agonist binding to the myocardial muscarinic acetylcholine receptor (mAChR). Two or three agonist sites are necessary to account for the complex binding isotherms obtained in the absence of GN (4,5); in the presence of the non-hydrolizable GTP analog Gpp(NH)p, the agonist sites predominantly convert to the low affinity form (1,2,3).

Recently we and others have suggested that the antagonist sites in the mAChR, long regarded as a homogeneous population, are in fact a heterogeneous group subject to regulation by ionic strength, GN, choline and Tris, and

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Abbreviations: mAChR, muscarinic acetylcholine receptor; QNB, quinuclidinyl benzilate; PBS, physiologic buffer solution; Gpp(NH)p, 5'guanylimidodiphosphate; GN, guanine nucleotides; R_T, maximal binding capacity.

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monovalent ions (6,7,8). These multiple interactions are complex and have not been completely elucidated.

Burgisser et al. (7) proposed an intriguing model to integrate some of these observations. Their model postulated a GN-mediated reciprocal interconversion of sites in which the receptor displays an increased affinity for antagonists and a decreased affinity for agonists in the presence of GN. The interconversion was presumably mediated by a guanine nucleotide regulatory protein linked to adenylate cyclase (9), although the nucleotide specificity of this effect was not defined.

Here we report that GN regulation of antagonist binding to the mAChR requires the occupation of a previously defined choline/Tris regulatory site (8), and that such regulation is not demonstrable in physiologic buffers in the absence of Tris or exogenous choline. This effect, present in choline or Tris solutions, follows a nucleotide specificity similar to that of the guanine nucleotide regulatory proteins linked to adenylate cyclase (9).

Materials and Methods

Hearts were excised from mongrel dogs, CD rats, CD-1 mice and Rana pipiens frogs and prepared as described (8). The homogenate was wire meshed filtered, centrifuged and 50 µl of the resuspended pellet was added to a final volume of 1 ml containing [^{3}H]-L-QNB, drugs, nucleotides and buffer. Buffers used included a physiologic salt solution (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), 50 mN NaP_i or 50 mM Tris-HCl, all at pH 7.4 at 25°C. Incubations, filtrations and counting were carried out as previously described (8). Adenine and guanine nucleotides were obtained as Na salts from Sigma.

Results

As previously described (8), Scatchard analysis of binding isotherms of [^{3}H]-L-QNB to myocardial tissue carried out in the presence of choline or Tris reveals a significantly reduced maximal binding capacity (R_T) compared to parallel experiments carried out in PBS or NaP_i buffer (Figure 1). Addition of GN restores the reduced R_T determined in Tris to the levels determined in PBS and NaPi. GN additions failed to alter R_T values determined in PBS or NaPi buffer. Membranes assayed in NaPi displayed an R_T of 213 $\pm$ 10 fmol/mg protein. Membranes assayed in Tris in the absence of exogenous GN exhibited a reduced apparent R_T (68°/o of control), which was

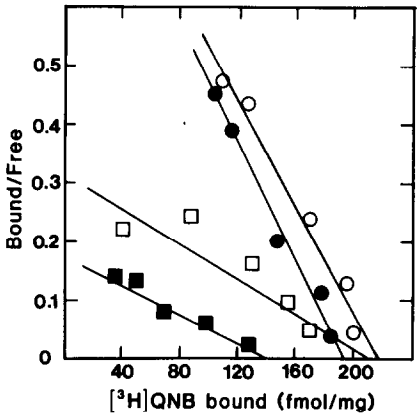

Figure 1 Scatchard plot of [^{3}H]-L-QNB binding to canine atrial membranes in NaPi buffer without Gpp(NH)p (●), or with 100 uM Gpp(NH)p (o); in Tris buffer without Gpp(NH)p (■) or with 100 μM Gpp(NH)p (□). Total and blank incubations were carried out in duplicate in the absence or presence of 1 μM atropine respectively, with 50 μl of identical membrane suspension in a total volume of 1 ml. [^{3}H]-L-QNB concentration was varied between 80 pM and 2 nM. These experiments were replicated 5 times with similar results.

restored to control values in the presence of 100 μM Gpp(NH)p. This concentration of GN increased the R_T determined in NaPi by only 9°/•. (Figure 1 and Table 1).

Tris caused a 3-fold increase in the K_D for [^{3}H]-L-QNB for the mAchR compared to K_D values determined in NaPi buffer of similar ionic strength (153 ± 20 vs. 52 ± 10 pM; Table 1). Gpp(NH)p at 10-100 μM increased the K_D in Tris to a value approaching that measured in NaPi, while not affecting the

Table 1

Effects of Gpp(NH)p on Affinity and Maximal Binding Capacity of Atrial Homogenates for [^{3}H]-L-QNB Assayed in NaPi and Tris Buffer

	K_D (pM)	R_T (°/• control)
NaPi (control)	52 ± 10	100
NaPi + Gpp(NH)p	46 ± 7	109 ± 3
Tris	153 ± 20	68 ± 3
Tris + Gpp(NH)p	81 ± 12	111 ± 5

K_D values are presented as mean ± S.E.M. for 5-6 experiments determined from Scatchard analysis of binding isotherms. R_T values are expressed as the mean ± S.E.M. of the percent of control R_T determined in NaPi buffer. The concentration of Gpp(NH)p was 100 μM.

