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Tissue-Dependent Association of Muscarinic Acetylcholine Receptors with Guanine Nucleotide-Binding Regulatory Proteins

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SUMMARY

The muscarinic acetylcholine receptors in heart and cerebellum form a stable association with guanine nucleotide-binding regulatory proteins (G proteins) in the presence of receptor agonists. This has been confirmed by purification of the muscarinic receptor-G protein complexes using an immunoprecipitation protocol. The isolated complexes were subjected to Western blotting to identify the G protein subunits present in the complexes. At saturating concentrations of carbachol, the muscarinic receptors in atrial membranes co-purified exclusively with G_o, whereas in cerebellar and ventricular membranes an association with both G_i and G_o was demonstrated. Further characterization of the G protein subunits allowed identification of the species of $G_{i\alpha}$ subunits present in the complexes of muscarinic receptor and G protein; in ventricle $G_{i\alpha 2}$ was the only subtype present, whereas in cerebellum both $G_{i\alpha 1}$ and $G_{i\alpha 2}$ were present. These results demonstrate that a single muscarinic receptor subtype, depending on the tissue studied, is capable of interacting with more than one G protein subtype. The concentrations of agonist required to promote receptor-G protein association in atrial and ventricular membranes correlated with the high affinity component of receptor occupancy by agonist, as measured in equilibrium binding assays. Furthermore, incubation of cardiac membranes with saturating concentrations of pilocarpine or McN A343 resulted in reduced amounts of receptor-G protein complexes, compared with carbachol. Overall, our results suggest that the specificity of cellular effects of muscarinic agonists may relate, in part, to the selective interaction of receptor with G proteins.

A variety of cellular responses are known to be mediated by acetylcholine and other mAChR agonists. These include inhibition of adenylyl cyclase, activation of phospholipase C, and activation or inhibition of ion channels (1–4). These mAChR-mediated responses are thought to occur after an initial interaction of the receptor with one of a family of G proteins (5–9). Included in the family of G proteins are structurally related members that can be grouped into G_s, G_i, G_o, transducin, and possibly other subfamilies, each containing multiple members. Expression of cDNA clones coding for mAChR subtypes (10–12) in clonal cell lines has allowed an evaluation of the ability of each receptor subtype to mediate cellular responses to mAChR agonists (2, 13, 14). The m_1 and m_3 [nomenclature of Bonner *et al.* (10)] subtypes efficiently promote polyphosphoinositide hydrolysis and less efficiently inhibit adenylyl cyclase. The m_2 and m_4 receptor subtypes less efficiently promote polyphosphoinositide hydrolysis and fully promote inhibition of adenylyl cyclase in the same cell lines. These studies suggest that there is some specificity in the ability of each receptor subtype to interact with cellular effector systems. It has been proposed that this specificity may be due to selective interaction of the receptor subtypes with G proteins mediating each response.

It was the purpose of the current study to characterize interactions of mAChRs with G proteins, to identify possible selective interactions of the receptors with various G proteins in different tissues. Our previous studies (15) have now been extended to examine in more detail the molecular components of the m_2 mAChR-G protein complexes in atrial, ventricular, and cerebellar tissues. The relative abilities of partial agonists to promote mAChR-G protein interactions in cardiac membranes, compared with carbachol, were also studied (16).

Experimental Procedures

Materials

All electrophoresis and blotting chemicals and Affigel-10 were obtained from Bio-Rad. Nitrocellulose was from Shleicher and Schuell.

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ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; G protein, guanine nucleotide-binding regulatory protein; Gpp(NH)p, 5′-guanylyl imidodiphosphate; m_1–m_4, muscarinic receptor subtypes identified by molecular cloning; McN A343, (4-hydroxy-2-butynyl)trimethylammonium chloride; NMS, *N*-methylscopolamine; oxo-M, oxotremorine M; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide.

Digitonin was obtained from Gallard-Schlesinger. Rat hearts and brains were obtained from Pel-Freeze. Carbamylcholine (carbachol), *N*-acetylglucosamine, goat anti-mouse IgG1-agarose, sodium cholate, and atropine were from Sigma. Biotinylated goat anti-rabbit antibodies, avidin-alkaline phosphatase, and avidin-horseradish peroxidase were from Vector Laboratories; wheat germ agglutinin was also obtained from Vector and was coupled to Affigel-10 at 10 mg/ml of resin. [^{3}H]NMS was purchased from Amersham and [^{3}H]oxo-M from DuPont/NEN. The anticardiac mAChR antibody 31-1D1 was a gift from Dr. Neil Nathanson (University of Washington, Seattle, WA).

