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Partial Agonist Effects on the Interaction Between the Atrial Muscarinic Receptor and the Inhibitory Guanine Nucleotide-Binding Protein in a Reconstituted System

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SUMMARY

The mechanism of action of the partial muscarinic agonist pilocarpine was analyzed in a reconstituted system consisting of the purified porcine atrial muscarinic receptor and the purified porcine atrial inhibitory guanine nucleotide-binding protein, G_i. When GTPase activity was measured as a function of receptor·agonist complex concentration at saturating concentrations of either the full agonist carbachol or pilocarpine, both ligands gave similar values of k_{cat} (4.3 ± 0.2 min^{-1} for carbachol and 5.4 ± 0.7 min^{-1} for pilocarpine); however, the observed dissociation constant for the ligand·receptor complex binding to G_i was about 4-fold lower for carbachol (0.81 ± 0.19 nM) than for pilocarpine (3.02 ± 0.83 nM). These results suggested that, in this system, the reduced activity of the partial agonist compared with the full agonist was the result of a decrease in affinity of the receptor·ligand complex for G_i, as opposed to differences in their relative abilities to activate the guanine nucleotide-binding protein. Several analogues of oxotremorine were also tested to determine their effects on the GTPase activity of G_i. Results from these studies indicate that the reconstituted system may be useful in determining structure-function relationships for muscarinic agonists with regard to receptor·G_i interactions.

The binding of agonists to the mAcChR initiates a variety of biochemical responses, including the inhibition of adenylyl cyclase, an increase in PI turnover, and the activation of inward rectifying potassium channels in the atria (1). mAcChRs are coupled to these physiological effector systems by G proteins. The mAcChR·agonist complex is thought to activate the G protein catalyzing the exchange of GDP for GTP (2–4).

There are at least five distinct mAcChR subtypes (5–7). Each of these subtypes shows selectivity in coupling to specific biochemical responses. The M1, M4, and M5 subtypes [the nomenclature used in this manuscript is that of Peralta *et al.* (6)] couple to the stimulation of PI turnover, whereas the M2 and M3 couple mainly to inhibition of adenylyl cyclase (7–10). Because the differing responses are mediated by G proteins, it would appear that mAcChR subtype-specific responses are based on a preference for different G proteins (8–11).

Muscarinic drugs can be classified as antagonists, partial agonists, or full agonists, depending on their relative ability to initiate a given physiological response. Antagonists may bind to the mAcChR with high affinity, but the antagonist-occupied mAcChR cannot activate the G proteins that couple the mAcChR to physiological effector system(s). Full agonists cause maximal activation of the requisite G protein(s), whereas partial agonists give rise to a response intermediate between those found for full agonists and antagonists.

Oxotremorine and pilocarpine are examples of drugs that act as partial agonists for the mAcChR-initiated stimulation of PI metabolism (12, 13). When these two drugs were tested for their relative ability to induce mAcChR-mediated inhibition of adenylyl cyclase in human heart auricles, oxotremorine gave the same maximal response as the full agonist carbachol but the maximal response to pilocarpine was significantly lower (14). A possible explanation for these observations is that the relative potency of some muscarinic agonists may depend on the particular muscarinic subtype and G protein involved in mediating a given biochemical response. Because it is now possible to obtain pure preparations of recombinant mAcChR subtypes and $G_{i\alpha}$ subunits, it is of interest to develop *in vitro*

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ABBREVIATIONS: mAcChR, muscarinic acetylcholine receptor; PI, inositol phospholipid; CHAPS, (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; buffer A, 10 mM Na 4-(2-hydroxyethyl)-1-piperazineethanesulfonate, pH 7.4, 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride; buffer B, 25 mM imidizole, 0.1 M NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride; G protein, guanine nucleotide-binding protein; G_i, the inhibitory guanine nucleotide-binding protein; G_s, the stimulatory guanine nucleotide-binding protein; Gpp(NH)p, 5′-guanylyl imidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; QNB, quinuclidinyl benzilate.

systems for studying the mechanisms of full and partial agonists in which agonist activity can be related to specific receptor subtypes and G proteins.

There were two main objectives of this study. The first was to use the reconstituted atrial M2 mAcChR-G_i system to examine a series of oxotremorine analogues for their ability to act as full or partial agonists in activating the GTPase activity of the reconstituted G_i. Because modification of the oxotremorine structure may result in drugs that function as full agonists, partial agonists, or antagonists, these experiments may give information concerning the structural requirements for M2 mAcChR-G protein interactions. Secondly, the reconstituted system was used to investigate the biochemical basis for partial and full agonist activity. The difference between a ligand behaving as a partial or a full agonist may depend on the relative affinity of the respective receptor·ligand complex for the G protein and/or on the ability of the drug·receptor complex to activate the G protein by promoting GDP/GTP exchange once the (agonist·receptor)·(G protein·GDP) complex has been formed. After comparison of the agonist activities of pilocarpine and several oxotremorine analogues with carbachol in the reconstituted system, pilocarpine was found to give the largest difference in relative activity and was used as a model partial agonist. By reconstituting G_i with increasing concentrations of mAcChR and measuring the GTPase activity at saturating levels of pilocarpine or carbachol, it was possible to determine the effects of a full and a partial agonist on both the turnover number of G_i and the observed dissociation constant of G_i for the ligand·receptor complex.

