Pharmacological and Biochemical Characterization of Complexes of Muscarinic Acetylcholine Receptor and Guanine Nucleotide-binding Protein*

Complexes of agonist-bound muscarinic acetylcholine receptor (mACHR) and guanine nucleotide-binding protein (G protein) were solubilized and isolated from rat heart. Heart membranes were incubated with mACHR agonists or antagonists, solubilized using digitonin and cholate, and subjected to chromatography over wheat germ agglutinin-Affi-Gel. Eluted fractions were precipitated using a cardiac-selective anti-mACHR antibody (Luetje, C. W., Brumwell, C., Norman, M. G., Peterson, G. L., Schiumerliik, M. L., and Nathanson, N. M. (1987) Biochemistry 26, 6892–6898). Using samples obtained from membranes initially incubated with carbachol (10 nM, 100 nM, or 1 mM), Gα immunoreactivity was detected on Western blots probed using antibodies with specificity for Gα subunits. The Gα immunoreactivity was not detected when atropine alone (10 nM or 1 μM) or when excess atropine (1 μM) plus carbachol (100 nM) was used during the membrane preincubation. Gα immunoreactivity, when detectable on Western blots, was present in substoichiometric amounts relative to that of Gα. The Gα immunoreactivity was not present if GTP was included during incubation of membranes with agonist and following membrane solubilization. Further results indicate that although agonist binding to receptors is rapidly reversed by GTP or GDP (tα < 10 min), the mACHR-G protein complex is reversed more slowly or not at all. It was also shown that at high agonist concentrations, the cardiac mACHR interacts with both Gα and Gα-like proteins. Together, these results demonstrate the utility of an immunoaffinity approach to the purification and biochemical study of receptor-G protein interactions.

It has been well established for many neurotransmitter receptors that the mechanism of the binding of agonists differs from that of antagonists. For example, high affinity agonist

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1 The abbreviations used are: mACHR(s) muscarinic acetylcholine receptor(s); G protein, guanine nucleotide-binding protein; QNB, quinuclidinyl benzilate; NMS, N-methylscopolamine; oxo-M, oxotremorine-M; Gpp(NH)p, guanyl-5’-O-(3-thiotriphosphate); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; NEM, N-ethylmaleimide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. In the text, "Gα" refers to a presumed complex of β and γ G protein subunits.

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mation, complete isolation, and detailed characterization of any given receptor–G protein complex have not been accomplished.

In this study, we report a combined lectin affinity chromatography and immunoprecipitation protocol, supported by pharmacological studies, which allows identification of the agonist-specific formation of a mAChR-G protein complex in cardiac membranes.

**EXPERIMENTAL METHODS**

**Materials**

All chemicals were of the highest grade commercially available. Digitonin was from Sigma (lot 16P-2081) or Gallard-Schlesinger (lot 1151B). Wheat germ agglutinin was obtained from Vector Laboratories. Carbamylcholine (carbachol), Gpp(NH)p, GTP, GDP, N-acetylglucosamine, goat anti-mouse IgG, and atropine, were from Sigma. All electrophoresis and blotting materials were from Bio-Rad, except nitrocellulose BA85 membranes were from Schleicher & Schuell. Rat hearts were obtained fresh or from Pel-Freez Biologicals (Rogers, AR). Identical results were obtained comparing fresh and Pel-Freez hearts. [3H]Oxotremorine-M (87 Ci/mmol), [3H]NMS (85 Ci/mmol), and rainbow molecular weight markers were from Amer sham Corp. [3H]QNB (33.6 Ci/mmol) was from Du Pont-New England Nuclear.

**Methods**

**Tissue Preparation**

All operations were performed at 4°C. Hearts were dissected from decapitated rats or thawed (Pel-Freez), minced, and homogenized at 5 ml/heart in 10 mM Tris (pH 7.5), 1 mM EDTA using a Biospec homogenizer (high setting, 10 s). Homogenates were filtered through two layers of cheesecloth and then centrifuged at 20,000 × g for 15 min. Membrane pellets were resuspended (high setting, 5 s) in 5 ml/heart 10 mM Tris (pH 7.5), 1 mM EDTA, and were stored at −80°C until use.

