

Pharmacology of Muscarinic Receptor Subtypes Constitutively Activated by G Proteins

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SUMMARY

We have examined the effects of raising G protein concentration on the pharmacology of a series of agonist and antagonist ligands at the m1, m3, and m5 muscarinic subtypes using a functional assay. Overexpression of $G_{\alpha q}$ induced constitutive activity of these receptors. The constitutive activity was reversed completely by every muscarinic antagonist tested, which indicates that they are all negative antagonists (inverse agonists). The potencies of antagonists for reversing G protein-induced activity and agonist-induced activity were identical, suggesting the same mechanism of action. Overexpression of $G_{\alpha q}$ increased the potencies of every tested agonist and the efficacies of all partial agonists. The fold-gains in potency were positively correlated with ligand efficacy with the most efficacious agonists displaying the greatest potency gains. In addition, the efficacies of partial agonists approached those of full

agonists. Constitutive activity of receptors has been explained by allosteric models in which receptors exist in spontaneous equilibrium between active and inactive conformations that are stabilized by agonists and antagonists, respectively. In this context, drug efficacy and potency are interrelated because they both depend on the same parameters, namely the absolute and relative affinities of a compound for receptors in active and inactive states and the ratio and concentrations of receptors in active and inactive states. All of our data are consistent with this model, in which raising G protein levels favors formation of the active conformation of receptors. Based on our findings, regulation of G protein concentration may be an important means of controlling receptor activity in vivo. These results define the functional relationship between G protein levels and muscarinic receptor pharmacology.

Classic pharmacological theory states that agonist ligands activate receptors by inducing conformational changes, whereas antagonists block the binding of agonists to receptors. Recently, this theory has been revised; currently, there is widespread acceptance of the idea that GPCRs spontaneously interconvert between R^* and R (1–3). This concept has been used to explain numerous observations that receptors are capable of activating G proteins and eliciting responses in the absence of agonists (3–13). In fact, constitutive activity of wild-type serotonin (7), bradykinin (8), opioid (5), muscarinic (9, 12), and β -adrenergic receptors (3) have been documented. In addition, many mutationally activated GPCRs have been described (10, 11). In general, activating mutations are believed to alter the equilibrium between R and R^* to increase the proportion of receptor that exists as R^* . In this context, agonists and antagonists are defined by how much, and in which direction they influence the equilibrium between R and R^* . Antagonists can be distinguished between those that

can block agonist-independent responses and those that only block agonist-dependent responses. These compounds are called negative and neutral antagonists, respectively, and the ability to decrease agonist-independent responses has been termed “negative antagonism” or “inverse agonism” (1–3).

An important implication of the concept that receptors spontaneously adopt an active conformation is that the basal activity of GPCRs *in vivo* may not be zero but may be set at some intermediate level, as is the case for the ligand-gated ion channels. Experimental support for this hypothesis is that muscarinic antagonists depress basal frequency of K^+ channel opening in rabbit atrial cells (13), reduce basal inhibition of cAMP production in rat myocytes and CHO cells transfected with muscarinic receptors (9), and lower basal binding of GTP γ S to porcine cardiac membranes treated with acetylcholinesterase to remove any endogenous agonist (4). Therefore, there could be an important contribution of endogenous receptor “tone” to cholinergic physiology.

Theoretically, raising G protein concentration should shift receptor equilibrium to favor formation of R^* , assuming that G proteins preferentially interact with the active conforma-

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ABBREVIATIONS: GPCR, G protein coupled receptor; R , inactive state; R^* , active state; R-SAT, receptor selection and amplification technology; QNB, quinuclidinyl benzilate; O-ethyl-THAO, O-ethyl-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-c]azepin-3-ol.

tion of receptors (1–3, 14). Given that expression levels of G proteins can be up-regulated or down-regulated *in vivo* (15–18), receptor activity could be controlled at the level of G proteins. Consistent with this idea, we have shown that raising the concentration of G proteins induces constitutive activity of preferred receptors (12, 19). Therefore, we have used a functional assay to examine the effects of raising the G protein concentration on the pharmacology of a series of agonist and antagonist muscarinic ligands at the m1, m3, and m5 muscarinic subtypes.

Materials and Methods

Cell culture. NIH/3T3 cells (American Type Culture Collection, Rockville, MD) and COS-7 cells were incubated at 37° in a humidified atmosphere (5% CO₂) in Dulbecco's modified Eagle's medium supplemented with 4500 mg/liter glucose, 4 mM L-glutamine, 50 U/ml penicillin G, 50 units/ml streptomycin (A.B.I.), and 10% calf serum for 3T3 cells or 10% fetal bovine serum for COS-7 cells (GIBCO, Grand Island, NY). *N*-methyl-scopolamine binding studies on COS-7 cells were performed as described previously (11).