Experimental Procedures

Materials. [^{35}S]GTPγS (1000 Ci/mmol) and [γ-^{32}P]GTP (20–30 Ci/mmol) were purchased from New England Nuclear. L-[^{3}H]QNB (30–50 Ci/mmol) was purchased from either New England Nuclear or Amersham. Carbachol, oxotremorine, pilocarpine, *l*-hyosycamine, CHAPS, digitonin, Gpp(NH)p, cholesterol, soybean L-α-phosphatidylcholine (type III-s), and L-α-phosphatidyl-L-serine (bovine brain) were purchased from Sigma. Unlabeled GTPγS was from Boehringer Mannheim. Oxotremorine analogues were a generous gift of Dr. Stephen Fisher, Neuroscience Lab, University of Michigan. Porcine atrial mAcChR (10 nmol of L-[^{3}H]QNB binding sites/mg) was purified as described by Peterson *et al.* (15), and atrial G_i (8 nmol of [^{35}S]GTPγS binding sites/mg) was purified as a by-product of mAcChR purification (3). Analysis of purified protein preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described previously (3), showed a single band for the mAcChR and three bands with apparent molecular weights of 41,000, 35,000, and 10,000, corresponding to the $G_{i\alpha}$, $G_{i\beta}$, and $G_{i\gamma}$ subunits. Western blot analysis of a similar atrial G_i preparation[2] using antiserum raised against G_α subunits (16) showed that the preparation contained approximately equimolar amounts of $G_{i\alpha_1}$ and $G_{i\alpha_3}$.

Reconstitution of mAcChR and G_i. The reconstitution of mAcChR and G_i was performed as described previously (3). The data in Tables 1 and 2 were from experiments using liposomes prepared from 10 pmol of mAcChR and 50 pmol of G_i. Any other differences in the amount of protein used will be indicated in the figure legends. Briefly, both proteins were added to a mixture of 7 mM CHAPS, 1 mg/ml lipid (1:1:0.1, w/w, phosphatidylcholine/phosphatidylserine/cholesterol), and 50 μM acetylcholine and were diluted 25-fold with buffer B. The liposomes were precipitated by the addition of polyethylene glycol 8000 to a final concentration of 10% (w/v). After incubation for 1 hr on ice, the mixture was centrifuged at 250,000 × *g* for 75 min. The pellet containing lipid plus mAcChR and G_i was resuspended in 250 μl of buffer B. The liposomes used for the binding studies in Fig. 2 were resuspended in 250 μl of buffer A.

Ligand binding. [^{35}S]GTPγS binding was performed as described by Northup *et al.* (17). Reconstituted G_i was diluted into buffer A and the binding assay was begun by the addition of [^{35}S]GTPγS to a final concentration of 100 nM. The reaction was terminated by dilution into 2 ml of ice-cold 10 mM Na HEPES, 0.1 M NaCl, 25 mM $MgCl_2$, followed by filtration over BA85 nitrocellulose filters. The filters were washed with 4 × 2 ml of the above ice-cold buffer, dried, and dissolved in 1 ml of methyl Cellusolve. Scintillation fluid was added and the samples were counted for ^{35}S.

mAcChR was quantitated in terms of L-[^{3}H]QNB binding sites using a DEAE filter disk assay (18). All binding assays for reconstituted material were performed in buffer A using 125 μl of sample. Nonspecific binding was determined in the presence of 10 μM *l*-hyoscyamine or 0.1 M carbachol. Total mAcChR concentration was determined by using 20 nM L-[^{3}H]QNB with or without 10 μM *l*-hyoscyamine or by using lipids without mAcChR to measure nonspecific binding.

GTPase activity. GTPase activity was determined as described by Sunyer *et al.* (19), with the following modifications. Reconstituted mAcChR and G_i were diluted with buffer A to the concentrations indicated. Muscarinic ligands were added as described in the text and allowed to equilibrate for 5 min at 32°. The GTPase reaction was initiated by the addition of [γ-^{32}P]GTP and was incubated at 32° for the desired time interval. The reaction (50 μl) was then quenched by the addition of 0.25 ml of an ice-cold suspension of activated charcoal (5%, v/w) in 20 mM sodium phosphate (pH 2.3). This mixture was placed on ice for 5 min and then centrifuged at 15,000 × *g* for 15 min at 4°. The ^{32}P in 0.1 ml of the clear supernatant was measured by Cerenkov counting. Where significant (Tables 1 and 2, Fig. 1), nonenzymatic hydrolysis of GTP was subtracted from each determination. GTP hydrolysis was linear as a function of time for all experiments.

Data analysis. Data used to calculate the K_{app} values and maximal stimulation relative to carbachol for muscarinic agonists in Fig. 1 and Table 2 were fit to Eq. 1 using Marquardt's algorithm, as described by Duggleby (20).

$$G = \frac{(M)[L]}{[L] + K_{app}} \quad (1)$$

In Eq. 1, G is the experimentally determined percentage of stimulation of the GTPase activity of G_i at ligand concentration $[L]$, relative to that observed for a saturation concentration of carbachol (2 mM). The data were fit to determine values of K_{app}, the concentration of $[L]$ at which stimulation of the GTPase activity was half-maximal, and M, the maximal fractional stimulation of the GTPase activity at saturation with $[L]$, relative to saturating concentrations of carbachol.

Ligand-binding data for the competitive binding of either carbachol or pilocarpine versus L-[^{3}H]QNB (Fig. 2) were fit to Eq. 2 using Marquardt's algorithm (20)

$$\bar{Y} = \frac{[Q]}{K}\left(\frac{F_1}{1 + \frac{[I]}{K_1} + \frac{[Q]}{K}} + \frac{F_2}{1 + \frac{[I]}{K_2} + \frac{[Q]}{K}}\right) \quad (2)$$

where $\bar{Y}$ is the fractional saturation of the mAcChR by L-[^{3}H]QNB and $[Q]$ and $[I]$ are the free radioligand and inhibitor concentrations. F_1 and F_2 are the fractions of binding sites with high and low affinity for the inhibitor, having dissociation constants K_1 and K_2, respectively, and K is the overall dissociation constant for L-QNB. Data were then normalized according to Eq. 3, where $\bar{Y}_o$ equaled the fractional saturation in the absence of inhibitor.

$$\%\text{ of L-[}^3\text{H]QNB specifically bound} = \frac{\bar{Y}}{\bar{Y}_o} \times 100 \quad (3)$$

Because the value of K varied slightly between experiments, its value was calculated for each curve from the law of mass action, using the concentration of free mAcChR and $[Q]$ determined in the absence of

[2] S. Mumby, unpublished data.

inhibitor. Data in Fig. 2B were analyzed assuming a single class of inhibitor binding sites, by setting F_2 equal to zero.