**Solubilization of Receptors**

Frozen membranes were thawed, placed on ice, and 1 mg MgCl2 was added to give a final concentration of 3 mM. Drugs (dissolved in water at 100 ×) were added to give the final concentrations indicated, and samples were incubated for 30 min (unlabeled drugs) or for 3 h at 4°C (labeled oxo-M, NMS). In studies using QNB as a radioligand, QNB was incubated for 30 min at 23°C and then 2.5 h at 4°C to allow equilibration of the ligand and receptors. Membranes were collected by centrifugation at 20,000 × g for 15 min and resuspended in 5 ml/heart 10 mM Tris (pH 7.5), 1 mM EDTA, 3 mM MgCl2, 0.1% digitonin, and 0.02% cholate (buffer A). Membranes were immediately centrifuged at 30,000 × g for 20 min, and pellets thus obtained were resuspended at 5 ml/heart in buffer A supplemented to 1% digitonin, 0.2% cholate. After a 30-min incubation at 4°C, homogenates were centrifuged at 40,000 × g for 40 min. The supernatant thus obtained was used as a solubilized receptor preparation. Identical results were obtained in preliminary studies in which all centrifugations were performed at 140,000 × g.

**Chromatography over WGA-Affi-Gel**

Wheat germ agglutinin (WGA) was coupled to Affi-Gel 10 as described by the manufacturer, using 10 mg of WGA/ml of resin and coupling buffer composed of 20 mM HEPES, pH 7.5, containing 10 mM N-acetylglucosamine. The resin was blocked after coupling using 0.1 M ethanolamine (pH 8). Solubilized proteins from one heart were incubated with 1 ml (packed resin) of WGA-Affi-Gel in minicolumns equilibrated to buffer A, with constant mixing for 1 h at 4°C. The resin was drained, washed three times with 1 ml of buffer A containing 150 mM NaCl, and then each column was eluted with 2.5 ml of buffer A, 150 mM NaCl, 0.3% N-acetylglucosamine. Samples were concentrated to 40 μl using Centricon 30 microfilters (Amicon, Danvers, MA).

**Immunoprecipitation of WGA-purified Proteins**

The immunoprecipitation studies employed several different receptor preparations. The conditions described below were developed to optimize receptor labeling and precipitation for each experimental condition.

**Precipitation of Oxo-M- or NMS-labeled Receptors—Antibody 31-1D1 (0.2 μl of ascites; Luetje et al., 1987; obtained as a gift from Dr. Neil M. Nathanson, Seattle, WA) was incubated with 25 μl of a 1:1 slurry of goat anti-mouse IgG-agaroase in 10 mM NaHPO4 (pH 7.5), 1 mM EDTA. 150 mM NaCl for 2 h at room temperature and then placed on ice. The resin was collected by centrifugation in a microfuge, and the supernatant was aspirated. Concentrated WGA-purified samples were added, and incubations were continued for 3 h on ice. Samples were then diluted with 0.5 ml of buffer A and the resin collected by centrifugation for 5 s. The supernatants were discarded, and the wash step was repeated. The final pellets were resuspended in Laemmli sample buffer (% SDS), and eluted proteins were subjected to SDS-PAGE.

**Precipitation of Carbachol- and Atropine-labeled Receptors—Receptors isolated from one heart were concentrated after WGA-Affi-Gel chromatography and incubated with 31-1D1 antibodies (final 1:200 dilution; 0.2 μl of ascites) overnight at 4°C. A slurry (25 μl) of goat anti-mouse IgG-agaroase beads was added, and the incubation was continued for 4 h at 4°C. Immune precipitates were collected as described above for radiolabeled receptors. Identical results were obtained if ligands were included during the overnight incubation rather than during membrane preparation only.

**Precipitation of Oxo-M- and QNB-labeled Receptors with Anti-G, and Anti-Gi Antibodies—Receptors were labeled with oxo-M or QNB and then subjected to chromatography over WGA-Affi-Gel as described above. Dilutions of anti-G, or anti-Gi antibodies were incubated for 3 h at 4°C with 0.1 ml or in some experiments 0.5 ml of WGA-purified receptors. Precipitation was effected by the addition of 50 μl of a 10% slurry of Pansorbin (Calbiochem) and incubation on ice for 30 min. Buffer A (0.5 ml) was added and the pellets collected by centrifugation. Pellets were washed with 0.5 ml of buffer A, resuspended, and counted. In some control experiments, subunits of purified G, were radioiodinated (Greenwood et al., 1983), and approximately 1000 cpm of radioiodinated subunits was used to spike 0.1- or 0.5-ml aliquots of WGA-purified receptors. Precipitations using radioiodinated proteins were performed in parallel with those described above.

