Constitutive activation of muscarinic receptors by the G-protein G_q

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Abstract In the absence of ligands, G-protein coupled receptors interconvert between active and inactive conformations. These conformations are stabilized by agonists and antagonists, respectively. Like agonists, G-proteins are thought to preferentially associate with receptors in the active conformation and should therefore be able to promote their formation in the absence of agonist. We show that over-expression of G_q induces constitutive activation of compatible muscarinic receptors and that this activity is blocked by muscarinic antagonists. G_q also increases the potency and efficacy of agonists. These results indicate that regulation of G-protein levels has a profound impact on receptor control of cellular physiology, even in the absence of agonist ligands.

Key words: Seven transmembrane receptor; G-protein; Constitutive activity

1. Introduction

Muscarinic receptors consist of five genetically defined subtypes (m1–m5) that belong to a superfamily of seven transmembrane receptors that couple to G-proteins [1–4]. The subtypes m1, m3 and m5 selectively couple the G-protein G_q [5,6]. Many receptors have recently been shown to activate G-proteins in the absence of agonist [7–11]. Constitutive activity has been explained by allosteric models in which receptors exist in equilibrium between two conformations, one of which interacts with G-proteins [12,13]. Accordingly, agonists are compounds that stabilize the active conformation, while antagonists preferentially interact with the inactive conformation, shifting receptor equilibrium to enhance or diminish activity, respectively. If G-proteins preferentially interact with the active conformation of receptors [14], then it follows from these models that increasing the G-protein concentration should stabilize the active conformation and thus induce constitutive activity. Based upon the ability of G_q-selective receptors to induce agonist-dependent transformation of NIH 3T3 cells [15], we developed a convenient assay of receptor activity using the reporter gene β-galactosidase (R-SAT, patents pending; [16]; H. Braüner-Osborne and M.R. Brann, submitted). We show that the G-protein G_q induces constitutive activation of compatible muscarinic receptor subtypes.

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2. Materials and methods

2.1. Cell culture

NIH 3T3 cells (ATCC no. CRL 1658) and COS7 cells were incubated at 37°C in a humidified atmosphere (5% CO_2) in Dulbecco's modified Eagles medium supplemented with 4500 mg/l glucose, 4 nM L-glutamine, 50 U/ml penicillin G, 50 U/ml streptomycin (ABI) and 10% calf serum for 3T3 cells or 10% fetal bovine serum for COS7 cells (Gibco).

2.2. Functional assays

Receptor-selection and amplification technology (R-SAT) assays were performed as described previously [16]. Briefly, cells were plated 1 day before transfection using 1×10^6 cells in 10 ml of medium per 10 cm plate. Cells were transfected by calcium precipitation as described [30] using 1–3 μg each of the human muscarinic receptor subtypes (m1, m2, m3, m5), pSV-β-galactosidase (Promega, Madison, WI), $G_{\alpha q}$ (generous gift from B. Conklin) or control vector, and 20 μg of salmon sperm DNA (Sigma, St. Louis, MO). One day after transfection media were changed, and after 2 days cells were trypsinized and aliquoted into the wells of a 96-well plate (100 μl/well). One 10 cm plate yields enough cells for 96 wells. Ligands were combined with the cells to a final volume of 200 μl/well. After 5 days in culture β-galactosidase levels were measured as described [31]. Media were aspirated from the wells and the cells rinsed with phosphate buffered saline (PBS). 200 μl of PBS containing 3.5 mM *O*-nitrophenyl-β-D-galactopyranoside and 0.5% nonidet P-40 (both Sigma, St. Louis, MO) were added to each well and the 96-well plate was incubated at room temperature. After 16 h the plates were read at 405 nm on a plate-reader (Bio-Tek EL 310 or Molecular Devices). Data from R-SAT assays were fitted to the equation:

$$R = D + (A - D)/(1 + (x/c))$$

where A = minimum response, D = maximum response and $c = EC_{50}$ (R = response, x = concentration of ligand). Curves were generated by least-squares fits using the program KaleidaGraph (Abelbeck Software).

