Volume 282, number 2, 289–292 FEBS 09673 May 1991

ADONIS 001457939100384S

High affinity quinuclidinyl benzilate binding to rat parotid membranes requires muscarinic receptor

G protein interactions

Valerie J. Horn, Indu S. Ambudkar and Bruce J. Baum

Clinical Investigations and Patient Care Branch, National Institute of Dental Research, NIH, Bldg. 10, Rm. 1N-113, Bethesda, MD 20892, USA

Received 1 February 1991; revised version received 1 March 1991

The binding of the non-selective muscarinic antagonist [^{3}H]quinuclidinyl benzilate (QNB) to rat parotid membranes was characterized. Under equilibrium conditions, [^{3}H]QNB bound to a homogenous population of muscarinic receptors (K_d, 118 ± 19 pM; B_{max}, 572 ± 42 fmol/mg membrane protein, $n = 12$). The addition of G protein activators AlF_4^- or guanosine-5'-*O*-(3-thiotriphosphate) (GTPγS) + Mg^{2+} increased the K_d by 77 ± 7% ($n = 4$, $P < 0.05$) and 83 ± 27% ($n = 7$, $P < 0.05$), respectively, without a change in the B_{max} or homogeneity of the binding site. GTPγS added without exogenous Mg^{2+} did not affect [^{3}H]QNB binding. Thus, optimal QNB binding requires a muscarinic receptor/G protein interaction.

Antagonist; Receptor; G protein; Parotid; AlF_4^-; GTPγS

1. INTRODUCTION

Previous studies have shown that the affinity of receptors for agonists is influenced by G proteins [1–4]. Conversely, antagonists are commonly believed to be unaffected by any G protein mediated changes in receptor characteristics [5]. Recently, we reported that the non-selective muscarinic receptor antagonist (NSMRA) atropine could affect β-adrenoreceptor-induced signal transduction events related to Ca^{2+} mobilization in intact rat parotid acinar cells via a mechanism which utilized the muscarinic receptor but which occurred at a step distal to ligand binding to the receptor [6]. This led to an hypothesis that atropine binding to the muscarinic receptor involves a G protein interaction. In the present study we have examined the equilibrium binding of the NSMRA QNB to parotid muscarinic receptors, under conditions that are known to influence receptor/G protein interactions.

2. MATERIALS AND METHODS

Parotid membranes were prepared from male rats as described [7]. All [^{3}H]QNB binding studies were carried out (37°C, 90 min) in a total volume of 1 ml 50 mM Tris, pH 7.4, containing 50 μg membrane protein. Equilibrium binding was achieved within 60 min in both control and experimental conditions (Fig. 1). Incubations were stopped by rapid filtration (45 μm Millipore filters). Values for all data points were determined in triplicate for each experiment. Specific binding was calculated as the difference between total binding and binding observed in the presence of 10 μM atropine and was ~70% of total binding at QNB concentrations near the K_d. Where indicated P values were determined by Student's t-test. [^{3}H]QNB (33 Ci/mmol) was obtained from New England Nuclear and nucleotides were from Sigma Chemical Co. All other reagents were of the highest available commercial grade.

Correspondence address: V. Horn, CIPCB, NIDR, NIH, Bldg. 10, Rm. 1N-113, Bethesda, MD 20892, USA. Fax: (1) (301) 4021228.

3. RESULTS AND DISCUSSION

The NSMRA [^{3}H]QNB showed high affinity, saturable binding to an apparently homogeneous population of muscarinic receptors in rat parotid gland membranes. Scatchard-Rosenthal analysis (Fig. 2, open circles), demonstrates binding with an average B_{max} of 572 ± 42 fmol/mg membrane protein (mean ± SEM; $n = 12$) and a K_d of 118 ± 19 pM. These data are in good agreement with previously published results [8–10].