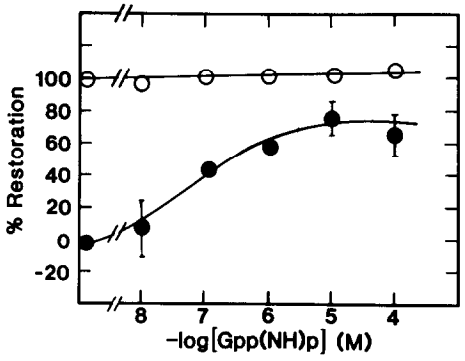

Figure 2 The influence of Gpp(NH)p on [^{3}H]-L-QNB binding to atrial membranes in the absence or presence of choline. Dose response curves for the effect of Gpp(NH)p on [^{3}H]-L-QNB binding to atrial membranes were constructed using 50 µl tissue homogenate in NaPi buffer in the absence (o) or the presence (•) of 1 mM choline. [^{3}H]-L-QNB was 2 nM throughout and total and nonspecific binding were determined for each condition by the absence or presence of 1 µM atropine. No effect on blank values was observed. Results are presented as the °/° restoration from binding observed in the presence of choline without Gpp(NH)p addition, to the binding observed in NaPi without choline addition. Typically, specific binding in the presence of choline was 1300 cpm/50 µl tissue (taken as 0°/° restoration) and was restored to 2000-2200 cpm/50 µl tissue (approximately 80°/° restoration). Binding in NaPi buffer alone was 2200-2400 cpm and is defined as 100°/° restoration. The values are the mean ± S.E.M. of 4 independent determinations.

K_D measured in NaPi (Table 1). Similarly, choline reduces the R_T and increases the K_D measured in Scatchard analysis of the binding isotherms (data not shown).

Studies were carried out with saturating concentrations of [^{3}H]-L-QNB (2 nM) to determine the influences of GN and adenine nucleotides on the R_T measured in Tris, PBS and NaPi buffer. Gpp(NH)p caused a dose dependent increase in [^{3}H]-L-QNB binding in PBS in the presence of 1 mM choline, reflecting an increase in R_T (Figure 2). Similar results were obtained with Gpp(NH)p in Tris, buffer, but not in NaPi.

We examined the nucleotide specificity for this GN-mediated restoration of R_T measured in Tris to the levels determined in NaPi buffer (Figure 3). GTP and GDP completely restore [^{3}H]-L-QNB binding measured in Tris to that measured in NaPi buffer, and do not alter binding measured in NaPi buffer alone. GTP and GDP produced their restoration at approximately 300 nM. The stable GTP analog Gpp(NH)p is about 10-fold more potent than GTP in its restoration of binding, with an EC_{50} of 30 nM (Figure 3). GMP fails to restore binding at 100 µM. The adenine nucleotides ATP, ADP and AMP have no

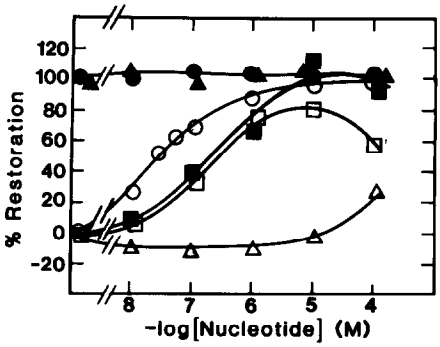

Figure 3 The influence of guanine nucleotides on [^{3}H]-L-QNB binding in NaPi buffer or Tris. Dose response curves for Gpp(NH)p (o), GTP (■), GDP (□), and GMP (▲) were constructed using 2 nM [^{3}H]-L-QNB with 50 µl membrane suspension incubated in a volume of 1 ml Tris Buffer. Dose-response curves constructed in NaPi buffer for Gpp(NH)p (●) and GTP (▲) showed no effect on total or blank binding levels. Results are presented as the percent restoration from binding in Tris to the binding observed in NaPi buffer alone, as described in Figure 2. Values presented are the mean ± SEM of 4 independent determinations.

effect on [^{3}H]-L-QNB binding in Tris or NaPi buffer at concentrations of 10^{-8} M to 10^{-4} M (data not shown).

Discussion

The major finding of the present study is that GN regulation of antagonist binding to the mAChR is absolutely dependent on the presence of choline or Tris. In PBS or NaPi buffer, GN failed to influence the binding of [^{3}H]-L-QNB to atrial membranes, whereas in the presence of choline or Tris, GN restored the R_T to levels observed in PBS alone. Scatchard analysis of the binding isotherms confirms that the action of GN in the presence of choline or Tris is on the apparent R_T, and the action of the nucleotides cannot be explained simply as a reduction in affinity of the receptor for the ligand.

We previously reported the presence of an allosteric site for choline and Tris which regulates antagonist binding to the mAChR (8). This site is distinct from previously described agonist or antagonist binding sites. Approximately one half of the antagonist sites appeared sensitive to choline and Tris regulation in a non-competitive manner (8). Based on these observations, we postulated a heterogeneity of antagonist sites within the mAChR.

In this report we show that occupation of this site by choline or Tris is an obligatory feature for the expression of GN regulation of antagonist binding to the mAChR. None of the mono- and di-valent ions present in PBS can substitute for choline or Tris in allowing this expression. This phenomenon, illustrated here for canine atrial membranes, is also observed in atria from rats and mice and in atria and ventricles of frogs (Rana pipiens; the species used by Burgisser et al. (7)). The widespread biologic expression of this effect is evident.

The obligatory involvement of the allosteric choline/Tris site in GN regulation of antagonist binding suggests a re-examination of available data and models (7, 8, 10), and imposes a further constraint on plausible models. We cannot determine from the present data the molecular location of the allosteric choline regulatory site nor the exact mode of interaction with the GN regulatory protein (9). More information is needed to determine if this site resides within the mAChR, with the GN regulatory protein, or another as yet undefined component of the functional receptor complex, as well as to clarify the role of GN and this allosteric site in cholinergic transmission.

Acknowledgements

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