Methods

Tissue preparation. All operations were performed at 4°. Hearts were thawed, minced, and homogenized, at 5 ml/g of tissue, in 10 mM Tris (pH 7.5), 1 mM EDTA, using a Biospec homogenizer (setting, high; 8–10 sec). Cerebella were suspended and homogenized (setting, low; 10 sec) in the same buffer at 5 ml/cerebellum. In most experiments shown, tissue homogenates were incubated for 15 min at 4° in buffer containing 0.1 mM GTP and 3 mM $MgCl_2$, to release any bound endogenous agonist, and then centrifuged at 20,000 × *g* for 15 min after addition of 3 mM EDTA. Membranes were resuspended in Tris/EDTA buffer and used immediately or stored frozen at −80° until use.

Solubilization of receptors. Membrane suspensions were placed on ice, and 1.0 M $MgCl_2$ was added to give a final concentration of 3.0 mM. Drugs were added at the indicated concentrations, and samples were incubated on ice for 30 min. Membranes were collected by centrifugation and resuspended in the original volumes of 10 mM Tris (pH 7.5), 1 mM EDTA, 0.1% digitonin, 0.02% cholate (TEDC buffer). The membranes were immediately centrifuged at 30,000 × *g* for 15 min, and the pellets thus obtained were resuspended in the original sample volume of TEDC supplemented to 1% digitonin and 0.2% cholate. After a 30-min incubation on ice, the soluble proteins were obtained after a 40,000 × *g* centrifugation. Previous studies established that centrifugation steps performed at 150,000 × *g* did not precipitate agonist- or antagonist-labeled mAChRs from this solublized preparation (15). Chromatography of solubilized receptors and immunoprecipitation using 31-1D1 antibody (17) were performed as previously described (15). This antibody immunoprecipitates receptors solubilized from cells expressing the m_2 but not other mAChR clonal subtypes.[2]

SDS-PAGE and Western blotting. For high resolution of G_α subunits, samples were treated with NEM (18) and subjected to SDS-PAGE on 0.75-mm-thick 10% polyacrylamide gels containing half the normal bis concentration (19). These gels allow resolution of 39-, 40-, and 41-kDa proteins representing the α subunits of G_o, G_{i2}, and $G_{i1\&3}$, respectively. In some experiments where resolution of G_α subunits was not required, proteins were separated on 12% acrylamide minigels without NEM treatment. Electrophoresed samples were transferred to nitrocellulose and incubated with antibody (1/100 final dilution of antiserum in all cases) as described (20, 21). Color development was achieved using biotinylated goat anti-rabbit antibody in conjunction with avidin-horseradish peroxidase (both at 1/1000 dilution) and 4-chloro-1-napthol/H_2O_2 as chromagen. In some cases, incubations with different primary antibodies were performed sequentially. After incubation and color development with the first primary antibody, photographs of the blot were taken immediately. The blot was then equilibrated to 3% gelatin and subsequently incubated with a second primary antiserum, as described above. After the second (or third) incubation and color development, photographs of the blots were aligned to allow identification of the sequence of immunoreactive band appearance. This allowed assignment of G protein band identity based on both molecular weight and predicted antibody specificity.

Rabbit antisera to G proteins have been described elsewhere (20–23). Antibodies to G_α (antiserum 1398) were raised against the peptide CGAGESGKSTIVKQMK conjugated to keyhole limpet hemocyanin and have the predicted specificity for $G_{i\alpha1\text{-}3}$ and $G_{o\alpha}$ as described (21, 22) (Fig. 2). Antibodies that recognize $G_{o\alpha}$ preferentially were raised against the synthetic peptide EYGDKERKADSK (antiserum 2918) (Fig. 2). Antibodies that recognize $G_{i\alpha}$ subunits were raised to the peptide KNNLLDCGLF (antiserum 8730) (Fig. 2); another antibody recognizing $G_{i\alpha2}$ was generated to the peptide LERIAQSDY (antiserum 1521) (Fig. 2). An antiserum specific for G_β subunits (antiserum 5357; $G_{\beta36} > G_{\beta35}$) has been previously characterized (20).

Ligand binding assay. Radiolabeled NMS (2 nM) or oxo-M (10 nM) and unlabeled drugs were incubated with atrial or ventricular membranes for 3 hr at 4° (assay volume, 0.5 ml), as described (15). Reactions were terminated by vacuum filtration (24).

Densitometry. Immunoblots were scanned on a Shimadzu CS-930 densitometer in reflectance absorbance mode at 600 nm, and relative densities were measured by comparing integrated peak areas. A standard curve using a partially purified G protein standard indicated that the densities measured were within a linear range on the immunoblots. Duplicate samples from individual experiments were averaged before background subtraction and mean calculations.