3. Results

3.1. $G_{\alpha q}$ induces constitutive activation of the m3 muscarinic receptor

As shown in Fig. 1A when NIH 3T3 cells transfected with the m3 muscarinic receptor and the β-galactosidase gene were cultured in the presence of the indicated concentrations of the muscarinic agonist carbachol, there was a dose-dependent increase in β-galactosidase activity. When we co-expressed the α subunit of $G_{\alpha q}$ [17–19] there was a dramatic increase in basal activity, representing approximately 25% of the total response (Fig. 1A). Besides inducing constitutive activity, $G_{\alpha q}$ also caused a 36-fold leftward shift of the carbachol dose–response curve (Fig. 1A, Table 1). The $G_{\alpha q}$ induced activity was reversed in a dose–dependent manner by the potent muscarinic antagonist atropine, indicating it is actually a negative antagonist (Fig. 1A). The compound McN-434 is a weak partial agonist, with only a third of the efficacy of carbachol (Fig. 1B, Table 1). $G_{\alpha q}$ co-expression increased the efficacy of McN-434 to 70% of carbachol, and also increased its potency, although to a lesser

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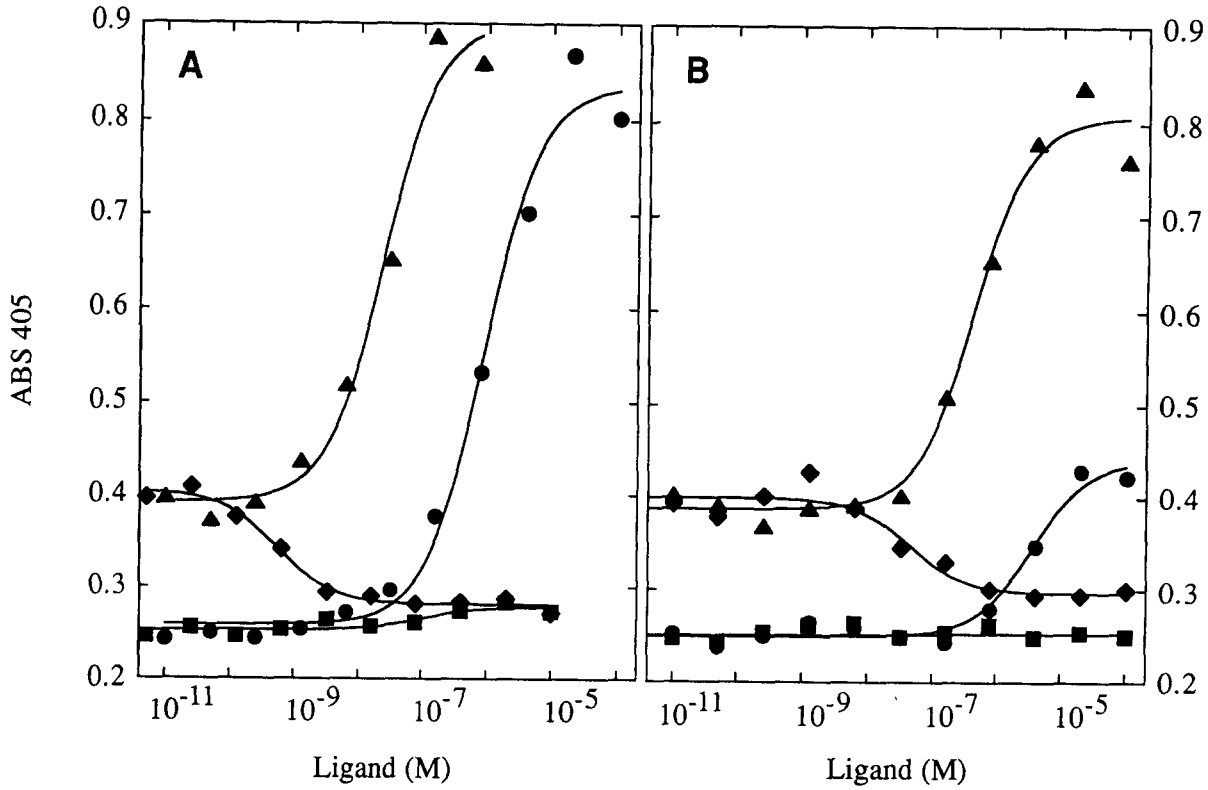

Fig. 1. Constitutive activation of the m3 muscarinic receptor by G_q. m3 and β-galactosidase were transfected into NIH 3T3 cells either with or without $G_{\alpha q}$ and cultured in the presence of the indicated concentrations of either (A) carbachol (▲ = m3 + G_q, ● = m3); atropine (◆ = m3 + G_q, ■ = m3) or (B) McN-434 (▲ = m3 + G_q, ● = m3); pirenzepine (▲ = m3 + G_q, ■ = m3).

extent than for carbachol (Fig. 1B, Table 1). We also tested the muscarinic antagonist pirenzepine and found that, like atropine, it is actually a negative antagonist, reversing the constitutive activity induced by $G_{\alpha q}$ (Fig. 1B). In contrast to the results for agonists, $G_{\alpha q}$ over-expression did not significantly alter the EC_{50} for either antagonist compared with their K_i values (Table 1).