Functional assays. R-SAT assays were performed essentially as described previously (11, 12, 20). Briefly, cells were plated 1 day before transfection using 2×10^6 cells in 22 ml of media per 15-cm³ plate. Cells were transfected by calcium precipitation as described using 3 µg each of the human muscarinic receptor subtypes (m1, m3, m5), pSI-β-galactosidase (Promega, Madison, WI), G_{αq} or control vector, and 20 µg of salmon sperm DNA (Sigma Chemical, St. Louis, MO). Previous experiments verified that the G_{αq} construct is expressed (21). One day after transfection media were changed, and after 2–3 more days, cells were trypsinized, resuspended in Dulbecco's modified Eagle's medium containing calf serum or 2% Cyto-Sf3 synthetic supplement (Kemp Laboratories) and aliquoted into the wells of a 96-well plate (100 µl/well). The extra time between transfection and addition of ligands was found to reduce an inhibitory effect of G_{αq} overexpression, which limits cellular responses at high carbachol concentrations (19). One 15-cm³ plate yields enough cells for 150–200 wells. Ligands were combined with the cells to a final volume of 200 µl/well. All ligands were from standard vendors except *O*-ethyl-THAO, which was synthesized as described (22). After 5 days in culture, β-galactosidase levels were measured as described (20). Media were aspirated from the wells and the cells were rinsed with phosphate-buffered saline. Phosphate-buffered saline (200 µl) containing 3.5 mM *O*-nitrophenyl-β-D-galactopyranoside and 0.5% nonidet P-40 (both from Sigma) was added to each well and the 96-well plate was incubated at room temperature. After 16 hr, plates were read at 420 nm on a plate reader (Molecular Devices, Menlo Park, CA). Agonist data from R-SAT assays were fitted to the equation $R = A + B \times x / (x + c)$, where *A* is basal response, *B* is maximum response, *c* is the EC₅₀ value, and *x* is the concentration of ligand. Antagonist data from R-SAT assays were fitted to the equation $R = A + B \times c / (x + c)$. Curves were generated by least-squares fits using the program KaleidaGraph (Abelbeck/Synergy, Reading, PA). Data were normalized according to the maximum responses to carbachol and atropine.

Results

We have developed a functional assay called R-SAT in which proliferative responses of NIH/3T3 cells are monitored using a reporter enzyme (11, 12, 20). The pharmacology of carbachol and several other muscarinic agonists and antagonists, as determined with R-SAT, has been shown previously to be very similar to that determined with traditional functional assays (23). We have exploited R-SAT to examine the functional interactions of receptors with G proteins. As

shown in Fig. 1, A, C, and E, when NIH/3T3 cells transfected with either the m1, m3, or m5 muscarinic receptors and the β-galactosidase gene were cultured in the presence of the indicated concentrations of the muscarinic agonist carbachol, there were dose-dependent increases in β-galactosidase activity. When we coexpressed G_{αq} (21) there were dramatic increases in basal activity, representing approximately 20 to 30% of the total response. G_{αq} does not constitutively activate the m2 receptor subtype that is coupled to inhibition of adenylate cyclase through PTX-sensitive G proteins (24) but also is able to activate the α1B and NK1 receptors which, like m1, m3, and m5, also are coupled to phosphatidylinositol turnover through PTX-insensitive G proteins.¹ A series of other G proteins are unable to constitutively activate m5, which indicates that induction of constitutive activity is restricted to preferred receptor/G protein pairs (19). The observed constitutive activity was not due to endogenous ligands present in the media, as this phenomenon was readily observable using synthetic media. Although large increases in total receptor number could result in observable constitutive activity (3), coexpression of G_{αq} had little or no effect on the expression levels of the receptors (1.6 ± 0.3 fmol/mg for m5 versus 1.9 ± 0.5 fmol/mg for m5 + G_{αq}); therefore, the constitutive activity is due to the increased levels of G_{αq}.

In addition to inducing constitutive activity, G_{αq} also caused 15- to 30-fold increases in the potency of carbachol (Fig. 1, A, C, and E; compare EC₅₀ values in Table 1). However, the maximum responses to carbachol were not significantly increased by G_{αq}. In contrast, maximum responses to the partial agonist McN-A-343 were greatly increased by G_{αq}, up to but not exceeding the level observed with carbachol (Fig. 1, B, D, and F). G_{αq} coexpression also increased the potency of McN-A-343 but to a much lesser extent than carbachol (Table 1). The G_{αq}-induced activity was reversed in a dose-dependent manner by the potent muscarinic antagonist atropine, which indicates that it is actually a negative antagonist (Fig. 1, A, C, and E). A small component of the response could not be reversed by atropine and represents interaction of G_{αq} with endogenous cellular components because this effect can be reproduced by transfection with G_{αq} alone (19). 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_{αq} (Fig. 1, B, D, and F). In contrast to the results for agonists, G_{αq} overexpression did not significantly alter p*K_i* values for either antagonist (Table 2).