Results

We recently described isolation of m_2 mAChR-G protein complexes formed in the presence of mAChR agonists (15). The mAChR-G protein complexes were purified from cardiac membranes by a combined wheat germ agglutinin affinity/immunoprecipitation protocol using cardiac-selective monoclonal antibodies. The conditions employed do not alter the relative levels of mAChRs existing in the high affinity state, based on [^{3}H]oxo-M and [^{3}H]NMS binding (15) (data not shown). G proteins that co-purified with mAChRs were visualized by immunoblotting with anti-G protein antibodies after SDS-PAGE, as shown in Fig. 1. Fig. 1, *lane 1*, shows G proteins that co-purified with mAChRs upon exposure of membranes to 1 mM carbachol; Fig. 1, *lane 2*, shows an absence of such G protein immunoreactivity when mAChRs were purified from membranes exposed to the antagonist atropine. G proteins also did not co-purify with mAChRs when GTP (1 mM) or Gpp(NH)p (100 μM) was present during incubation with agonist (15). These results demonstrate a physical interaction between mAChR and G protein, which is reversed by GTP, and

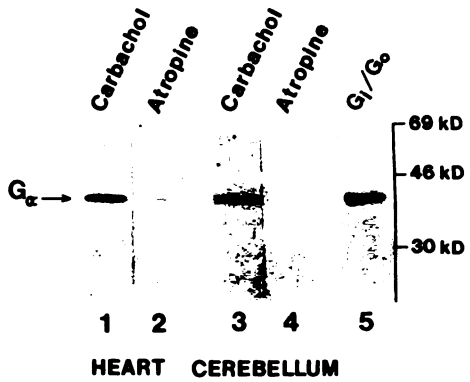

Fig. 1. Co-purification of mAChRs and G_α subunits from heart and cerebellum. Membranes were incubated with 1 mM carbachol or 1 μM atropine, solubilized, and processed through chromatography and immunoprecipitation with an anti-mAChR antibody, as described in the text. Samples were subjected to SDS-PAGE and Western blotting using 1398 antibody to visualize co-precipitated G_α subunits. This experiment was performed using a minigel; resolution of proteins in the 39–41-kDa range was not attempted. *Lanes 1* and *2*, G_α subunits that co-precipitated with mAChRs from heart membranes incubated with carbachol or atropine; *lanes 3* and *4*, G_α subunits isolated similarly from cerebellar membranes; *lane 5*, immunoreactivity of a partially purified G protein fraction from rat brain containing G_i and G_o.

[2] B. B. Wolfe, personal communication.

support the hypothesis that an interaction of mAChRs with G protein(s) supports high affinity agonist binding. A full description of this effect has been described in detail (15). The mAChR-G protein complexes were also isolated from cerebellar membranes exposed to the agonist carbachol but not atropine (Fig. 1, *lanes 3* and *4*).

A partially purified preparation of G protein from rat brain (18) was used to demonstrate our ability to resolve by molecular weight and to identify immunologically various members of the G protein family. As shown in Fig. 2A (*lane 4*), the use of low bis-containing gels allows resolution of 39-, 40-, and 41-kDa bands of protein that are identified as G_α subunits using 1398 (anti-G_α) antiserum. G proteins that co-purified with agonist-labeled mAChRs from atrium, ventricle, and cerebellum were similarly resolved and identified on Western blots using 1398 antibodies. With atrial receptors, a single major immunoreactive G_α band was observed in the 39-kDa region of the gel (Fig. 2A, *lane 1*). With ventricular and cerebellar receptors, additional immunoreactive bands in the 40-kDa (ventricle) and 40/41-kDa (cerebellum) region of the gel were also observed (Fig. 2A, *lanes 2* and *3*). These initial results suggested possible interactions of the activated mAChRs in atrium with $G_{o\alpha}$ and in ventricle and cerebellum with both $G_{o\alpha}$ and $G_{i\alpha}$ subunits. As reported in a previous study, G_β subunits were undetectable on the Western blots (15).

