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GUANINE NUCLEOTIDE-INDUCED POSITIVE COOPERATIVITY IN MUSCARINIC-CHOLINERGIC ANTAGONIST BINDING

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The effect of guanine nucleotides on the binding of [^{3}H]Quinuclidinyl benzilate to heart muscarinic receptors was studied. It was observed that GTP and Gpp(NH)p increased antagonist binding. Scatchard transformation of the data resulted in curvilinear plots (downward concavity) consistent with positive cooperativity. Graphic analysis for cooperativity indicated that guanine nucleotides: a) increased slightly but consistently the affinity for the antagonist (independent of receptor occupancy) and b) induce positive cooperativity in the binding of [^{3}H]QNB (dependent of receptor occupancy).

Heart muscarinic-cholinergic receptors are responsible of modulating heart rate. Activation of these receptors result in inhibition of adenylate cyclase, decreased levels of cyclic AMP and changes in ion conductance (1). It is well known that receptors coupled to adenylate cyclase exist in two interconvertible affinity states for agonists. Such interconversion of the affinity state for agonists seems to reflect the interaction with the guanine-nucleotide regulatory proteins and therefore is modulated by guanine-nucleotides (2). Thus, it has been shown that guanine nucleotides shifts the equilibrium between the affinity states of the receptors towards the low affinity state for agonists (3,4). This has also been shown for cholinergic muscarinic receptors (5-9) although the existence of 3 affinity states for agonists has been proposed (10). Interestingly, guanine nucleotides seem to modulate not only

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the state of affinity of the muscarinic cholinergic receptors for agonists but also for antagonists, in a reciprocal fashion (9, 11). We have studied the effect of guanine nucleotides on the binding of [^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB), a cholinergic antagonist, to heart muscarinic receptors; our results indicate that guanine nucleotides changes the interaction of the ligand with the receptor in a complex fashion consistent with the induction of positive cooperativity.

MATERIALS AND METHODS

Guanyl-5-yl imido diphosphate (Gpp(NH)p), GTP and atropine sulfate were obtained from Sigma. (-)[^{3}H]QNB (37.2 Ci/mmole) was from New England Nuclear.

Male Wistar rats (200-250 g) were sacrificed by decapitation and exanguinated. The heart was quickly removed and washed with ice-cold homogenization buffer (containing 20 mM tris-maleic, 5 mM $MgCl_2$, 0.25 M sucrose, 1 mM EDTA, 12.5 mM β-mercaptoethanol, pH 7.4), minced and homogenized in 15 volumes of homogenization buffer using a polytron (Brinkmann Instruments) two times for 15 seconds at mild speed. The homogenate was filtered through four layers of cheesecloth, and centrifuged at 700 x g for 15 min; the resultant supernatant was then centrifuged at 30,000 x g for 15 min. The pellet was resuspended in incubation buffer (containing 50 mM tris, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4) to a protein concentration of 3 mg/ml.

Plasma membranes (200-250 ug/tube) were incubated with 0.008 to 2.0 nM [^{3}H]QNB in a final volume of 3.5 ml at 25°C during 60 min. The membrane suspensions were filtered over Whatman GF/C filters and the filters washed with 20 ml of ice cold incubation buffer. Non specific binding was defined as the binding of [^{3}H]QNB in the presence of 1 μM atropine, and it was less than 10% of the total binding. Where indicated Gpp(NH)p or GTP were present at 100 μM. Protein was measured by the method of Lowry et al (12) using bovine serum albumin as standard. Analysis of the data was performed by the method of Scatchard (13) and the possibility of cooperativity by the graphic analysis of De Meyts and Roth (14).