We then compared the pharmacology of a series of compounds at each receptor expressed with or without G_{αq}. As we saw above, the potencies of all agonists and the efficacies of partial agonists were enhanced with increased G protein levels (see Fig. 2). In general, the shifts in the EC₅₀ value were greater for the stronger, more efficacious agonists than for the partial agonists, and the maximum response was increased more for weak partial agonists than full agonists. For example, arecoline, which displayed intermediate efficacy (maximum response 70 to 90% of carbachol), also exhibited intermediate gains in potency (8- to 14-fold) compared with the stronger or weaker agonists, which had greater or lesser increases in potency, respectively (Table 1). Plotting the relationship between agonist efficacy and G protein ef-

¹ E. S. Burstein and M. R. Brann, unpublished observations.

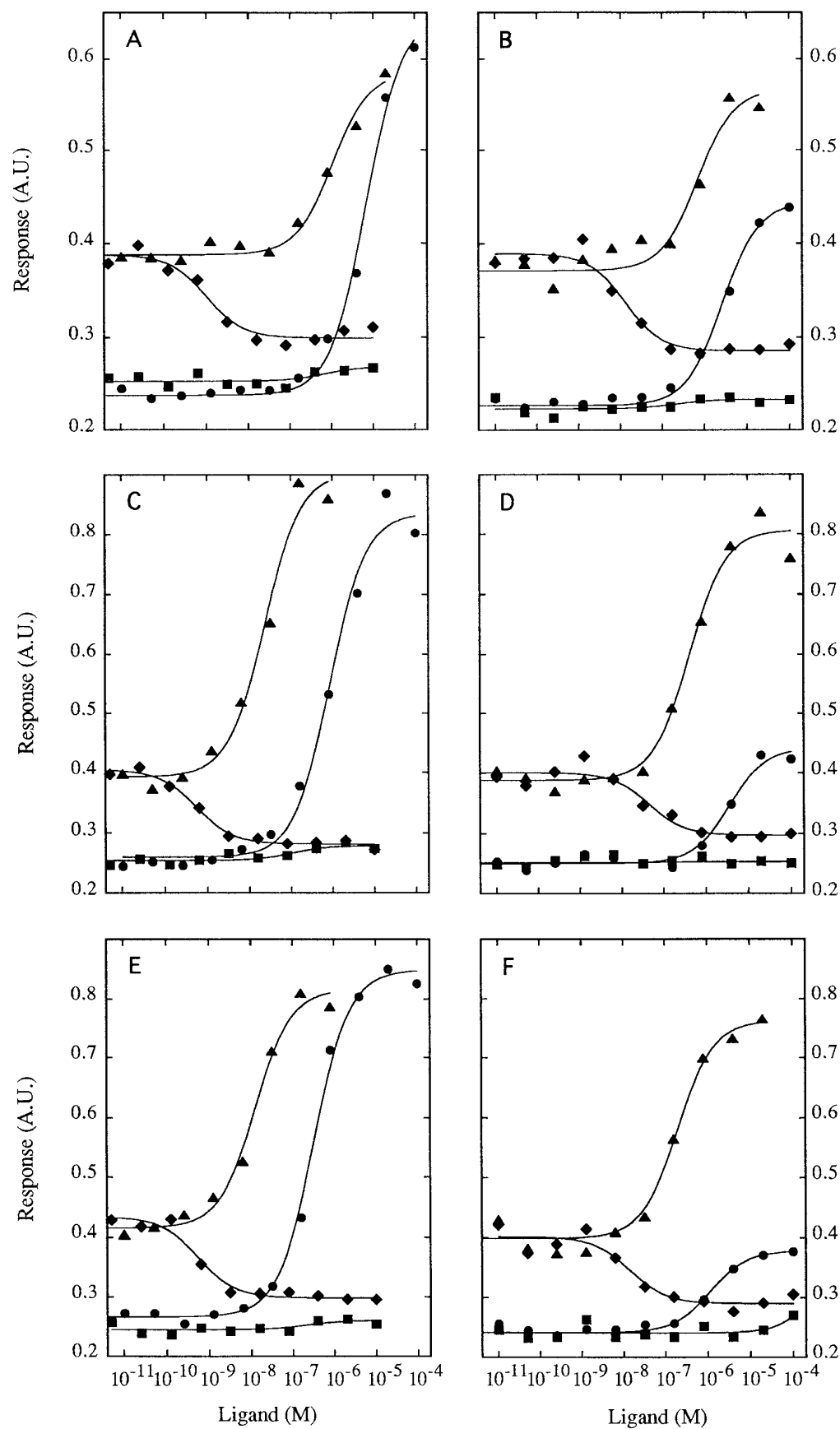


Fig. 1. Constitutive activation of m1, m3, and m5 muscarinic receptors by $G_{\alpha q}$. Either m1, m3, or m5 and β -galactosidase were transfected into NIH/3T3 cells either with or without $G_{\alpha q}$ and cultured in the presence of the indicated concentrations of ligands. Ligands are represented by the same symbols in A, C, and E and in B, D, and F. A, C, and E, carbachol (\blacktriangle , m1 + $G_{\alpha q}$; \bullet , m1); atropine (\blacklozenge , m1 + $G_{\alpha q}$; \blacksquare , m1). B, D, and F, McN-434 (\blacktriangle , m1 + $G_{\alpha q}$; \bullet , m1); pirenzepine (\blacklozenge , m1 + $G_{\alpha q}$; \blacksquare , m1). C and D are m3, E and F are m5. Plotted are absorbance of the β -galactosidase substrate O-nitrophenyl- β -D-galactopyranoside at 420 nm versus ligand concentration. Points, average of two determinations.