Data in Fig. 3 were analyzed by a fit to Eq. 4 (see Appendix) using Marquardt's algorithm (20)

$$v = V_{max}([RAG]/[G]_0) \quad (4)$$

where $[RAG]$ is the concentration of agonist·mAcChR·G_i complex and is equal to

$$\frac{([RA]_0 + [G]_0 + K_d) - \sqrt{([RA]_0 + [G]_0 + K_d)^2 - 4[RA]_0[G]_0}}{2}.$$

$[RA]_0$, the total concentration of agonist·mAcChR complex, $[G]_0$, the total G_i concentration, and v, the observed steady state velocity of the GTPase activity of G_i, were used as input values to determine K_d, the observed dissociation constant of the receptor agonist complex from G_i, and V_{max}, the maximal steady state GTPase activity.

Results

The muscarinic ligands listed in Table 1 differed in their ability to stimulate the GTPase activity of atrial G_i in the reconstituted system. Oxotremorine (compound **4**) and oxotremorine analogues **2** and **3** were as effective as carbachol in activating the GTPase activity of G_i and were considered full agonists. Compounds **5** and **6** were slightly less effective, whereas the remaining compounds (**7** through **10**) were significantly less active than carbachol. The antagonist *l*-hyoscyamine (compound **11**) was included as a control to determine the unstimulated GTPase activity. Analysis of dose-response curves for compounds **3** and **7–10** (Fig. 1) indicated that, even when compounds **7–10** were present at saturating concentrations, they exhibited a submaximal stimulation of atrial G_i (Table 2). The K_{app} for these agonists was between 10^{-7} and 10^{-5} M, a value comparable to the K_{app} for carbachol derived under the same conditions [2.1 μM (3)]. Thus, although these ligands bound to the muscarinic receptor with about the same affinity as the full agonist carbachol, they behaved as partial agonists with respect to the stimulation of atrial G_i in the reconstituted system. It should be noted that, although there was no detectable difference seen between compounds **1**, **2**, **3**, and **4**, this assay does not preclude the possibilty that differences in agonist efficacy do exist for those compounds.

Analysis of tertiary and quaternary amine analogues of oxotremorine (Table 1, compound **2** versus **3** and compound **6** versus **7**) in this reconstituted system suggested that replacement of a methyl group with a proton at pH values near neutrality did not affect the maximal GTPase activity in this assay. These results differ from those found for the stimulation of PI metabolism, where oxotremorine-M elicited a full agonist response but compound **2** was less effective (13).

Reduction of the triple bond of oxotremorine-M (compound **3**) to give the fully reduced molecule (compound **9**) led to a decrease in agonist activity and affinity (Tables 1 and 2). It has been proposed that this region of high electron density interacts with the same electrophilic site that binds to the ester oxygen of acetylcholine (21). Although no data are available regarding the effects of compound **9** on PI metabolism, the fully reduced analogue of oxotremorine was inactive when tested for its effects on guinea pig ileum and cat blood pressure (21).

Methylation of the 5′ position on the pyrrolidinone ring (compounds **7** versus **3**, **6** versus **2**, and **8** versus **4**) led to a 10–30% reduction in agonist activity. This modification also resulted in a reduction of agonist efficacy for the contractile responses in isolated guinea pig ileum (22) and the inhibition of adenylyl cyclase activity in rabbit myocardium (23). No agonist activity for compound **8** was observed for the stimulation of PI metabolism in the cerebral cortex (13).

The oxotremorine-M analogue which the pyrrolidinone ring was open (Table 1, compound **5**) showed essentially the same agonist activity as the parent compound. Similar results were found for the stimulation of PI turnover in cerebral cortex (13). These results suggest that modification of the pyrrolidinone ring of the oxotremorine-M molecule from the cyclic to the open chain form does not effect its agonist activity in either system.

Pilocarpine (compound **10**), which showed partial agonist activity (Tables 1 and 2), was subsequently used as a model compound to characterize the behavior of partial agonists in this system. The displacement of the antagonist L-[^{3}H]QNB by pilocarpine displayed a steeper curve than displacement by carbachol, but both K_H and K_L were within a factor of 6 for the two ligands (Fig. 2A). Both agonists displayed about the same fraction of high affinity sites (20%) but the ratio of K_L to K_H for carbachol was 900, whereas the ratio of the K_L to K_H for pilocarpine was only 90. In the presence of Gpp(NH)p, both agonists showed a rightward shift; however, the rightward displacement was more pronounced for carbachol (Fig. 2B). The K_d for carbachol in the presence of Gpp(NH)p was 10-fold higher than the K_d for pilocarpine under the same conditions.