RESULTS

[^{3}H]QNB binds rapidly and reversible to cardiac membranes. A saturation isotherm is presented in Figs. 1 and 2 panel A. Scatchard analysis of the data (Figs. 1 and 2 panel B) indicated that the K_D was 63 ± 3 pM and the Bmax 196 ± 14 fmol/mg of protein (mean ± S.E.M. of 6 experiments in duplicate in each case). Linear regression analysis was consistent with a straight line ($p < 0.001$) and correlation coefficients of 0.98-0.99. Interestingly, addition of GTP (Fig. 1) or the hydrolysis resistant analogue Gpp(NH)p (Fig. 2) markedly affect the ligand receptor interaction; in both cases guanine nucleotides increased [^{3}H]QNB binding and the ascending part of the saturation curves became much steeper. Similar results have been observed by Burgisser *et al* (9). In none of these cases the Scatchard transformation of the data gave

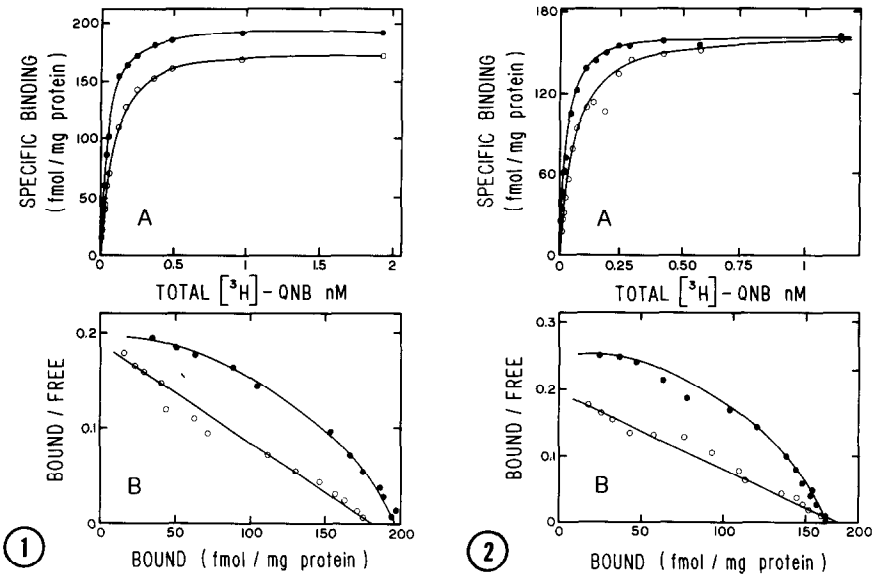

Figure 1. SATURATION ISOTHERM (A) AND SCATCHARD PLOT (B) OF [^{3}H]QNB BINDING TO CARDIAC MEMBRANES IN THE ABSENCE (O) AND PRESENCE (●) OF GTP. Plotted is a representative experiment out of 10 with identical results. Each point is the average of duplicate determinations with less than 10% variation.

Figure 2. SATURATION ISOTHERM (A) AND SCATCHARD PLOT (B) OF [^{3}H]QNB BINDING TO CARDIAC MEMBRANES IN THE ABSENCE (O) AND PRESENCE (●) OF Gpp(NH)p. Other indications as in Figure 1.

straight lines but were clearly curvilinear, i.e. downward concave (Figs. 1 and 2 panel B), suggesting the existence of positive cooperativity in the presence of guanine nucleotides (15).

In order to overcome the problems due to the possible existence of site-site interactions and to obtain meaningful information the data were replotted using the graphic analysis of De Meyts and Roth (14). In this analysis the average affinity ($\bar{K}$) calculated as (Bound/free)/(Bmax-B) is plotted as a function of the factional occupancy (B/Bmax). Using this graphical analysis the differences seen in this Scatchard plots were more clearly observable. In the absence of guanine nucleotides the $\bar{K}$ was constant indicating the absence of cooperativity (Fig. 3). On the contrary in the presence of GTP (Fig. 3, panel A) or Gpp(NH)p (Fig. 3 panel B) the average affinity varied markedly (from 20 to 100 nM^{-1} i.e. the KDs varied between 50 to 10 pM) as a function of receptor occupancy. As shown in Fig. 3, in the presence of guanine nucleotides, as the receptor occupancy increased the average affinity increased. Calculation