Fig. 2B shows the composition of the total G_α subunit pool present in atrial, ventricular, and cerebellar tissues. In both atrium and ventricle, this composition differs from that of G proteins that co-purify with mAChRs after treatment with agonist (Fig. 2A). Specifically, G_i is present in atrial membranes in approximately equivalent amounts as G_o (Fig. 2B, *lane 1*), whereas G_o exclusively co-purifies with mAChRs treated with carbachol (Fig. 2A, *lane 1*). In ventricular membranes, G_o is present in levels far below those of G_i (Fig. 2B, *lane 2*), whereas agonist-induced mAChR-G protein complexes contain G_i and G_o in approximately equivalent amounts (Fig. 2A, *lane 2*). Thus, there appears to be an ability of cardiac mAChRs to interact with G_o in both tissues, suggesting that the specificity of the mAChR interaction with G protein is not governed exclusively by the relative abundance of the G_α protein subunit(s) present within the tissue. In cerebellar membranes, $G_{o\alpha}$ is the predominant subtype present in isolated mAChR-G protein complexes, as in atrial membranes. In this tissue, G_o is also the predominant subtype present in the total G protein pool (compare Fig. 2A, *lane 3*, with Fig. 2B, *lane 3*).

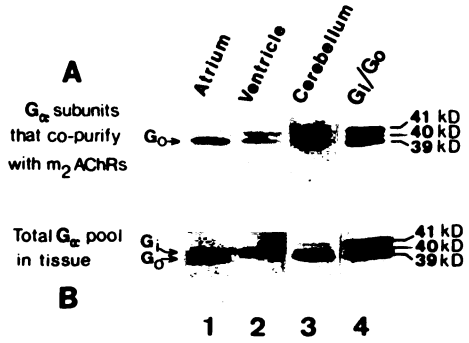

Fig. 2. A, Resolution of G_α subunits present in isolated mAChR-G protein complexes by molecular weight. NEM-treated samples of mAChR-G protein complexes isolated from carbachol-treated atrial (*lane 1*), ventricular (*lane 2*), or cerebellar (*lane 3*) membranes were subjected to SDS-PAGE on low-bis gels, followed by Western blotting using 1398 antibodies. The number of receptors immunoprecipitated from each tissue was measured in initial experiments using [^{3}H]oxo-M, and approximately equivalent amounts (5 pmol of estimated agonist binding) were used in each gel lane. This corresponds to the receptors solubilized from six atria, three ventricles, and three cerebella for the gel lanes shown. *Lane 4*, resolution of G_α subunits present in the partially purified rat brain G protein fraction into 39-, 40-, and 41-kDa bands. B, Resolution of G_α subunits present in atrial (*lane 1*), ventricular (*lane 2*), or cerebellar (*lane 3*) membranes. Membranes were solubilized in 1% SDS, 10 mM Tris, 1 mM EDTA, for 15 min, at 5 ml/g of tissue, and were centrifuged at 120,00 × *g* for 40 min to remove insoluble material. Supernatants were treated with NEM and separated on low-bis gels, as described for A. *Lane 4*, resolution of G_2 subunits present in the partially purified rat brain G protein fraction.

In addition to resolution of the G protein subunits by apparent molecular weight on SDS-PAGE, the identities of G_α subunits were further characterized by their reactivity with antibodies to specific sequence determinants on the G proteins. The specificities shown in Table 1 have been previously determined for these antibodies (21, 23). Sequential incubation of a single immunoblot with the different antibodies allowed correlation of G_α subunit molecular weight with immunoreactivity with each antibody. Thus, using partially purified G_i/G_o from rat brain, the results shown in Fig. 3 were obtained after sequential probing and development with 1521 ($G_{i\alpha2}$ specific), 2918 ($G_{o\alpha}$ specific), and 8730 ($G_{i\alpha1,2,\&3}$ specific) antibodies. A single band appeared at 40 kDa (Fig. 3, *lane 1*) upon incubation with 1521 antiserum, consistent with published reports of the molecular weight of $G_{i\alpha2}$ (19). A second immunoreactive band appeared at 39 kDa (Fig. 3, *lane 2*) upon incubation of the same blot with 2918 antiserum, consistent with the expected molecular weight and immunoreactivity of $G_{o\alpha}$. An additional band was detectable at 41 kDa (Fig. 3, *lane 3*) upon subsequent incubation with 8730 antiserum, consistent with the reported molecular weight of $G_{i\alpha1}$ or $G_{i\alpha3}$ (18, 19). The bands are shown in comparison with the same standard proteins developed by incubation with 1398 (G_α) antibodies (Fig. 3, *lane 4*). Antibodies specific for G_β (5357) were also present in the sample in Fig. 3, *lane 4*. Using this sequential immunoblot approach, the subunit identities of G proteins that co-purified with agonist-bound mAChRs in heart and cerebellum were assigned. These data are summarized in Table 2. For atrial receptors, a single immunoreactive G_α band was found at 39 kDa, which could be identified using 1398 and 2918 antisera but not 1521 or 8730 antisera. This protein is, thus, likely to be $G_{o\alpha}$. The same band was identified in mAChR-G protein complexes isolated from ventricular and cerebellar membranes. Thus, in all three tissues, a stable complex of mAChR and $G_{o\alpha}$ could be identified. In addition to $G_{o\alpha}$, an immunoreactive G_α band at 40 kDa was identified with purified ventricular mAChRs (Fig. 2A, *lane 2*). This band was reactive with 1398, 1521, and 8730 antibodies but not 2918 antibodies and, thus, likely represents $G_{i\alpha2}$. This protein band was also found in mAChR-G protein complexes isolated from cerebellar membranes. An additional immunoreactive G_α band was found with isolated cerebellar receptors,