The effects of the full agonist carbachol and the partial agonist pilocarpine on the mAcChR-stimulated GTPase activity of G_i were examined in the reconstituted system (Fig. 3). By reconstituting the preparation with varying amounts of mAcChR at a fixed concentration of G_i and maintaining saturating concentrations of either carbachol or pilocarpine, the dependence of the GTPase activity on agonist·mAcChR concentration could be determined. Under the experimental conditions used, the concentration of [γ-^{32}P]GTP was saturating and the background (unstimulated) GTPase activity was small (<5%), compared with the mAcChR-stimulated activity, and could be neglected. The agonist·mAcChR-stimulated GTPase activity behaved as a saturable function of agonist·mAcChR concentration and was analyzed according to a simplified kinetic model that assumed formation of the agonist·mAcChR·G_i ternary complex (see Appendix). A fit of the data in Fig. 3 to Eq. 4 permitted calculation of the values for V_{max} and the observed K_d for binding of the respective mAcChR·agonist complexes to G_i. The calculated value of V_{max} in the presence of pilocarpine, $4.36 \pm 0.54 \times 10^{-5}$ mmol/min/mg, was 1.3 ± 0.2-fold greater than that determined in the presence of carbachol, $3.45 \pm 0.19 \times 10^{-5}$ mmol/min/mg. In contrast, the observed K_d for agonist·mAcChR binding to G_i in the presence of pilocarpine, 3.02 ± 0.83 nM, was 3.7 ± 1.3-fold greater than the value determined in the presence of carbachol, 0.81 ± 0.19 nM. Thus, substitution of the full muscarinic agonist carbachol with the partial agonist pilocarpine decreased the affinity of the mAcChR·ligand complex for G_i but did not decrease the steady state GTPase activity of the G_i under conditions where the mAcChR·agonist complex was saturating.

Discussion

The studies presented above utilized a reconstituted system consisting of purified atrial mAcChR and purified atrial G_i to

TABLE 1

Muscarinic agonist activation of atrial G_i reconstituted with atrial muscarinic receptors

Liposomes were diluted to a concentration of 2.4 nM G_i and 0.4 nM mAcChR. The samples were incubated for 5 min at 32° with either 10 μM *l*-hyoscyamine, 2 mM carbachol, or 100 μM levels of the indicated muscarinic ligand. The GTPase reaction was initiated by addition of [γ^{32}P]GTP to a final concentration of 500 nM and was allowed to proceed for 1 hr at 32°. Liberated ^{32}P was measured as described in Experimental Procedures. Values are the average ± standard deviation of triplicate determinations.

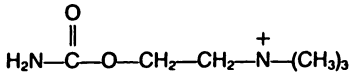
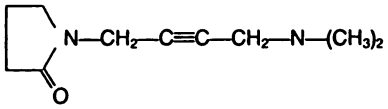
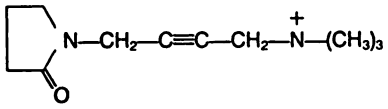
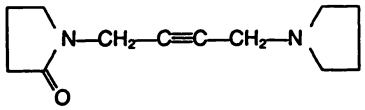
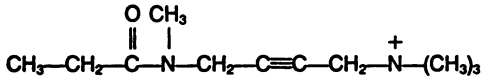
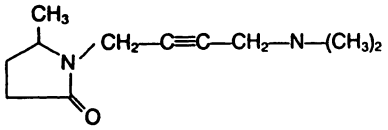
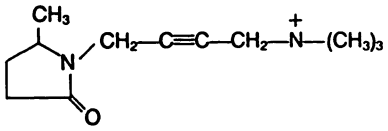
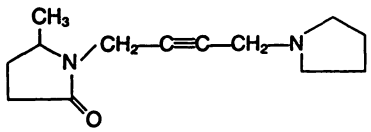
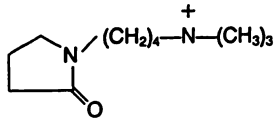
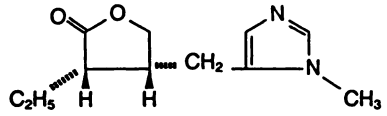
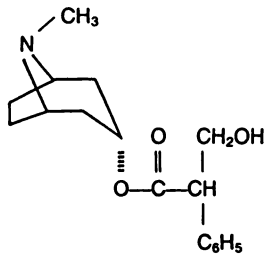
	Agonist	GTPase activity	GTPase activity relative to Carbachol
		mmol/min/mg × 10^6	%
1	Carbachol 	6.61 ± 0.28	100 ± 11
2	1-[4-(Dimethylamino)-2-butynyl]-2-pyrrolidinone 	6.67 ± 0.33	101 ± 12
3	Oxotremorine-M 	6.61 ± 0.56	100 ± 16
4	Oxotremorine 	6.56 ± 0.33	99 ± 12
5	*N,N,N*-Trimethyl-4-[methyl-(1-oxopropyl)amino]-2-butyn-1-aminium 	6.28 ± 0.44	92 ± 13
6	1-[4-(Dimethylamino)-2-butynyl]-5-methyl-2-pyrrolidinone 	6.11 ± 0.33	89 ± 12
7	*N,N,N*-Trimethyl-4-(2-methyl-5-oxo-1-pyrrolidinyl)-2-butyn-1-aminium 	5.50 ± 0.50	76 ± 14
8	5-Methyl-1-[4-(1-pyrrolidinyl)-2-butynyl]-2-pyrrolidinone 	5.11 ± 0.44	67 ± 12
9	*N,N,N*-Trimethyl-2-oxo-1-pyrrolidinebutanaminium 	5.00 ± 0.28	65 ± 10

TABLE 1
Continued

	Agonist	GTPase activity	GTPase activity relative to Carbachol
		mmol/min/mg × 10^6	%
10	Pilocarpine 	4.69 ± 0.22	58 ± 9
11	*l*-Hyoscyamine 	2.00 ± 0.28	0

[a] Calculated from 100 $\left(\frac{x-a}{y-a}\right)$, where x and y are the GTPase activities observed at 100 μM ligand and 2 mM carbachol and a is the background GTPase activity in the presence of 10 μM *l*-hyoscyamine.