3.2. *Constitutive activation of receptors by G-proteins is specific to compatible pairs*

Similar effects of $G_{\alpha q}$ were observed on m1 and m5, but not the m2 muscarinic receptor subtypes (not shown). Unlike $G_{\alpha q}$, the G-protein $G_{\alpha 12}$ [20], which also mediates proliferative responses in NIH 3T3 cells [21–23], had no effect on muscarinic receptor activity. Expression of either $G_{\alpha q}$ or $G_{\alpha 12}$ alone caused slight cellular responses which were neither atropine- nor carbachol-sensitive. We recently isolated constitutively activated m5 muscarinic receptors from a library of receptors with mutations randomly incorporated into the sixth transmembrane domain (T.A. Spalding et al., submitted). The phenotypes of these mutant receptors were almost indistinguishable from those induced by $G_{\alpha q}$ in the present study. The mutant receptors had constitutive activity that was suppressed by the antagonists atropine and pirenzepine, the potency of the antagonists was unchanged, and the potencies of agonists were increased in proportion to their efficacy (i.e. carbachol exhibited a greater shift in potency than McN-434). To exclude the possibility that the constitutive activities were due to trace amounts of the endogenous agonist acetylcholine, we repeated our experiments in synthetic media and obtained similar results (not shown). Furthermore, acetylcholine is totally ineffective in our cellular assay, presumably due to cholinesterases in the serum, as is its breakdown product choline (T.A. Spalding et al., submitted).

4. Discussion

The model shown below both predicts and explains all of our observations.

$$R \rightleftharpoons R^* + G \rightleftharpoons R^*G \rightarrow \text{RESPONSE}$$

In this model receptors exist in equilibrium between inactive (R) and active (R*) conformations. Agonists and G-proteins have higher affinity for R* while antagonists preferentially bind R. When compatible receptor/G-protein combinations are co-expressed we observe constitutive activity. Agonists acquire increased potency and efficacy whereas the potencies of antagonists are unaffected. All these results can be explained if G-proteins increase the proportion of R* independently of agonist, assuming the receptor exists primarily as R in the absence of added agonists.

These observations have implications for the regulation of cellular physiology by receptors. Basal tone may be an important component of muscarinic (and other receptor) physiology. In fact, it has been shown that treatment of atrial cell membranes with muscarinic antagonists blocks spontaneous K^+ channel opening and G-protein activity [24,25]. Our observations imply that the control of G-protein expression may profoundly contribute to these phenomena. It is known that the

Table 1
Effects of G_q on m3 muscarinic receptor pharmacology

	EC_{50} (nM)		Ratio
	m3	m3 + G_q	
Carbachol	790 ± 190 (100)	22 ± 6 (84)	36
McN-434	3300 ± 120 (32)	400 ± 90 (70)	8.2
Atropine	n.r.	1.3 ± 0.3 (−22)	1.0*
Pirenzepine	n.r.	100 ± 80 (−18)	2.5*

The maximum responses (above basal) are shown in parentheses and were calculated as percent maximum response (above basal) of m3 to carbachol. A negative value indicates suppression of basal activity and was also normalized to the maximum response of m3 to carbachol. Ratios were calculated as $EC_{50,m3}/EC_{50,m3+Gq}$. *For comparing the EC_{50} values of antagonists we used K_i values determined for antagonism of carbachol responses at m3 (Jørgensen and Brann, manuscript submitted). Values are reported as the mean ± S.E.M. and are taken from an experiment done in duplicate which is representative of four separate experiments.

subcellular locations and levels are tightly regulated by physiological stimuli [26–28], and regulation of $G_{\alpha q}$ levels by compatible muscarinic receptors has been documented [29]. Thus constitutive receptor activity induced by G-proteins may be an important component of normal cellular physiology.

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