TABLE 1
Specificities of antisera for G_α subunits

Antiserum	G_α subunit specificity
1398	$G_{i\alpha}$, $G_{o\alpha}$
2918	$G_{o\alpha}$
8730	$G_{i\alpha1}$, $G_{i\alpha2} > G_{i\alpha3}$
1521	$G_{i\alpha2} > G_{z\alpha}$

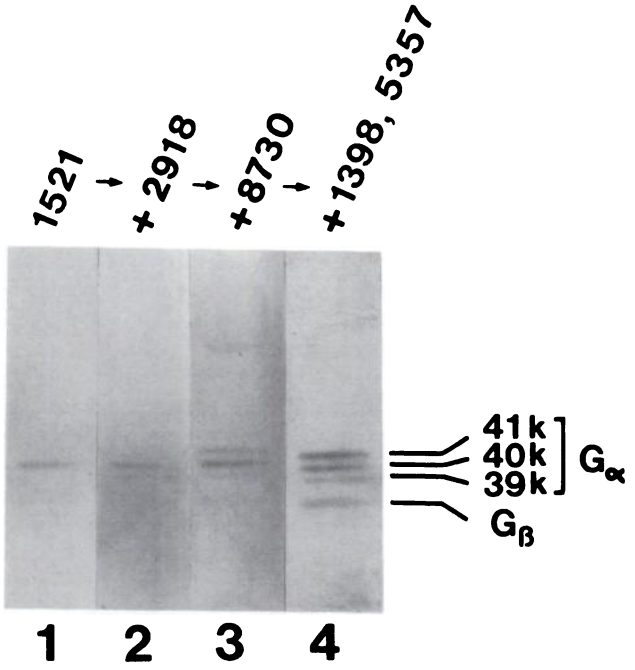

Fig. 3. Resolution of G_α subunits by molecular weight and identification by sequential antiserum incubations. NEM-treated samples of rat forebrain G_i/G_o were separated on low-bis SDS gels, followed by Western blotting. *Lanes 1*, *2*, and *3*, results of sequential incubations of the resolved G_α subunits with 1521, 2918, and 8730 antisera, respectively. *Lane 4*, same sample subsequently incubated with 1398 antiserum and Gβ-specific 5357 antiserum. Photographs were taken after incubation with each antibody and color development, thus showing the sequential appearance of immunoreactive bands.

TABLE 2

Immunoreactivity of G_α subunits present in mAChR-G protein complexes

G_α subunits were resolved on SDS 10% acrylamide gels containing a low bis content and were transferred to nitrocellulose. +, Detectable immunoreactivity of G_α subunit(s) present in mAChR-G protein complexes isolated from carbachol-treated tissue; −, no detectable immunoreactivity above background, where background is defined as G_α immunoreactivity present in mAChR-G protein complexes isolated from atropine-treated tissue.

Tissue	Immunoreactivity			
	1398 (G_α)	2918 ($G_{o\alpha}$)	8730 ($G_{i\alpha}$)	1521 ($G_{i\alpha 2}$)
Atrium	+	+	−	−
Ventricle	+	+	+	+
Cerebellum	+	+	++[a]	ND[b]

[a] ++, two bands detected.
[b] ND, not determined.

which had a molecular mass of 41 kDa and was reactive with 1398 and 8730 antibodies but not 2918 or 1521 antibodies. This band likely represents $G_{i\alpha 1}$ or $G_{i\alpha 3}$. These results support the concept of interaction of the atrial mAChRs with a single member of the G protein family, G_o, and of ventricular and cerebellar mAChRs with both G_i- and G_o-like proteins.