**SDS-PAGE and Western Blotting**

SDS-PAGE over 10% acrylamide gels (Laemmli, 1970) and Western blotting (Towbin et al., 1979) were as described previously (Woolkalis et al., 1986). Transfer of proteins to nitrocellulose was performed for 30 min at 100 V using a Bio-Rad miniblot apparatus. All primary antisera were used at 1:100 final dilution except as indicated in the text. For SDS-PAGE of NEM-treated samples, alkylation was performed by sequential treatment of samples with dithiothreitol (1 mM, 100°C for 3 min) and NEM (5 mM, 35°C for 20 min) prior to electrophoresis (Sternweis and Robishaw, 1984).

**Antibodies to G Protein Subunits**

Rabbit antisera to G proteins have been described elsewhere (Carlson et al., 1988; Woolkalis and Manning, 1987). Antibodies to G, (antisera 1398) were raised against the peptide Cys-Gly-Ala-Gly-Glu-Ser-Gly-Lys-Ser-Thr-Ile-Val-Lys-Gln-Met-Lys conjugated to keyhole limpet hemocyanin and have a predicted specificity for G, G0, G1, and Gi, as reported (Carlson et al., 1988; see also Fig. 8). Antibodies to G, (antiserum 556E) used in this study were raised against G, purified from bovine brain; these antisera recognize preferentially (but not exclusively) the 36-kDa form of G, (Woolkalis and Manning, 1987; see also Fig. 8). These antibodies are referred to in the text as anti-G, and anti-Gi, respectively. G, and G, were purified as described (Sternweis and Robishaw, 1984; Bokoch et al., 1984). Tranexulin was purified as described by Baeuerle et al. (1979).

**Binding Assay**

Heart membranes (100 μg of protein), N-[3H]methylscopolamine (2 nm), and the indicated drugs were incubated in a final volume of 0.5 ml of 10 mM NaHPO4 (pH 7.5), 1 mM EDTA (buffer B) for 2 h at 4°C. Samples were diluted with 5 ml of ice-cold buffer B, 150 mM NaCl, filtered over no. 30 filters (Schleicher & Schuell). They were washed with 5 ml of buffer B, 150 mM NaCl. Filters were counted at an efficiency of 35% after addition of 4 ml of Budget-solve (RPI).

**RESULTS**

GTP Sensitivity of Agonist Binding to Solubilized Receptors—[3H]Oxo-M was used to label muscarinic receptors in
membranes prepared from whole rat heart. Oxo-M binding to membranes was inhibited by co-incubation with 0.1 mM GTP (data not shown) as reported previously (Harden et al., 1983; Berrie et al., 1984). The o xo-M binding was reversed by the addition of GTP (or 50 μM Gpp(NH)p; not shown) following solubilization from the membranes (Fig. 1A). QNB binding was insensitive to GTP (or Gpp(NH)p; not shown); identical results were obtained using NMS instead of QNB (not shown). Following membrane solubilization, QNB- or oxo-M-binding activity was subjected to chromatography over WGA-Affi-Gel. Material eluted from the resin with N-acetylglucosamine (generally >50% of applied oxo-M- or QNB-binding activity) retained its sensitivity to GTP (or 50 μM Gpp(NH)p; not shown) when the agonist oxo-M was used to label receptors (Fig. 1B). The time course for GTP effects on oxo-M binding demonstrated complete dissociation of radioligand within 10 min at 4 °C (Fig. 2).