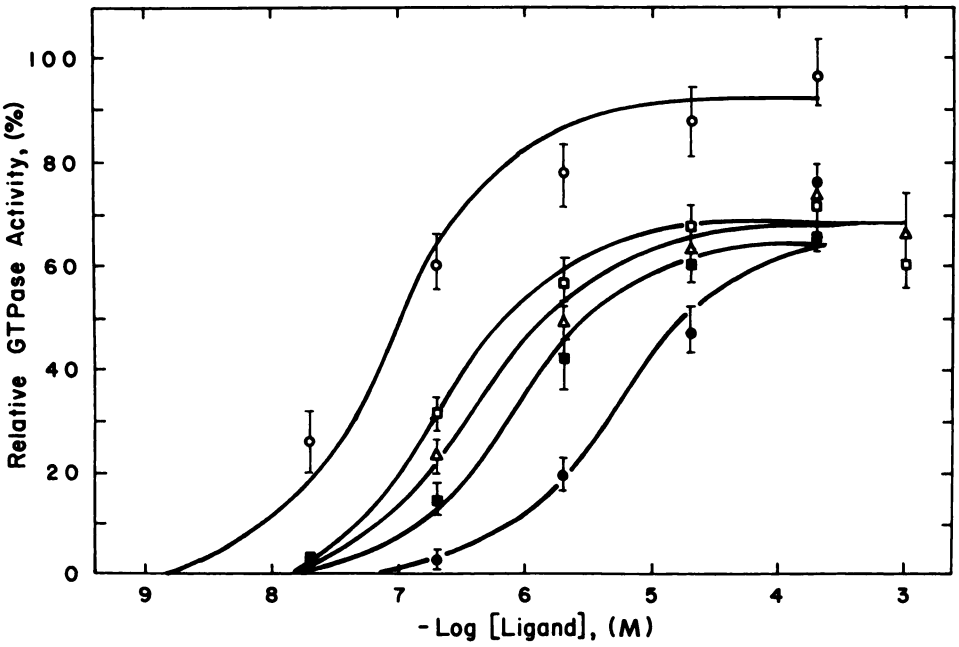


Fig. 1. Dependence of mAcChR-stimulated GTPase activity on agonist concentration. The final concentrations of reconstituted mAcChR and G_i were 0.6 and 4 nM, respectively. GTPase activity was determined as described in Table 1. Each *data point* is the average of three values and the *error bars* represent the standard deviation. The agonists used were compounds **3** (○), **7** (△), **8** (□), **9** (●), and **10** (■), where the numbers refer to the list in Table 1. The GTPase activity of G_i in the presence of 2 mM carbachol was 5.44 ± 0.21 × 10^{-6} mmol/min/mg (three experiments), whereas the GTPase activity in the absence of ligands was 1.60 ± 0.04 × 10^{-6} mmol/min/mg (five experiments). Data were fit to Eq. 1 in the text and a summary of the calculated values for K_{app} and M is given in Table 2. The theoretical curves were calculated using the values for K_{app} and M.

examine structural and mechanistic features of partial muscarinic agonists. Initially, partial agonists were identified by a decrease in agonist-stimulated GTPase activity at saturating or near saturating ligand concentrations, compared with the full agonist carbachol (Table 1). Using this criterion, pilocarpine (Table 1, compound **10**) and oxotremorine analogues **7–9** were identified as partial agonists, whereas oxotremorine (Table 1, compound **4**) and oxotremorine analogues **2, 3, 5,** and **6** appeared to elicit full agonist activity. Measurement of the fractional stimulation of the GTPase activity of G_i as a function of ligand concentration for compounds **3** and **7–10** (Fig. 1, Table 2) permitted calculation of their K_{app} values as well as a more accurate determination of their maximal stimulation of the GTPase activity of G_i, relative to carbachol. These latter results confirmed the partial agonist assignment for these drugs and were consistent with data from human heart specimens, where carbachol and oxotremorine elicited a maximal or near maximal agonist response with respect to inhibition of adenylyl cyclase activity (14, 23) and muscle contraction, whereas pilocarpine was less effective (14).

A comparison of the agonist activities elicited by saturating concentrations of selected muscarinic ligands revealed some of