We wished to establish the relationship between relative receptor occupancy and formation of mAChR-G protein complexes. It is predicted by current models that the high affinity component of agonist binding is that which reflects receptor-G protein complex formation. The fact that continual agonist occupancy is not required to stabilize mAChR-G protein complexes was established in a prior study (15). To establish the concentration-response curve for mAChR-G protein complex formation in heart, membranes were incubated with low (2–10 nM) and high (1 mM) concentrations of carbachol and then receptors and their associated G proteins were isolated as described above. Low (2 and 10 nM) concentrations of agonist supported mAChR-G protein interaction in both atrial (data not shown) and ventricular membranes (Fig. 4), and the interaction appeared maximal at ~100 nM. Thus, G_α immunoreactivity was similar in blots taken from tissue exposed to 100 nM (data not shown) or 1 mM carbachol (Fig. 4). Using the amount of G protein present at 1 mM to reflect maximal formation of mAChR-G protein complexes, we estimated the agonist concentration required for half-maximal effect from densitometric scans of G_α-immunoreactive bands. In both atrial and ventricular membranes, this value was ~10 nM. In five experiments using cardiac membranes, 10 nM carbachol induced formation of 50.8 ± 8.7% of the maximal number of mAChR-G protein complexes induced by 1 mM carbachol, whereas 2 nM carbachol induced 33.2 ± 14.3% of the maximal number.

The dose of carbachol required to promote maximal mAChR-G protein interactions (100 nM) was similar to the dose of carbachol required to fully saturate the high affinity receptor binding sites in cardiac membranes, based on [^{3}H]NMS competition binding curves (Fig. 5A). A concentration-response curve for the highest affinity component of carbachol binding to cardiac mAChRs in the absence of GTP was also obtained using competition for 2 nM [^{3}H]oxo-M, which binds exclusively to the high affinity state of mAChRs at this concentration (25). These results (not shown) showed that oxo-M binding was completely inhibited at 100 nM carbachol. Because higher concentrations of carbachol (1 mM) did not promote formation of additional mAChR-G protein complexes, compared with 100 nM carbachol, these data, taken together, directly demonstrate that the high affinity component of agonist binding, but not the low affinity component, reflects those receptors that are associated with G protein(s).

The relative abilities of the muscarinic agonists pilocarpine and McN A343 to support cardiac mAChR-G protein interactions were compared with that of the agonist carbachol. First, dose-response curves for inhibition of [^{3}H]NMS binding to cardiac membranes in the absence or presence of GTP were generated for each ligand (Fig. 5). These data suggest that the number of high affinity binding sites in the absence of GTP is

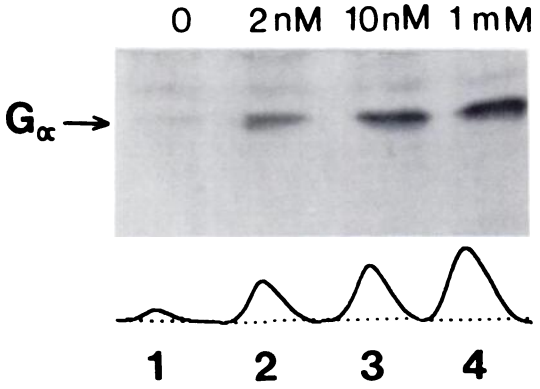

Fig. 4. Dose response for formation of complexes of mAChRs and G proteins by carbachol. Ventricular membranes were incubated with the indicated concentrations of carbachol, solubilized, and immunoprecipitated with mAChR-specific antibodies, as described in the text. Co-precipitated G_α subunits were visualized on Western blots of minigels using 1398 antibodies. Densitometric scan traces (*bottom*) show relative peak areas of immunoreactive bands.