**Immunoprecipitation of mAChR Agonist-binding Sites with G Protein-directed Antibodies—**Membranes were labeled with oxo-M or QNB and then solubilized and chromatographed over WGA-Affi-Gel. Receptors thus obtained were incubated with antibodies to G, or G, subunits. Oxo-M-binding activity was precipitated from the WGA-purified preparation upon incubation with anti-G, antibodies (Fig. 3A). In five experiments, a maximum of 27 ± 4% of oxo-M-labeled receptors could be precipitated by a 1:32 dilution of anti-G, antibodies (after subtracting blank (no antibody) value). No further activity could be precipitated using higher (e.g. 1:10, 1:5) concentrations of antibodies. Anti-G, antibodies were ineffective in precipitating oxo-M-binding sites at all dilutions tested. QNB-binding activity was not precipitated by either of the anti-G protein antibodies (Fig. 3B). To determine the efficiency of the antibodies to precipitate G, and G, subunits, radiiodinated G, or G, subunits were used to spike samples of WGA-purified heart proteins, and these samples were incubated in parallel using the same dilutions of anti-G, or anti-G, antibodies. Table I shows that 85% of G, and 45% of G, subunits were precipitated under these conditions using the appropriate antibodies. These data illustrate that the antibodies used in this study exhibit efficacy to precipitate G protein subunits. It is not clear, however, that G proteins solubilized from heart membranes are recognized by the antibodies in a manner identical to that for the purified subunits under these conditions.

**Immunoprecipitation of G Protein Subunits with Antibodies Directed to the Cardiac mAChR—**Oxo-M- or NMS-labeled WGA-purified mAChRs were further purified by incubation with an antibody generated to the porcine heart mAChR (Luetje et al., 1987; Luthin et al., 1988) or were incubated with an unrelated ascites fluid as a control. Goat anti-mouse IgG beads were used to precipitate complexes of receptor and antibody, and the immunoprecipitated proteins were subjected to SDS-PAGE and Western blotting. The Western blots were developed with anti-G, antibodies. Fig. 4, lane 1, shows the relative position on Western blots of purified G,.

When oxo-M-labeled membranes were subjected to this protocol, immunoreactivity in the molecular mass 39–41-kDa region of the gel could be identified in the Western blots (Fig. 4, lane 2). This immunoreactive band was not seen when NMS-labeled membranes were used (Fig. 4, lane 3) or when oxo-M-labeled material was incubated with an unrelated ascites fluid instead of S1–D1 ascites (not shown). Because the band of G, immunoreactivity was seen using oxo-M- and not NMS-labeled mAChRs, the co-purification of mAChRs and G protein subunits appears to be specific for the presence of...
mACHR agonist. Similarly, this band is not due to simple carryover of G protein during mACHR purification, as evidenced by the control experiments using NMS or an unrelated ascites fluid during precipitation.

**Pharmacology of mACHR-G Protein Interaction**—The studies using radiolabeled agonists and antagonists showed that high affinity mACHR agonist- and antagonist-binding sites could be solubilized from heart membranes and subsequently processed through WGA-Affi-Gel and immunoprecipitation protocols. Radiolabeled drugs were used in these initial studies to verify at every step of the protocol the relative concentrations of agonist- or antagonist-bound receptor and in this way to estimate the stability of the mACHR-G protein complex. Detection of G\(_4\) immunoreactivity in these highly purified complexes was shifted to the right. Similar data have been used to support the concept of high and low affinity agonist binding to mAChRs, interconverted by GTP (Harden et al., 1982, 1983; Berrie et al., 1984; McMahon and Hosey, 1985). In the experiments below, we used the immunoprecipitation protocol to establish the mACHR pharmacology associated with mACHR-G protein complex formation and then characterized the effects of guanine nucleotides on the mACHR-G protein complexes.

mAChR agonist. Based on calculations of the number of receptors labeled by oxo-M in the membranes and surviving solubilization (1.5 pmol), WGA-Affi-Gel chromatography (0.8–1 pmol), and immunoprecipitation (0.5–0.8 pmol), and given the amount of purified G\(_4\) loaded on the gel lane (1 pmol, 40 ng; Fig. 4, lane 2), we estimate that approximately 39–41-kDa immunoreactive bands represent a mixture of G\(_4\) subunits, studies are in progress to clarify the stoichiometry of mAChR and G protein subunits present in the gel lanes.