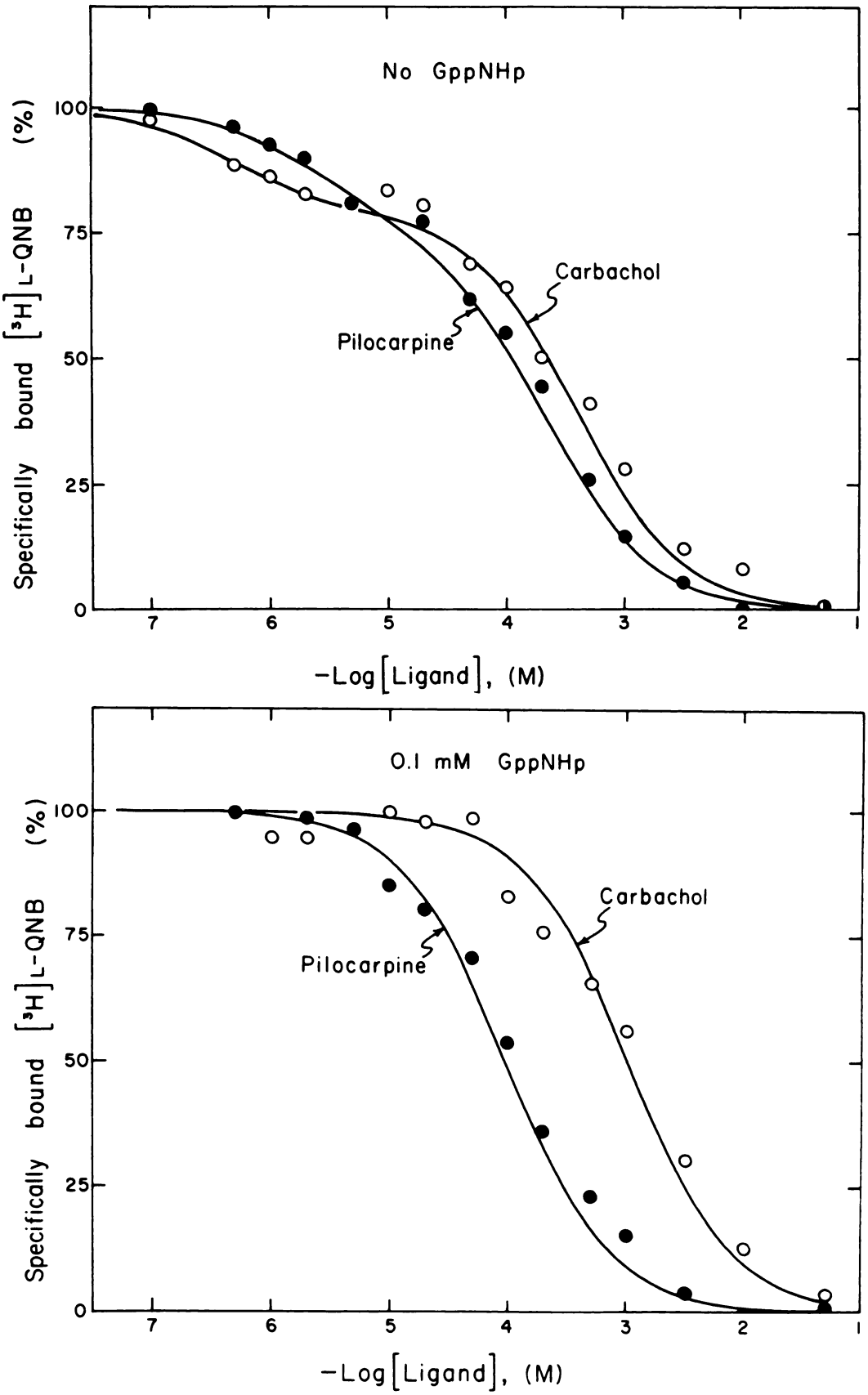


Fig. 2. Competitive binding of L-[^{3}H]QNB and carbachol or pilocarpine. The reconstituted preparation containing 49 nM L-[^{3}H]QNB binding sites and 53 nM [^{35}S]GTPγS binding sites was diluted into buffer, as described in Experimental Procedures, to give the indicated mAcChR concentration. The samples were then incubated for 30 min at 32° with the indicated concentrations of carbachol (○) or pilocarpine (●). L-[^{3}H]QNB was added to a final concentration of 650 pM and the incubation was continued for 2 hr before termination. *Data points* are the average of triplicate determinations (the standard deviations were less than or equal to 10% for each point). A, The binding data for carbachol were analyzed according to Eq. 2 in Experimental Procedures, with 76 pM L-[^{3}H]QNB binding sites and K = 264 pM. The calculated values were F_1 = 0.20 ± 0.02, K_1 = 1.51 ± 0.76 × 10^{-7} M, F_2 = 0.80 ± 0.02, and K_2 = 1.36 ± 0.15 × 10^{-4} M. The pilocarpine binding data were analyzed using 198 pM L-[^{3}H]QNB binding sites and K = 271 pM. The analysis gave F_1 = 0.24 ± 0.03, K_1 = 8.14 ± 2.37 × 10^{-7} M, F_2 = 0.76 ± 0.03, and K_2 = 7.34 ± 0.67 × 10^{-5} M. B, Binding curves in the presence of 0.1 mM Gpp(NH)p were analyzed assuming competition at a single class of sites. The competition curves for carbachol were analyzed using the concentrations of L-[^{3}H]QNB binding sites equal to 266 pM and K = 239 pM and gave a value of K_1 = 3.38 ± 0.34 × 10^{-4} M. For pilocarpine, the concentration of L-[^{3}H]QNB binding sites was 266 pM and K = 261 pM. The calculated value for K_1 = 3.40 ± 0.22 × 10^{-5} M.

TABLE 2

Summary of kinetic data for partial agonists

Values of K_{app} and percentage of maximal stimulation relative to carbachol (*M*) were calculated from the fit of the data in Fig. 1 to Eq. 1 in the text.

Compound number[a]	K_{app}	Maximal stimulation relative to carbachol
	M	%
3	$8.3 \pm 2.3 \times 10^{-6}$	91.2 ± 4.1
7	$4.4 \pm 1.2 \times 10^{-7}$	67.2 ± 3.4
8	$2.3 \pm 0.3 \times 10^{-7}$	67.8 ± 2.4
9	$5.3 \pm 1.5 \times 10^{-6}$	65.8 ± 5.6
10	$7.5 \pm 1.2 \times 10^{-7}$	64.3 ± 2.0

[a] Numbers refer to compounds listed in Table 1.

the structural requirements for distinguishing a full agonist from a partial agonist. The results (Tables 1 and 2) suggested that the electrophilicity of the triple bond was important for eliciting the full GTPase activity of G_i, whereas opening of the pyrrolidine ring did not strongly affect the agonist activity in this system or in mAcChR-mediated stimulation of PI turnover in the brain (13). Methylation of the 5′ position of the pyrrolidinone ring (compound **8**) reduced agonist activity in this study and was also shown to reduce the agonist activity of mAcChRs coupled to adenylyl cyclase inhibition in rabbit heart (23). In a previous study (13), compound **8** was shown to lack agonist activity with respect to stimulation of PI turnover in the brain.