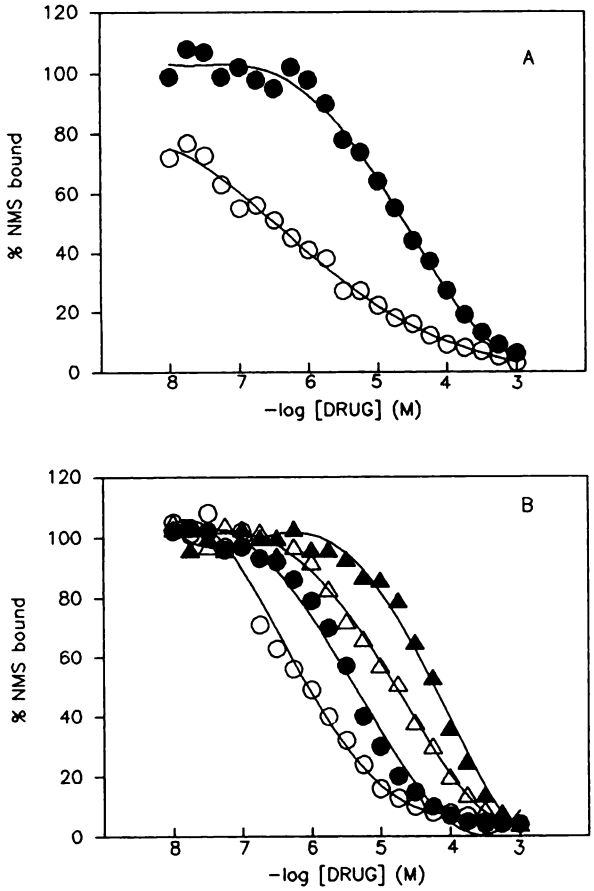

Fig. 5. A, Dose response for carbachol inhibition of [^{3}H]NMS binding to cardiac membranes in the presence (●) or absence (○) of 100 μM GTP. Values are the average of three determinations at each concentration. Concentration of NMS was 2 nM. B, Dose response for pilocarpine (*circles*) or McN A343 (*triangles*) to inhibit [^{3}H]NMS binding to cardiac membranes in the presence (*closed symbols*) or absence (*open symbols*) of GTP. Values are the average of two determinations at each concentration. These results have been replicated in at least three independent experiments.

similar for all three ligands, despite differences in their apparent affinities. In the presence of GTP, the curves are displaced to the right, reflecting only the low affinity component of binding. These data suggest that, at 1 mM concentration and in the absence of GTP, all three ligands promote full occupancy of mAChRs. Next, the relative numbers of mAChR-G protein complexes isolated from cardiac membranes incubated with 1 mM carbachol, pilocarpine, or McN A343 were compared on immunoblots (Fig. 6). These results show reduced levels of G_α subunits that co-purified with pilocarpine- or McN A343-treated mAChRs, compared with carbachol-treated mAChRs. Densitometric scans of these and other immunoblots indicated that the number of mAChR-G protein complexes isolated from pilocarpine- or McN A343-treated membranes was 59.7 ± 10.5% and 50.2 ± 20.1%, respectively, of the number isolated from carbachol-treated membranes (six experiments). These reduced levels of G protein complexed with pilocarpine- or McN A343-activated mAChRs were detected even when the ligands were maintained at saturating concentrations throughout the entire mAChR isolation procedure (data not shown). However, the binding data in Fig. 5 show that similar numbers of high affinity binding sites are formed with all three ligands

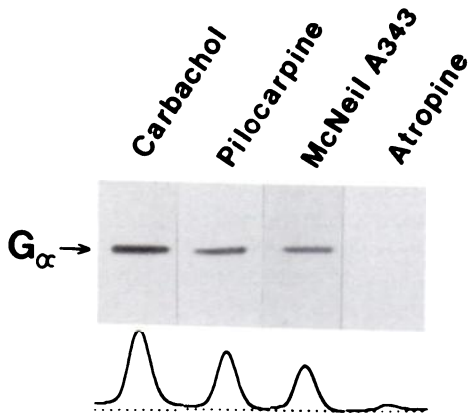

Fig. 6. Relative effect of carbachol, pilocarpine, and McN A343 on mAChR-G protein coupling in cardiac membranes. Membranes were incubated with 1 mM carbachol, pilocarpine, or McN A343 (*lanes 1–3*, respectively) or 10 μM atropine (*lane 4*) for 30 min. mAChRs and associated G proteins were then solubilized from the membranes and immunoprecipitated as described in the text. Western blots were developed with 1398 antiserum. *Traces below each lane*, relative peak areas from densitometric scans of immunoblots.

at saturating concentrations. The lower levels of mAChR-G protein complexes detected upon isolation of pilocarpine- or McN A343-activated receptors suggest that those receptors may have a lower affinity for G proteins than do carbachol-activated receptors.

Discussion

We have described our attempts to determine the specificity with which a single subtype of mAChR (11, 17) interacts with G proteins. The described assay system allows discrimination of interactions of the m_2 subtype with G proteins, because the monoclonal antibody employed specifically immunoprecipitates this and not other subtypes of mAChR (17).[3] Because the m_2 receptor subtype can provoke similar cellular responses in several tissues, it was of interest to determine whether interaction with the same G protein could be demonstrated in a variety of tissues expressing this receptor subtype. On the other hand, the variety of cellular responses (e.g., K^+ channel opening, adenylyl cyclase inhibition) provoked by the same receptor subtype suggested that this could be due to selective interactions of the same receptor subtype with different G proteins, linked to different cellular effector enzymes.