TABLE 1
Agonist efficacy and effect of $G_{\alpha q}$ on agonist potency

| Agonist | EC ₅₀ (% response) | | EC ₅₀ /EC ₅₀ (+G) | EC ₅₀ (% response) | | EC ₅₀ /EC ₅₀ (+G) | EC ₅₀ (% response) | | EC ₅₀ /EC ₅₀ (+G) |
|--------------|-------------------------------|------------------------|--|-------------------------------|--------------------------|--|-------------------------------|-------------------------|--|
| | m1 | m1 + G _q | | m3 | m3 + G _q | | m5 | m5 + G _q | |
| | <i>nm</i> | | | <i>nm</i> | | | <i>nm</i> | | |
| Carbachol | 1506 ± 166 (100%) | 87 ± 15 (100%) | 17 | 693 ± 36 (100%) | 20 ± 4 (100%) | 34 | 249 ± 21 (100%) | 11 ± 2 (100%) | 22 |
| Pilocarpine | 4718 ± 311 (91 ± 12%) | 264 ± 31 (75 ± 9%) | 18 | 2796 ± 474 (64 ± 9%) | 156 ± 72 (113 ± 4%) | 18 | 2476 ± 135 (96 ± 15%) | 192 ± 47 (103 ± 18%) | 13 |
| Arecoline | 514 ± 85 (84 ± 6%) | 64 ± 28 (86 ± 1%) | 8 | 593 ± 90 (70 ± 3%) | 64 ± 14 (126 ± 0%) | 9 | 261 ± 41 (79 ± 12%) | 18 ± 11 (94 ± 9%) | 14 |
| McN-A-343 | 1862 ± 46 (62 ± 36%) | 273 ± 11 (93 ± 17%) | 7 | 4440 ± 81 (38 ± 6%) | 1090 ± 75 (105 ± 26%) | 4 | 1729 ± 49 (56 ± 19%) | 341 ± 17 (98 ± 11%) | 5 |
| O-ethyl-THAO | 126 ± 3 (46 ± 17%) | 40 ± 3 (78 ± 11%) | 3 | 193 ± 7 (23 ± 9%) | 46 ± 2 (101 ± 38%) | 4 | 346 ± 1 (80 ± 30%) | 71 ± 1 (93 ± 33%) | 5 |

fects on agonist potency reveals a clear positive correlation between fold-change in potency and agonist efficacy (Fig. 3).

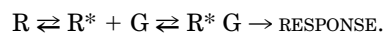
As shown in Fig. 2 and Table 2, all muscarinic antagonists tested were able to suppress constitutive activity induced by $G_{\alpha q}$, which indicates that these compounds are negative antagonists. With only minor exceptions, this suppression of activity was complete, in contrast to results for β -adrenergic antagonists, which possess a range of inverse efficacies (3). The same compounds also were able to block the actions of agonist ligands (Table 2). The inhibition constants for reversal of agonist and G protein-induced activity were nearly identical, suggesting that binding site was the same in both cases (Fig. 4). This was true even for compounds that displayed selectivity between the receptor subtypes (see pirenzepine, Table 2).

Discussion

We have examined the functional effects of raising G protein concentration on the pharmacology of an array of agonist and antagonist muscarinic ligands at the m1, m3, and m5 muscarinic subtypes using a functional assay. Overexpression of $G_{\alpha q}$ induced constitutive activation of these receptors. The constitutive activity was completely reversed by every muscarinic antagonist tested, which indicates that they were all negative antagonists. The inhibition constants for reversal of both agonist and G protein-induced activity were similar, which suggested the same mechanism of action. We examined the pharmacology of a series of muscarinic agonists with various efficacies ranging from weak partial agonists to full agonists and observed that in every case their potencies and efficacies were increased by raising $G_{\alpha q}$. The fold-gains in potency were positively correlated with ligand efficacy; the strongest agonists displayed the greatest potency gains. In addition, the efficacies of partial agonists approached those of full agonists at high G protein concentration.