The partial agonist pilocarpine was used in the reconstituted system in an attempt to elucidate the mechanistic difference between full and partial agonists. The curve obtained for the displacement of L-[^{3}H]QNB by pilocarpine showed approximately the same occupancy of low and high affinity sites as that found for carbachol (Fig. 2A); however, the ratio of K_L/K_H for carbachol was about 10-fold greater than for pilocarpine. The observation that the K_L/K_H ratio is larger for full agonists was first made by Birdsall and colleagues (24, 25) and agrees with the results obtained for mAcChR-initiated stimulation of PI metabolism (12, 13). These results also agree with earlier observations (12, 13) that muscarinic agonists show a more biphasic behavior in competitive binding curves as they increase in efficacy. Partial and full agonists have also been differentiated on the basis of ligand binding methodology by using a binding assay with [^{3}H]*N*-methylscopolamine and [^{3}H] oxotremorine-M (26).

Addition of 0.1 mM Gpp(NH)p converted the mAcChR into the low affinity state for both agonists (Fig. 2B), with dissociation constants that agreed with the values for K_L within a factor of 2 to 3. As a result of the larger ratio of K_L/K_H, a greater rightward displacement in response to addition of Gpp(NH)p was observed for the full agonist carbachol than for pilocarpine. Thus, the relative displacement in going from the high to the low affinity agonist state may be the predominant indicator of agonist activity. Recently, mutant β-adrenergic receptors that lacked the ability to interact with G_S were found to have a single class of agonist binding sites with an affinity that was intermediate between the high and low affinity states of the wild-type receptor (27). These results also suggest that the transition from high to low affinity binding may provide energy for the interaction of the receptor with the G protein.

The reduced agonist activity of pilocarpine could be attributed either to a decreased affinity of the mAcChR·ligand complex for G_i or to a decrease in the activity of the agonist·mAcChR·G_i complex. If the former alternative was correct, the partial agonist activity of the pilocarpine·mAcChR·G_i complex should be overcome by an increase in the amount of agonist-bound receptor. The dependence of the GTPase activity of G_i on mAcChR concentrations was determined under conditions where both [γ-^{32}P]GTP and agonist concentrations were saturating (Fig. 3). The value calculated for V_{max} of the GTPase activity of G_i at saturating mAcChR·agonist levels was 1.3-fold higher for pilocarpine than for carbachol (1.2 ± 0.1; average of two experiments), suggesting that the GTPase activity of G_i was not differentially affected by the partial versus full agonist. The turnover number, k_{cat} (equal to $V_{max}/[G_i]_{total}$), derived from the data in Fig. 3 was 4.3 min^{-1} in the presence of a saturating concentration of carbachol and 5.4 min^{-1} in the presence of a saturating concentration of pilocarpine. A previous determination of k_{cat} for the GTPase activity of G_i in this system in the presence of saturating carbachol gave a value of 2.1 min^{-1}

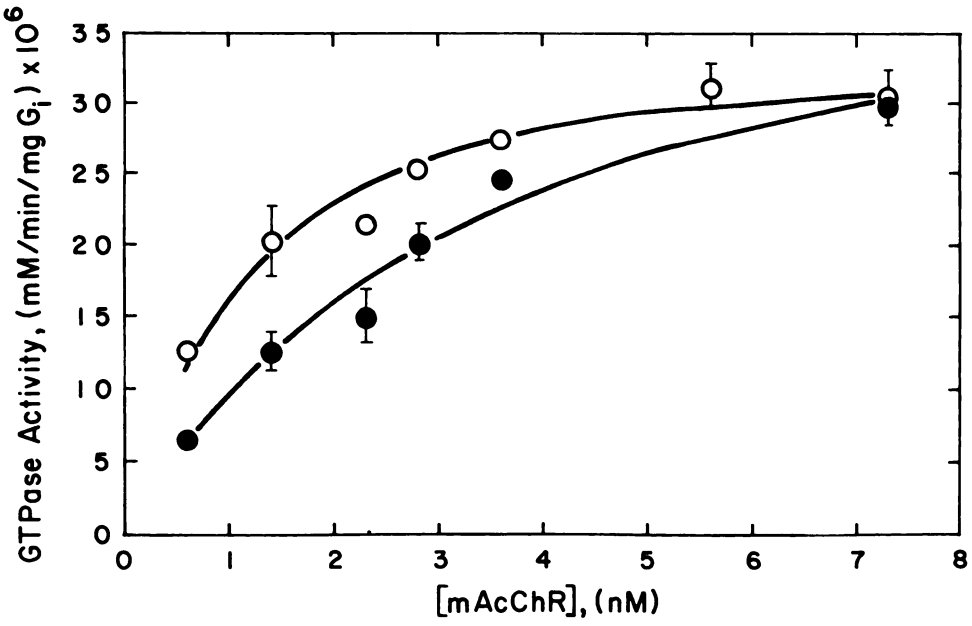


Fig. 3. Dependence of the agonist-stimulated GTPase of G_i on mAcChR concentration. Varying amounts of mAcChR (2–30 pmol) were reconstituted with a constant amount of G_i (5 pmol). The recovery of G_i was about 5 ± 1%. The lower recovery of G_i compared with previous work [30% (3)] may be the result of the suboptimum G protein concentration used for reconstitution. The reconstituted preparations were then diluted to give an average G_i concentration of 0.57 ± 0.11 nM and the indicated concentration of mAcChR. GTPase assays were then performed as described in Experimental Procedures, in the presence of 2 mM carbachol (○) or 2 mM pilocarpine (●). The data were fit according to Eq. 4 in Experimental Procedures, where $[G]_o$ equalled 0.57 nM. The carbachol data gave the fitted parameters of V_{max} = 3.45 ± 0.19 × 10^{-5} mmol/min/mg and an observed K_d equal to 0.81 ± 0.19 nM, whereas the pilocarpine data gave a V_{max} equal to 4.36 ± 0.54 × 10^{-5} mmol/min/mg and an observed K_d of 3.02 ± 0.83 nM.