We found evidence that the m_2 subtype of mAChR is capable of selective interactions with G proteins. Thus, in atrial membranes, muscarinic agonists promoted interaction apparently with G_o exclusively, whereas in ventricular membranes agonists promoted interactions with both G_o and G_i. In cerebellum, interactions with G_{i1}, G_{i2}, and G_o were found. There did not appear to be a predictable relationship between the tissue abundance of a G protein α subtype and the levels of G protein α subtype found in association with the agonist-activated mAChR. In brain G_o is the most abundant G protein expressed, as measured on Western blots. In rat atrium there is more G_i than G_o, whereas in ventricle very little G_o is detectable using Western blot analysis. Nonetheless, G_o was one of the predominant G protein subtypes found in association with agonist-activated mAChRs in all tissues examined.

[3] G. R. Luthin and B. B. Wolfe, unpublished data.

The doses of agonist required to promote receptor-G protein interactions were similar to those that comprise the high affinity component of agonist binding, measured by competition of carbachol for [^{3}H]NMS binding. It appears from this that the low affinity component of agonist binding reflects those receptors that do not form a stable association with G proteins. This is consistent with the observation that GTP or Gpp(NH)p, which inhibit high affinity agonist binding, can reverse receptor-G protein association (15).

The ability of a single mAChR subtype to interact with any of several G proteins may account, in part, for the ability of this receptor subtype to mediate multiple cellular responses. It is not clear that coupling of the receptor to a single G protein type is always directed to the production of a single cellular response, such as attenuation of adenylyl cyclase. In atrium, for example, activation of mAChRs can promote inhibition of adenylyl cyclase and, in addition, open potassium channels. Both of these activities occur independently and are thought to require a G protein intermediary. Under the conditions of mAChR-G protein complex purification we describe, we can recover the majority (>50%) of agonist-labeled receptors throughout the purification, as documented using [^{3}H]oxo-M and [^{3}H]NMS to measure agonist- and antagonist-labeled receptors. Thus, unless the receptor-G protein complex associated with one of the responses is selectively refractory to this purification protocol or present in levels lower than those detectable by this protocol, it is possible that both responses are mediated through a mAChR-G_o intermediary. On the other hand, in ventricle and cerebellum there is evidence that at least two independent pathways may exist, consisting of receptor-G_i and receptor-G_o coupling mechanisms. The exact relationship between a given receptor-mediated cellular event and the receptor-G protein intermediary through which the event is produced remains to be clarified.

Gil and Wolfe (4) have demonstrated that, in rat heart, carbachol is a full agonist (100% efficacy) in promoting adenylyl cyclase inhibition. Pilocarpine and McN A343 show roughly 50% and 0% efficacies, respectively, in the same assay. We compared the ability of carbachol, pilocarpine, and McN A343 to promote mAChR-G protein interactions in heart membranes. Our hypothesis was that a direct relationship between agonist efficacy in the adenylyl cyclase assay and the ability to promote mAChR-G protein interactions might provide a molecular basis for the actions of partial agonists. Our findings suggest that pilocarpine and McN A343 are both capable of promoting high affinity agonist-mAChR interactions at saturating concentrations, as evidenced by GTP shifts of radioligand binding curves and direct measurement of mAChR-G protein complexes. The relative affinity of these activated mAChRs for G protein may be reduced, compared with the affinity of carbachol-activated mAChRs. This interpretation is consistent with results reported by Tota and Schimerlik (26), which demonstrate a reduced affinity of mAChRs activated by pilocarpine for G protein, compared with those activated by carbachol, using a reconstituted system of purified proteins in liposomes. Despite the apparent high affinity binding of McN A343 to mAChRs in heart membranes, McN A343 shows no efficacy in the adenylyl cyclase assay (4) but, rather, exhibits the functional properties of an antagonist in this system. We have recently shown that the antagonist atropine promotes dissociation of mAChR-G protein complexes in cardiac membranes (27). The observation in the current study that McN A343 supports mAChR-G protein interaction shows that McN A343 is functionally different from atropine and, thus, cannot be classified as an antagonist. One possible explanation for the lack of efficacy of McN A343 is that this drug promotes efficacious coupling of mAChRs to as yet unidentified enzymes or ion channels. Another possibility is that the biochemical determinants of agonist efficacy lie downstream from the receptor-G protein interaction, such that the ability of a compound to promote mAChR-G protein interaction does not predict its efficacy in functional assays.

In summary, we have demonstrated an agonist-induced association of the m_2 mAChR with at least three G proteins. The number and type of associated G protein(s) varies in each of the three tissues studied. Additional studies using related immunoaffinity purification techniques should allow a more detailed analysis of the relationship between the ability of an agonist to promote a cellular response and the specificity of interaction with G proteins within the tissue.

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