Constitutive activity of receptors has been explained by allosteric models in which receptors exist in spontaneous equilibrium between active and inactive conformations (1–3). Accordingly, agonists are compounds that stabilize the active conformation, whereas antagonists preferentially interact with the inactive conformation, shifting receptor equilibrium to enhance or diminish activity, respectively. Assuming that G proteins interact preferentially with the active conformation of receptors, it follows that increasing the G protein

concentration will shift the equilibrium to increase the proportion of receptor in the active state. This would both induce constitutive activity and augment the potencies and efficacies of agonists. In this context, antagonists do not merely block the actions of agonists but also can possess an entire spectrum of efficacies, ranging from negative antagonism to neutral antagonism (1–3, 5, 7). Our results are entirely consistent with these predictions. The following model both predicts and explains all of our observations:



In this model, receptors exist in equilibrium between inactive R and R^* conformations. Agonists and G proteins have higher affinity for R^* , whereas antagonists preferentially bind R. Assuming that the receptor exists primarily as R in the absence of added agonists, coexpression of compatible receptor/G protein combinations causes constitutive activity by shifting this equilibrium to favor R^* . Recently, others have shown that coexpression of $G_{\alpha 16}$ induces constitutive activation of the fMLP receptor, which indicates that this may be a general phenomenon (25). However, in that study, no antagonists were used to verify that observed elevations in basal activity were specifically due to fMLP receptor/ $G_{\alpha 16}$ coupling.

Historically, potency has been defined as the amount of a drug needed to produce a half-maximal effect and efficacy is defined as the maximum effect a drug can generate; only agonists can be described in these terms. In the context of a two-state model of receptor activation, efficacy and potency are interrelated because they both depend on the same parameters, namely the absolute and relative affinities of a compound for R and R^* and the ratio and concentrations of R and R^* (2). One would predict that by raising [G], and thus R^*G , agonist potency will increase because, by definition, agonists preferentially bind R^* . Furthermore, there will be a greater effect of [G] on the potency of full agonists relative to partial agonists due to the greater selectivity of full agonists for R^* . As increased [G] shifts receptor equilibrium in favor of R^*G , the differences between the abilities of partial agonists and full agonists to promote the formation of R^*G will become obscured; i.e., the maximum responses to partial agonists will increase relative to the maximum responses to full agonists. Although this model also predicts that the potencies of antagonists to reverse activity will decrease as the fraction

TABLE 2

Antagonist inhibition of $G_{\alpha q}$ -induced (constitutive) and agonist-induced activity

| Antagonist | pK_i (% inhibition) | | pK_i | | pK_i | |
|------------------------------------|------------------------------------|-------------------------------------|------------------------------------|------------------------------------|-----------------------------------|------------------------------------|
| | m1 + Cch | m1 + G_q | m3 + Cch | m3 + G_q | m5 + Cch | m5 + G_q |
| Atropine | 8.78 ± 0.45 (100%) | 8.66 ± 0.33 (100%) | 9.11 ± 0.68 (100%) | 8.99 ± 0.23 (100%) | 8.66 ± 1.10 (100%) | 9.00 ± 0.40 (100%) |
| Pirenzepine | 7.40 ± 0.42 (108 \pm 13%) | 7.64 ± 0.18 (84 \pm 31%) | 6.69 ± 0.13 (109 \pm 2%) | 6.83 ± 0.11 (72 \pm 10%) | 6.65 ± 0.36 (98 \pm 22%) | 7.03 ± 0.47 (86 \pm 8%) |
| N-methyl scopolamine | 9.39 ± 0.05 (104 \pm 7%) | 9.49 ± 0.26 (101 \pm 45%) | 9.70 ± 0.45 (105 \pm 7%) | 9.57 ± 0.21 (121 \pm 18%) | 8.82 ± 0.44 (94 \pm 18%) | 8.59 ± 0.07 (82 \pm 11%) |
| QNB | 10.58 ± 0.20 (91 \pm 11%) | 10.71 ± 0.65 (116 \pm 45%) | 11.09 ± 0.68 (61 \pm 37%) | 10.71 ± 0.02 (103 \pm 4%) | 10.33 ± 0.06 (93 \pm 8%) | 11.04 ± 0.10 (94 \pm 17%) |
| Trihexyphenidyl | 8.02 ± 0.27 (117 \pm 21%) | 8.22 ± 0.18 (107 \pm 30%) | 8.15 ± 0.38 (106 \pm 29%) | 7.91 ± 0.38 (67 \pm 15%) | 7.57 ± 0.18 (86 \pm 14%) | 7.48 ± 0.41 (100 \pm 19%) |
| 4-Diphenylacetoxy-N-methylperidine | 8.09 ± 0.11 (104 \pm 16%) | 8.33 ± 0.17 (112 \pm 22%) | 9.04 ± 0.17 (96 \pm 3%) | 9.08 ± 0.27 (70 \pm 30%) | 7.97 ± 0.24 (94 \pm 5%) | 8.11 ± 0.29 (70 \pm 5%) |