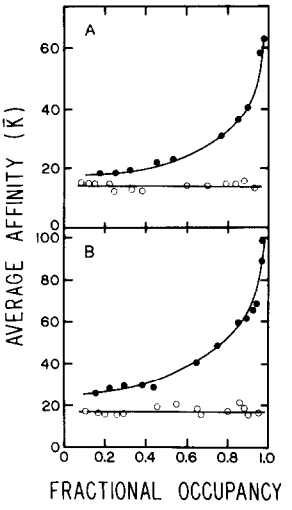

Figure 3. GRAPHIC ANALYSIS FOR COOPERATIVITY OF THE DATA PRESENTED IN FIG. 1 (A) AND FIG. 2 (B). Open symbols indicate the absence of guanine nucleotides and closed symbols indicate the presence of 100 μM GTP (panel A) or 100 μM Gpp(NH)p (panel B). ($\bar{K}$ units are: nM^{-1})

of the Hill coefficients n_H as indicated by De Lean and Rodbard (15) gave the following results: 1.0 in the absence of guanine-nucleotides, 1.46 in the presence of GTP and 1.50 in the presence of Gpp(NH)p. These results are clearly consistent with guanine nucleotide-induced positive cooperativity.

DISCUSSION

The main finding of our studies is the effect of guanine nucleotides on the binding of a cholinergic antagonist, [^{3}H]QNB, to the heart muscarinic-cholinergic receptors. Our results clearly show a downward concavity in the Scatchard analysis of the saturation isotherm consistent with positive cooperativity. Previously other authors had studied the action of guanine nucleotides on antagonist binding to muscarinic cholinergic receptor (5, 7-9, 11, 16). In most instances an increase in antagonist affinity by guanine nucleotides has been reported without detecting changes in the shape of the Scatchard plots (probably due to a small range of radioligand concentrations used in their assays). However, in some cases in which straight Scatchard plots were presented, careful inspection of the data evidenced downward concavity (17,18).

A graphic analysis (14) was successfully used to further document the underlying phenomenon responsible for the curvilinearity of the Scatchard

plots. The data are markedly similar to the theoretical plots for positive cooperativity (14). It should be mentioned that, as expected, the process of positive cooperativity is dependent on receptor occupancy (see Fig. 3) and not just an increase in receptor affinity for antagonists. Burgisser et al (9) have proposed that guanine nucleotides modulate in an opposite fashion the affinity of muscarinic receptors for agonists and antagonists, i.e. decrease the affinity for agonists whereas increase that for antagonist. This phenomenon does not depend on receptor occupancy. Our results do not allow us to discard the proposed reciprocal modulation of affinity. On the contrary, we consistently observed that the average affinity ($\bar{K}$) at the lowest receptor occupancies (5-15%), was higher in the presence of guanine nucleotides than in their absence. The change in affinity due to guanine nucleotides was small (i.e. from a K_D of 60 pM to a K_D of 50 pM in the absence and presence of guanine nucleotides, respectively). Other authors have mainly interpreted their data on the effect of guanine nucleotides as the results of an increase in affinity. As already mentioned, it is very possible that both processes (i.e. increase in affinity and positive cooperativity) may coexist.

The effects of guanine nucleotides on the affinity of receptors seem to take place through the guanine nucleotide regulatory proteins (Ni, Ns and Nx) (2). Current ideas indicate that the receptor-N (regulatory protein) complex is the form of the receptor with high affinity for agonists (and low affinity for antagonist in the case of the muscarinic receptors). Guanine nucleotides seem to dissociate the receptor-N complex and decrease the affinity for agonist (and increase that for antagonists in muscarinic receptors). It is possible that the induction of positive cooperativity by guanine nucleotides observed in the present study may result from receptor dissociation of the guanine nucleotide regulatory protein. However, more direct approaches are required to address this possibility.

After this research was finished, a paper appeared describing similar effects of guanine nucleotides on antagonist binding (19).

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