The ability of guanine nucleotides to decrease the stability of the agonist/receptor/G protein complex is well known [3,5]. However, the effects of guanine nucleotides on the antagonist/receptor/G protein complex have not been extensively studied, and are not necessarily predictable [11–15]. We found that the addition of 100 μM GDP, GTP, inosine 5'-triphosphate, guanosine 5'-*O*-(2-thiodiphosphate), GTPγS or 5'-guanylylimidodiphosphate in the absence of Mg^{2+}, had no significant effect on QNB binding to rat parotid membranes either when added simultaneously with the

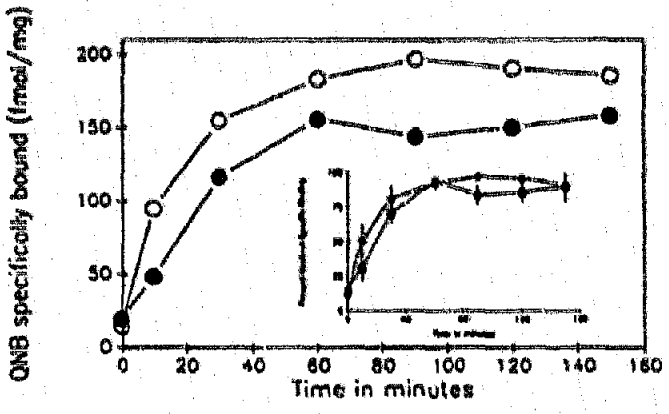

Fig. 1. Time course of specific [^{3}H]QNB binding to rat parotid membranes. Open circles represent the specific binding of [^{3}H]QNB to control rat parotid membranes (average of 3 experiments, each performed in triplicate with different membrane preparations) at the time points indicated. The solid circles represent the specific binding of [^{3}H]QNB in the presence of 10 mM NaF and 10 μM $AlCl_3$ determined from experiments parallel to those shown as control conditions. The inset shows the same experimental data expressed as a percentage of the maximal binding in control (●) and experimental (■) conditions, respectively.

[^{3}H]QNB, or if pre-incubated with the membranes for 30 min prior to the addition of the radioligand.

However, a significant change in ^{3}[H]QNB binding was observed in the presence of Mg^{2+} + GTPγS (Fig. 2, triangles). The affinity of the muscarinic receptor for the ligand was reduced (K_d increased by

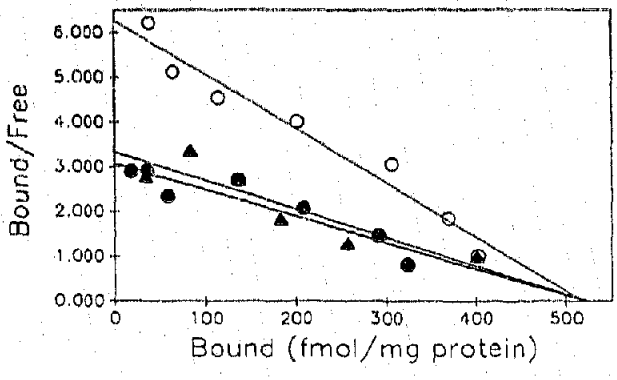

Fig. 2. Effect of G-protein activators on specific [^{3}H]QNB binding to rat parotid membranes. These Scotchard-Rosenthal plots are from representative experiments. The open circles represent the specific binding of [^{3}H]QNB under control conditions, (K_d 83 pM, B_{max} 518 fmol/mg membrane protein). The solid circles represent the specific binding of [^{3}H]QNB in the presence of 10 mM NaF and 10 μM $AlCl_3$ from the same experiment as shown for the control (K_d 156 pM, B_{max} 522 fmol/mg membrane protein). The triangles are the average of 2 experiments with one membrane preparation in which the membranes were pretreated with $MgSO_4$ and GTPγS before the addition of QNB (K_d 170 pM, B_{max} 522 fmol/mg membrane protein). For simplicity the control QNB binding isotherm from these experiments is not indicated as it is comparable to the control results shown. These results are representative of 4 experiments with 3 separate membrane preparations for AlF_4^- and 7 experiments with 4 separate membrane preparations for $MgSO_4$ and GTPγS.

Table I

Effects of Mg^{2+} and AlF_4^- on [^{3}H]QNB binding to parotid membranes

Addition	% Maximal QNB Bound
control (none)	100
10 mM $MgSO_4$	93 ± 4.04 (3)[a]
10 mM NaF plus 10 μM $AlCl_3$	67.5 ± 2.97 (13)[a,b]
10 mM NaCl plus 10 μM $AlCl_3$	95.5 ± 5.95 (4)
10 mM $MgSO_4$ plus 10 mM NaF plus 10 μM $AlCl_3$	56.7 ± 1.32 (3)[a,b]

Binding studies were performed as described in the text. The number of experiments for each condition is indicated in parentheses. At least 2 different membrane preparations were utilized. Each was done in triplicate at 100 pM QNB. Data were tested for significance by Student's t-test.