of R^*G increases (2), the observed constitutive activity levels are low enough that only a negligibly small increase in the IC_{50} values of antagonists would be expected. In agreement with these predictions, we observed that the fold-gain in agonist potency was positively correlated with ligand efficacy (Fig. 3) and that the efficacies of partial agonists approach the efficacies of full agonists when $[G]$ is increased (Figs. 1 and 2, Table 1). In contrast, the antagonist potencies for reversal of G protein- or agonist-induced activity were identical (Table 2). The observed pharmacological effects of raising $[G]$ also could be caused by increasing the total number of receptors under conditions in which the maximum attainable response to agonists is limited by factors downstream of the receptor or when the maximum response to agonists occurs at a submaximal level of receptor occupancy, i.e., when spare receptors exist (26, 27). Under these conditions, $[R^*G]$ increases due to the gain in total receptor number. Conversely, by raising $[G]$, the fraction of receptors that exist as R^*G is increased rather than the total number of receptors.

It was surprising to discover that all of the antagonists tested in this study were negative antagonists because antagonists ranging from completely negative to neutral have been described with activity at the δ opioid receptor (5), the β_2 -adrenergic receptor (3), and the 5-hydroxytryptamine receptor (7). In agreement with our findings, Jakubik *et al.* (9) found that atropine, N-methyl scopolamine, and QNB were negative antagonists although they did not observe negative activity of QNB in every condition examined. Our findings reconcile earlier observations that the binding affinities of N-methyl scopolamine, QNB, and pirenzepine increase in the presence of nonhydrolyzable analogs of GTP (14, 28). In theory, it should be possible to synthesize neutral muscarinic antagonists. Possibly, the therapeutic effects of the muscarinic compounds are derived from their negative efficacy. Differential physiological effects of neutral versus negative antagonists have been demonstrated *in vivo* using transgenic mice that overexpress the β_2 -adrenergic receptor in heart tissue (29). γ -Aminobutyric acid receptor ion channel ligands have been described with intrinsic activities ranging from agonist to negative antagonist (30). The pharmacological properties of these compounds correlate well with their physiological effects *in vivo*. γ -Aminobutyric acid receptor agonists have anticonvulsive effects, whereas inverse agonists promote convulsions. The high degree of safety associated with the use of these drugs stems in part from the fact that

they act allosterically. When neutral muscarinic compounds become available, it will be interesting to evaluate their physiological effects.

Our results emphasize the importance of efficacy in drug design. For example, use of partial agonists might reduce the risk of overdose associated with use of full agonists. Potentially, use of agonists and antagonists with limited efficacy would decrease some of the problems associated with chronic drug use, such as receptor down-regulation or up-regulation and desensitization or hypersensitization. Adjusting the drug efficacy also would be a useful strategy for improving selectivity and reducing side effects. As shown in this paper and discussed previously (27), increases in the concentration of either G proteins and/or receptors will significantly augment the efficacy of a partial agonist. Therefore, if a target tissue is enriched in a particular receptor subtype (and compatible G proteins), a weak partial agonist with selectivity for that subtype would have fewer side effects in other tissues that may express fewer receptors and/or other subtypes. For example, the heart is enriched in the m2 subtype of muscarinic receptor (31); therefore, it might be possible to design m2-selective partial agonists with efficacy for heart disorders and reduced cholinergic side effects. One strategy that has been proposed to adjust the efficacy of drugs is to use mixtures of agonists and antagonists (32), although pharmacokinetic issues may limit the clinical use of this approach.

Negative antagonists with high inverse efficacy would be desirable if it were necessary to lower basal receptor activity, such as for a constitutively activated receptor. Constitutive activation of receptors (by mutation) has been implicated in a number of diseases (33–35), and many GPCRs are proto-oncogenes (36–38). To date, random saturation mutagenesis of the human m5 muscarinic receptor has identified more than 40 constitutively activating mutations (11, 20),² which indicates that such events are likely to be common in nature and may be responsible for many more diseases than currently appreciated.

The subcellular locations and levels of G proteins are known to be tightly controlled by physiological stimuli (15–18, 39), and regulation of $G_{\alpha q}$ levels by compatible muscarinic receptors has been documented (15). Like receptors, G proteins undergo agonist-induced down-regulation (15, 16). In

² E. S. Burstein, T. A. Spalding, and M. R. Brann, unpublished observations.