(3). The value determined in the present study is probably more accurate, because it was derived by extrapolation of the mAcChR·carbachol complex to an infinitely high concentration, whereas the previous value was estimated from a fit to a two-component Michaelis-Menton plot and an estimation of the fraction of G_i that was coupled to the mAcChR in the steady state. The value calculated from the data in Fig. 3 should be considered a lower limit of k_{cat}, however, because the assumption inherent in our analysis is that all of the available G_i detected by the binding of [^{35}S]GTPγS can effectively couple to mAcChRs in the reconstituted system.

The most significant difference between pilocarpine and carbachol was the value of the observed dissociation constant of the mAcChR·ligand complex for G_i. Because the interactions between the reconstituted proteins may be taking place within a two-dimensional lipid system, rather than in solution, the observed dissociation constant may not represent a true dissociation constant. However, the ratio between two observed dissociation constants should still be valid for determining relative affinities. The affinity of the mAcChR for G_i was 3- to 4-fold lower (3.4 ± 0.4; average of two experiments) at saturating concentrations of the partial agonist than in the presence of saturating concentrations of carbachol. These results suggest that, in this system, pilocarpine and carbachol induce different conformations of the mAcChR such that the pilocarpine·mAcChR complex binds more weakly to G_i, resulting in a lower steady state level of activated G_i. At this time, it is not known whether this result is a general one, applying to all partial agonists, or is unique for pilocarpine. Although the reconstituted system described above shows several features of mAcChR interactions with G_i observed *in vivo*, such as guanine nucleotide-sensitive high affinity agonist binding, muscarinic agonist-stimulated GTPase activity of G_i, and uncoupling of the mAcChR from G_i by pertussis toxin treatment (3), the observed turnover number for G_i is 30- to 50-fold slower than that observed in electrophysiological measurements (28). Therefore, it is important that the proposed mechanism for the action of partial agonist(s) be validated with an *in vivo* experimental system.

In summary, a reconstituted system containing the purified porcine atrial M2 mAcChR and purified atrial G_i was used to examine the mechanism of partial agonist action and to conduct structure-function studies using a series of oxotremorine analogues. The use of an experimental system containing a defined mAcChR subtype and G protein permitted the correlation of structural modifications of the oxotremorine molecule with a specific mAcChR-G protein interaction. Experiments with pilocarpine and carbachol showed that the difference between the full and partial agonist activities of these two drugs in the reconstituted system was the reduction in affinity of the mAcChR·partial agonist complex for G_i and not the relative abilities of the agonist·mAcChR complex to activate G_i, as measured by the stimulation of GTPase activity. The lower affinity results in a decrease in the steady state level of active G_i and, under conditions where the agonist·mAcChR complex is not saturating, would decrease the fraction of G_i available to bind GTP, resulting in a decrease in the observed GTPase activity. The methodology described above may also prove useful in analyzing the interactions of drugs like oxotremorine with different muscarinic subtypes and G proteins, as well as in structure-function studies with mutant mAcChRs.

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Appendix: Dependence of the Observed Steady State GTPase Activity of G_i on the Concentration of Agonist·mAcChR Complex

The experimental observation (Fig. 3) that the agonist·mAcChR-stimulated GTPase activity of G_i behaved as a saturable function of agonist·mAcChR complex concentration suggested that the steady state GTPase activity could be analyzed in terms of a simplified model in which the agonist·mAcChR complex (RA) formed a ternary complex with G_i (G), promoting exchange of GDP for GTP, followed by hydrolysis of GTP to GDP and a return to the initial state.

$$RA + G \underset{}{\overset{K_d}{\rightleftharpoons}} RAG \xrightarrow{k_{cat}} RA + G \qquad \text{GTP} \quad \text{GDP} + P_i \tag{1}$$

From the law of conservation of mass

$$[RA]_o = [RA] + [RAG] \tag{2}$$

$$[G]_o = [G] + [RAG] \tag{3}$$

where $[RA]_o$ and $[G]_o$ are the total concentrations of agonist·receptor complex and G_i, respectively. The steady state GTPase activity (v) for the model in Eq. 1 is given by

$$v = k_{cat}[RAG] = k_{cat}[G]_o\left(\frac{[RAG]}{[G]_o}\right) \tag{4}$$

The dissociation constant for agonist·mAcChR binding to G_i, K_d, and the maximal steady state GTPase activity, V_{max}, are defined as follows:

$$K_d = \frac{[RA][G]}{[RAG]} = \frac{([RA]_o - [RAG])([G]_o - [RAG])}{[RAG]} \tag{5}$$

$$V_{max} = k_{cat}[G]_o \tag{6}$$

Substitution of Eq. 6 into Eq. 4 gives the relationship between the experimentally observed steady state GTPase activity (v), V_{max}, $[RAG]$, and $[G]_o$.

$$v = V_{max}\left(\frac{[RAG]}{[G]_o}\right) \tag{7}$$

Expanding and rearranging Eq. 5 gives

$$[RAG]^2 - [RAG]([RA]_o + [G]_o + K_d) + [RA]_o[G]_o = 0 \tag{8}$$

which can then be solved for $[RAG]$ using the quadratic formula to give

$$[RAG] = \frac{y - \sqrt{y^2 - 4x}}{2} \tag{9}$$

when $y = ([RA]_o + [G]_o + K_d)$ and $x = [RA]_o[G]_o$. Substitution for $[RAG]$ from Eq. 9 into Eq. 7 permits analysis of the data in Fig. 3, using the experimental concentrations of $[RA]_o$ and $[G]_o$ and the experimentally determined value of v to solve for V_{max} and K_d.

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