[a] Indicates a result significantly different from the control, ($P < 0.005$), and [b] indicates a result significantly different other results so marked ($P < 0.05$). Under these control conditions maximal QNB binding was 215 ± 7 fmol/mg protein.

83 ± 27%, $n = 7$, $P < 0.05$) without a significant change in the B_{max} (109 ± 14% of control). It is well known that Mg^{2+} is needed for optimal activation of G proteins, and facilitates the release of G proteins from the receptor [16–19]. Indeed, in the presence of Mg^{2+} alone, (10 mM $MgSO_4$), [^{3}H]QNB binding to parotid membranes was slightly, but significantly reduced (Table I). These data suggest that, in the absence of Mg^{2+}, the rat parotid muscarinic receptor/antagonist complex retains a 'relatively' high affinity for G proteins regardless of the guanine nucleotide present. Conversely, in the presence of 10 mM Mg^{2+}, GTPγS can apparently activate the G protein and decrease the receptor's affinity for the antagonist.

We next evaluated the effect of AlF_4^- (10 mM NaF and 10 μM $AlCl_3$), on [^{3}H]QNB binding to rat parotid membranes. AlF_4^-, by interacting with bound GDP on G_α subunits, is believed to activate G proteins and, thus, decrease the association of G proteins with cell surface receptors [20–22]. At a [^{3}H]QNB concentration near the K_d, AlF_4^- markedly decreased specific equilibrium ligand binding to parotid membranes (−35%, Table I) without affecting non-specific binding (not shown). The time to reach equilibrium binding was unchanged by the addition of AlF_4^- (Fig. 1). This inhibitory effect was specific for the fluoride complex, (Table I). The reduction in [^{3}H]QNB binding was dependent on the concentration of NaF present (IC_{50} ~ 3.5 mM NaF, Fig. 3). The effect of AlF_4^- on QNB binding was similar to the effect of Mg^{2+} and GTPγS (Fig. 2). In the presence of 10 mM NaF/10 μM

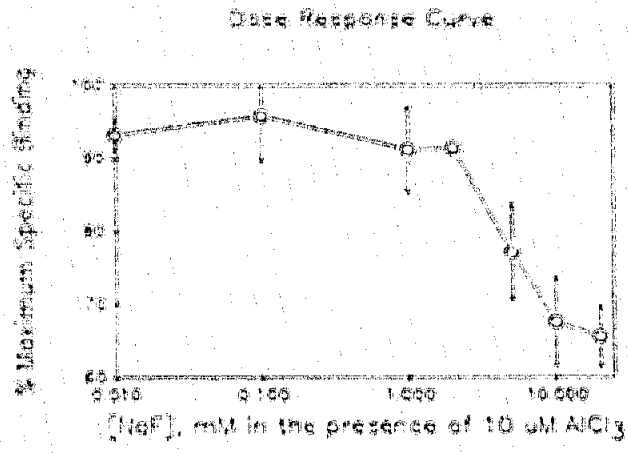

Fig. 3. The effect of different concentrations of NaF on [^{3}H]QNB binding. Each data point represents the average ± SEM of 3 or 4 experiments with at least 3 separate membrane preparations, performed in triplicate using 100 pM [^{3}H]QNB. For these experiments the specific binding at 100 pM QNB in control membranes represents 100% of maximal binding, and was 209 ± 9 fmol/mg protein.