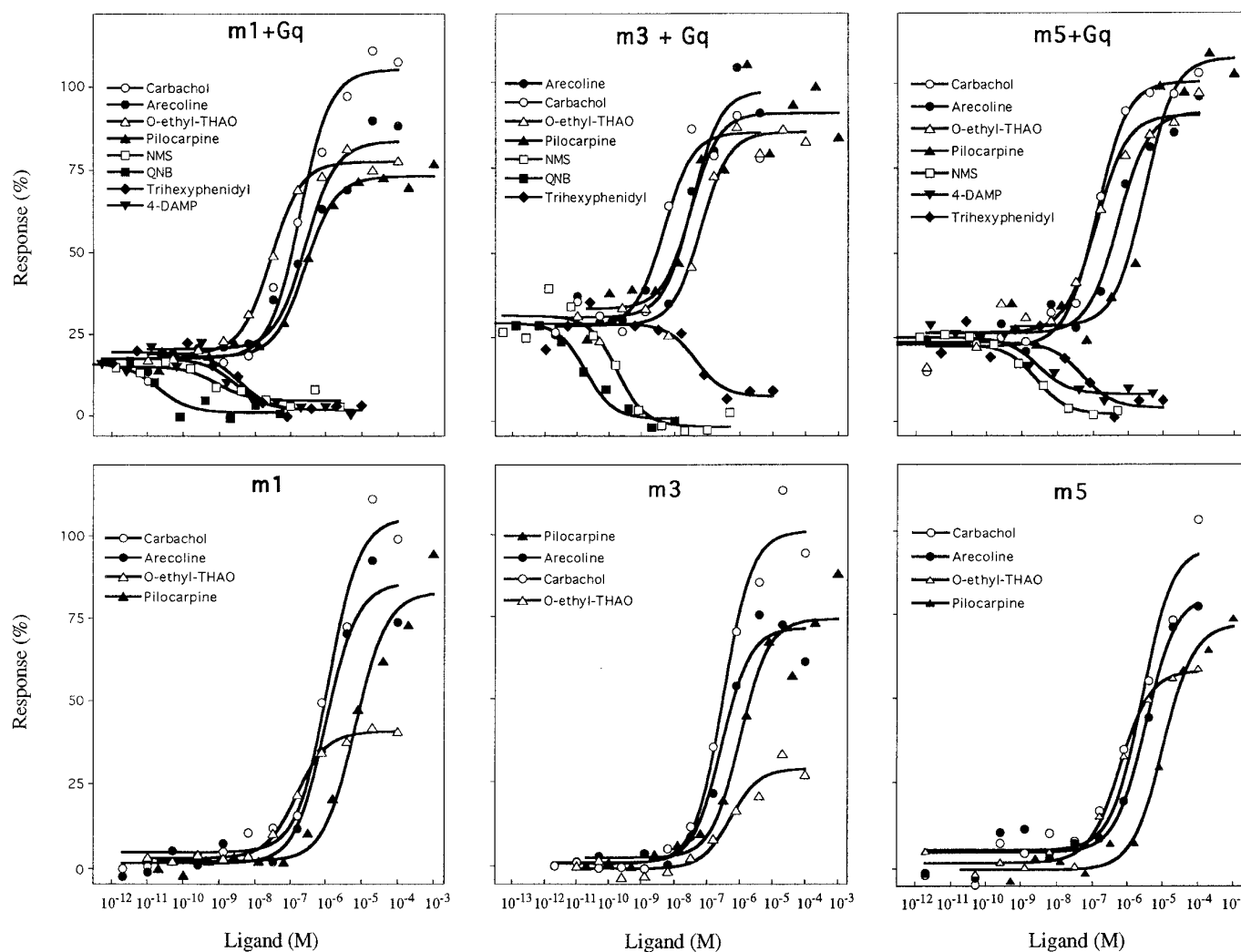


Fig. 2. Pharmacology of agonist and antagonist ligands at muscarinic subtypes constitutively activated by $G_{\alpha q}$. Functional assays were performed as described in Materials and Methods. Ligands are represented by the same symbol in each condition (\circ , carbachol; \bullet , arecoline; \triangle , O-ethyl-THAO; \blacktriangle , pilocarpine; \square , N-methyl scopolamine (NMS); \blacksquare , QNB; \blacklozenge , trihexyphenidyl; \blacktriangledown , 4-diphenylacetoxy-N-methylpiperidine (4-DAMP). Points, average of two determinations. Responses were normalized to the response to carbachol, which was assigned a value of 100%. Responses of receptors alone to antagonists were not significantly different from zero at all antagonist concentrations (not shown).