**TABLE I**

<table>
<thead>
<tr>
<th>Antibody used (^a)</th>
<th>G protein subunit (^b)</th>
<th>% in pellet (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-G(_4)</td>
<td>G(_4)</td>
<td>84 ± 7</td>
</tr>
<tr>
<td>None</td>
<td>G(_2)</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Anti-G(_2)</td>
<td>G(_2)</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>None</td>
<td>G(_2)</td>
<td>21 ± 4</td>
</tr>
</tbody>
</table>

\(^a\) Anti-G\(_4\) and anti-G\(_2\) were used at 1:10 final dilution. "None" indicates normal rabbit serum or no added antibodies.

\(^b\) G protein subunits were radiiodinated and precipitated as described under "Experimental Procedures." Immune pellets were dissolved and subjected to SDS-PAGE. Bands of radioactive subunits were located by autoradiography, excised, and counted. Distinctions between G\(_2\) and G\(_4\) were not made.

\(^c\) Data are expressed as percentage of added radioactivity and are the mean ± S.E. of four independent experiments.

**FIG. 4. Immunoprecipitation of oxo-M- and NMS-labeled receptors and co-precipitation of G proteins using anti-receptor antibodies.** Membranes were labeled with 10 nM oxo-M or 10 nM NMS, solubilized, and subjected to WGA-Affi-Gel chromatography. WGA-purified receptors were then incubated for 3 h at 4 °C with 31-1D1 antibody, preadsorbed to goat anti-mouse IgG1-agarose. Precipitated material was collected by centrifugation and then dissolved in Laemmli sample buffer and subjected to SDS-PAGE and Western blotting using anti-G\(_4\) antibodies. Lane 1, purified G\(_4\) (1 pmol); lane 2, oxo-M-labeled receptors (0.8 pmol) precipitated with 31-1D1 antibodies; lane 3, NMS-labeled receptors (1.5 pmol) precipitated with 31-1D1 antibodies. These results are representative of three independent experiments.
Bound and free ligand were separated by filtration. Results shown are the average of duplicate determinations; these experiments have been repeated three times with similar results.

**Fig. 5.** Effect of GTP on the ability of carbachol to inhibit NMS binding to heart membranes. NMS (2 nM) and the indicated concentrations of carbachol were incubated with heart membranes for 3 h at 4 °C in the absence (O) or presence (●) of 0.1 mM GTP. Bound and free ligand were separated by filtration. Results shown are the average of duplicate determinations; these experiments have been repeated three times with similar results.

**Fig. 6.** Pharmacology of mAChR-G protein coupling. Unlabeled carbachol or atropine was incubated with cardiac membranes, and the receptors were solubilized, purified over WGA-Affi-Gel, and immunoprecipitated with 31-1D1 antibodies as described under "Methods." Samples were subjected to SDS-PAGE on minigels and Western blotting using anti-G, antibodies (Fig. 6). Lane 2, molecular mass markers. Membranes were incubated with the following concentrations of drug: lane 3, 1 mM carbachol; lane 4, 1 µM atropine; lane 5, 100 nM carbachol; lane 6, 10 nM atropine plus 1 mM carbachol; lane 7, 1 µM atropine plus 100 nM carbachol. Under these conditions of SDS-PAGE and Western blotting, the α subunits of G, and G, are not resolved.

In the presence of 100 nM or 1 mM carbachol, a G, immunoreactive protein co-purified with mAChRs through WGA affinity chromatography and immunoprecipitation with a cardiac-selective anti-mAChR antibody, as detected on Western blots developed using anti-G, antibodies (Fig. 6, lanes 3 and 5). The G, immunoreactivity was not seen on similar Western blots of receptors purified from membranes initially incubated with the antagonist atropine (1 µM; Fig. 6, lane 4) or in the absence of added agonist (not shown). Incubation of membranes with a high concentration (1 mM) of carbachol resulted in the same effect in the presence or absence of a lower concentration (10 nM) of atropine (compare lanes 3 and 6). Conversely, a high concentration (1 µM) of atropine inhibited the ability of carbachol (100 nM) to promote the appearance of the band of G, immunoreactivity (lane 7). These results would be predicted based on the data in Fig. 5 showing relative receptor occupancy by carbachol and given the Kd of atropine under these conditions (~1 nM).