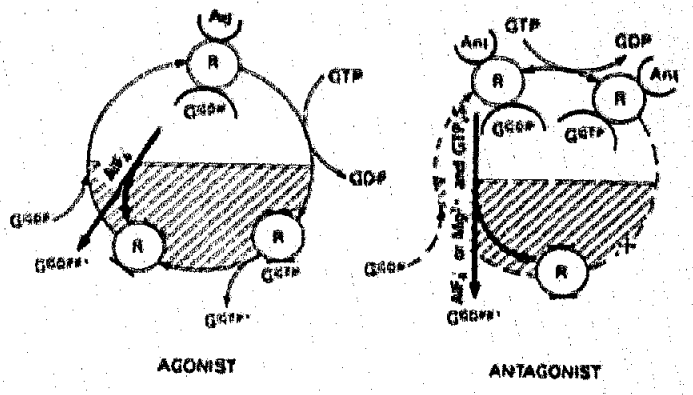

Fig. 4. Model of ligand/muscarinic receptor/G protein interactions in rat parotid acinar membranes. R stands for the muscarinic receptor. Ag stands for a muscarinic agonist, Ant stands for a muscarinic antagonist. The shaded areas reflect the part of the G protein cycle where the receptor has low affinity for the ligand. A curved line between the receptor and the ligand or G protein reflects a high affinity state of the receptor for that component. The* indicates the active form of the G protein. The 'X' between the receptor possessing high affinity binding of the antagonist and G protein, and the low affinity receptor, indicates that this change does not occur.

$AlCl_3$, the affinity of the antagonist for the receptor was significantly reduced, its K_d increased by 77 ± 7% ($n = 4$, $P < 0.05$) (Fig. 2, solid circles). No significant change was observed either in the maximum number of binding sites (95 ± 7% of control), or in the apparent homogeneity of these sites. Interestingly, Mg^{2+} also significantly enhanced the ability of AlF_4^- to inhibit [^{3}H]QNB binding (Table I). These aggregate results suggest that in parotid membranes the binding of QNB is reduced when G protein/receptor interactions decrease.

It has been widely demonstrated that GTP or non-hydrolyzable GTP analogues (such as GTPγS) change the affinity of a receptor for an agonist [1–7]. For this to occur, GTPγS *must* interact with the G protein, leading to its activation and dissociation from the receptor. Our data demonstrate that GTPγS alone cannot alter the affinity of the parotid muscarinic receptor for an antagonist. This indicates either that (i) GTPγS by itself, is unable to activate G proteins associated with the antagonist/receptor complex or (ii) antagonist binding to the receptor does not require an interaction with the G protein and, thus, GTPγS activation is irrelevant. By experimental manipulation (e.g. inclusion of Mg^{2+} with GTPγS in reaction mixtures) it was possible to induce G protein dissociation from the receptor with a subsequent decrease in ligand binding, thus supporting the first supposition. Furthermore, this can be mimicked, without addition of GTPγS, by utilizing a receptor-independent tool for G protein activation, AlF_4^-. Indeed, we have shown that under 4 incubation conditions which promote receptor/G protein dissociation (Mg^{2+} alone; GTPγS plus Mg^{2+}; AlF_4^- alone; and AlF_4^- plus Mg^{2+}) the binding of QNB to muscarinic receptors is significantly reduced. While this affinity change (~2 fold) is much smaller than the change in agonist affinity (~5–100 fold) which has been observed as a consequence of G protein/receptor dissociation, it nonetheless demonstrates some unexpected similarities between stimulatory and inhibitory ligand/muscarinic receptor/G protein interactions in rat parotid membranes.

These similarities are illustrated in Fig. 4. Agonist binding to receptors on whole cells is measured almost entirely as low affinity binding (left panel) because the agonist very rapidly promotes G protein binding and G protein activation. This activation, in turn, very rapidly decreases the association of the G protein with the receptor and the affinity of the receptor for the agonist (i.e. resulting in the low affinity state of the receptor for the agonist). In membranes where endogenous guanine nucleotides are limited or absent, the agonist cannot activate the G protein and the agonist remains bound with high affinity to its receptor [23–26].

On the other hand, antagonists are known to bind with high affinity to receptors in whole cells and in membranes [23–26]. The model shown in Fig. 4, right panel, can explain this phenomenon by virtue of the unchanged affinity of the receptor for the antagonist regardless of the guanine nucleotide bound to the G protein when the G protein stays bound to the receptor. The data, herein, demonstrate a lower affinity binding of the antagonist to the receptor when the G proteins have been dissociated from the receptor either using AlF_4^- or Mg^{2+} and GTPγS. Thus, we suggest that for highest affinity [^{3}H]QNB binding to muscarinic receptors in rat parotid membranes, an interaction between the muscarinic receptor and G protein is required.