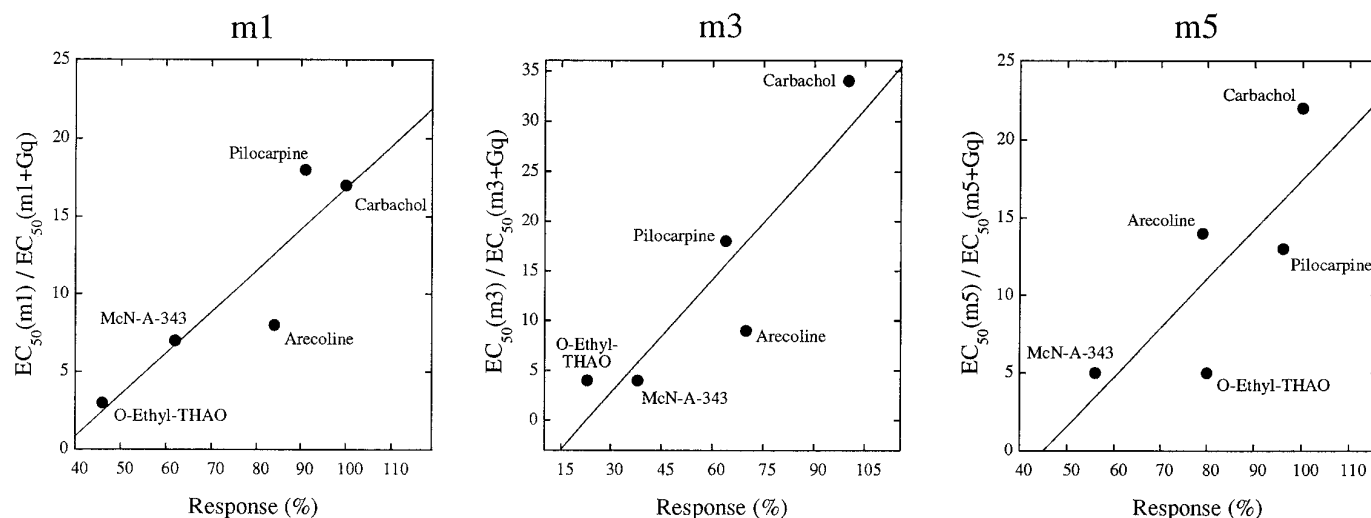


Fig. 3. Correlation between agonist efficacy and effect of $G_{\alpha q}$ on agonist potency. The ratio of the EC_{50} value at receptor to the EC_{50} value at receptor plus $G_{\alpha q}$ was plotted against the maximum response at receptor. Ligands are indicated on the graphs. Responses were normalized to the response to carbachol, which was set at 100%. Data were taken from Table 1.

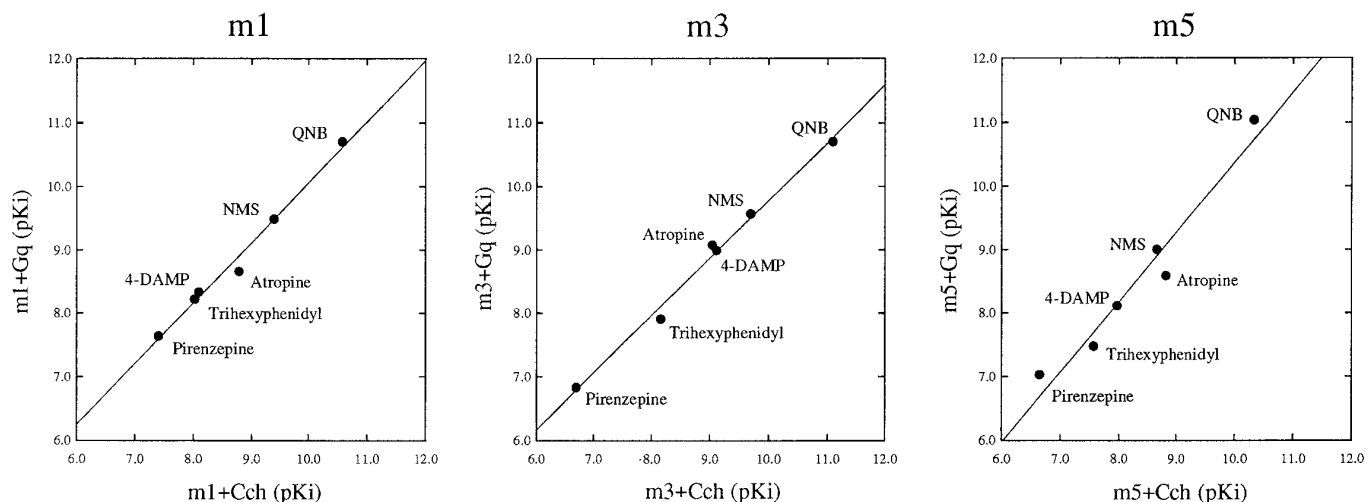


Fig. 4. Correlation between antagonist inhibition of $G_{\alpha q}$ -induced (constitutive) and agonist-induced activity. The pK_i values for receptor plus carbachol were plotted against the pK_i values for receptor plus $G_{\alpha q}$. Data were taken from Table 2.

some cases, G protein levels are up-regulated to provide increased sensitivity (17) or to enhance an alternative signaling pathway (18). For example, embryonic chick hearts acquire sensitivity to muscarinic cholinergic inhibition of adenylate cyclase during development by increasing expression of G_i -type G proteins and not by changing muscarinic receptor levels that remain constant (17). Previous studies (4, 9, 13) imply that the endogenous levels of constitutive receptor tone may be higher than appreciated previously and may constitute an important part of normal cholinergic physiology. This is plausible because the localized concentrations of receptors and G proteins are likely to be very high in specialized structures such as neuronal synapses. Based on our findings, regulation of G protein levels may be an important means of controlling receptor activity *in vivo*.

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