The G protein-promoted, high affinity muscarinic antagonist/receptor binding characteristics described by us for parotid membranes are clearly not common to every cell, or receptor type. For example, in many studies, no evidence for such antagonist/receptor/G protein interactions has been found. The factors which determine the specific nature of this interaction are not presently understood.

REFERENCES

[1] Asano, T., Brandt, D.R., Pedersen, S.E. and Ross, E.M. (1985) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 19, 47–56.
[2] Casey, P.J. and Gilman, A.G. (1988) J. Biol. Chem. 263, 2577–2580.
[3] Cerione, R.A., Codina, J., Benovic, J.L., Lefkowitz, R.J., Birnbaumer, L. and Caron, M.G. (1984) Biochemistry 23, 4519–4525.
[4] Freissmuth, M.P., Casey, P.J. and Gilman, A.G. (1989) FASEB J. 3, 2125–2131.
[5] Aguilar, J.S., Ochoa, E.L. and de Robertis, E. (1987) Neurochem. Res. 12, 83–91.
[6] Horn, V.J., Baum, B.J. and Ambudkar, I.S. (1989) FEBS Lett. 258, 13–16.
[7] Baum, B.J., Ambudkar, I.S., Helman, J., Horn, V.J., Melvin, J.E., Mertz, L.M. and Turner, R.J. (1990) Methods Enzymol. 192, 26–37.
[8] Dehaye, J.P., Marino, A., Soukias, Y., Poloczek, P., Winand, J. and Christophe, J. (1988) Eur. J. Pharmacol. 151, 427–434.
[9] Putney Jr, J.W. and Van De Walle, C.M. (1980) J. Physiol. 299, 521–531.
[10] van der Ven, P.F., Takuma, T. and Baum, B.J. (1986) J. Dent. Res. 65, 382–386.
[11] Bergstrom, A. and Wikberg, J.E. (1986) Acta Pharmacol. Toxicol. 59, 270–278.
[12] Boyer, J.L., Carcamo, M.M., Monroy-Sanchez, J.A., Posadas, C. and Garcia-Sainz, J.A. (1986) Biochem. Biophys. Res. Commun. 134, 172–177.
[13] Burgisser, E., De Lean, A. and Lefkowitz, R.J. (1982) Proc. Natl. Acad. Sci. USA 79, 1732–1736.
[14] Lang, P.H. and Lemmer, B. (1985) J. Cyclic Nucleotide Protein Phosphorylation Res. 10, 341–360.
[15] Ramkumar, V. and Stiles, G.L. (1988) J. Pharmacol. Exp. Ther. 246, 1194–1200.
[16] Cech, S.Y., Broaddus, W.C. and Maguire, M.E. (1980) Mol. Cell. Biochem. 33, 67–92.
[17] Higashijima, T., Ferguson, K.M., Sternweis, P.D., Smigel, M.D. and Gilman, A.G. (1987) J. Biol. Chem. 262, 762–766.
[18] Katada, T., Northup, J.K., Bokoch, G.M., Ui, M. and Gilman, A.G. (1984) J. Biol. Chem. 259, 3578–3585.
[19] Smigel, M.D., Ferguson, K.M. and Gilman, A.G. (1985) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 19, 103–111.
[20] Sternweis, P.C. and Gilman, A.G. (1982) Proc. Natl. Acad. Sci. USA 79, 4888–4891.
[21] Boyer, J.L., Waldo, G.L., Evans, T., Northup, J.K., Downes, C.P. and Harden, T.K. (1989) J. Biol. Chem. 264, 13917–13922.
[22] Chabre, M. (1989) Biochem. J. 258, 931–933.
[23] Gilman, A.G. (1987) Ann. Rev. Biochem. 56, 615–649.
[24] Pittman, R.N. and Molinoff, P.B. (1980) J. Cyclic Nucleotide Res. 5, 421–435.
[25] Sladeczek, F., Bockaert, J. and Mauger, J.-P. (1983) Mol. Pharmacol. 24, 392–397.
[26] Tota, M.R., Kahler, K.R. and Schimerlik, M.I. (1987) Biochemistry 26, 8175–8182.