**Fig. 7.** Effect of GTP on co-purification of mAChRs and G proteins. Membranes were incubated with the additions indicated below and processed through solubilization, WGA chromatography, and immunoprecipitation protocols in three separate experiments (A-C). Carbachol (1 mM) was present in initial incubations with membranes for all samples. Nucleotides were added to membranes or throughout the purification as indicated in parentheses below. Western blots were developed using anti-G, antibodies (α subunits of G, and G, are not resolved under these conditions). A: lane 1, carbachol alone; lane 2, carbachol plus 0.1 mM GTP (membranes only); lane 3, carbachol plus 0.1 mM GTP (present throughout); lane 4, carbachol plus 50 µM Gpp(NH)p (membranes only). B: lane 5, carbachol alone; lane 6, carbachol plus 0.1 mM GTP (following solubilization). C: lane 7, carbachol alone; lane 8, carbachol plus 50 µM Gpp(NH)p (membranes only); lane 9, carbachol plus 50 µM Gpp(NH)p (following solubilization). The results shown in each panel have been reproduced in at least three independent experiments.
may be more rapid than the rate of dissociation of mAChR from G protein.

Characterization of the G Protein Subunits—Using either radiolabeled or unlabeled agonists, we have been unable to establish the agonist-specific co-purification of G, subunits with mAChR through WGA chromatography and immunoprecipitation. In cases in which G, was seen in the Western blots, it appeared to be present in substoichiometric concentrations relative to the levels of G, and was not different in samples prepared from agonist- or antagonist-labeled membranes. This is represented in Fig. 8, in which stoichiometric amounts of α and β subunits of transducin, G, or G, were run in parallel lanes to carbachol-labeled mAChRs processed through the immunoprecipitation protocol. It can be seen that at the dilutions of antibodies used, the anti-G, antibodies stained G, subunits with equal intensity as anti-G, antibodies stained G, subunits when carbachol-labeled samples were processed with a mixture of anti-G, and anti-G, antibodies, each at 1:100 final dilution. With respect to G, subunits in the Western blots (data not shown). We cannot preclude, however, the presence of forms of G, only poorly recognized by the antibody employed.

Treatment of samples of purified G proteins with NEM prior to electrophoresis resulted in resolution of 40–41 kDa (G,-like) from 39 kDa (G,-like) G, subunits (Fig. 9, lane 1). A similar NEM treatment of immunoprecipitated samples allowed tentative molecular mass assignments to the G protein subunits that co-purified with the heart mAChR in carbachol-treated preparations. In atrium, a single α subunit could be identified which co-migrated on SDS-PAGE with G, (Fig. 9, lane 3). In ventricle, two G, subunits were detected (Fig. 9, lane 2) which co-migrated with the 40–41 kDa and 39 kDa G, subunits of G, and G, respectively (Fig. 9, lane 1).

Discussion

Our results demonstrate in two ways that mAChRs present in heart membranes are capable of a direct physical interaction with, minimally, the α subunit of a G protein. That the interaction of mAChR and G protein is not an experimental artifact is supported by the following observations related to its specificity. The co-purification of G, with mAChRs is agonist specific, as demonstrated by observations that (a) anti-G protein antibodies could successfully precipitate agonist- but not antagonist-labeled mAChRs; and (b) in the presence of agonist but not antagonist, anti-mAChR antibodies could precipitate the α subunit(s) of a G protein(s). The mAChR pharmacology associated with mAChR-G protein interaction was established using both labeled and unlabeled agonists and antagonists to the mAChR. In particular, the use of labeled oxo-M and NMS suggests that the agonist-bound mAChR and G protein may be immunoprecipitated in roughly equivalent molar amounts. The interaction of mAChR and G protein was supported at low (nM) concentrations of either oxo-M or carbachol, which presumably reflects the highest affinity binding state for these agonists. These data support the concept that high affinity agonist binding to mAChRs, and perhaps other receptors sharing sequence homology with mAChRs, reflects an interaction of the agonist-labeled receptor with a G protein.

Based on models for receptor-G protein interactions (Gilman, 1987), we reasoned that if the high and low affinity states of agonist binding reflect those receptors associated and non-associated with G protein, respectively, GTP might reduce the measured levels of mAChR-G protein complex. To test this possibility, it was first established that under the assay conditions employed, added guanine nucleotides completely abolished the high affinity component of agonist binding. The effects of GTP on agonist binding to cardiac membranes have been demonstrated previously (Harden et al., 1983; Berrie et al., 1984) and were reproduced in this laboratory using both labeled and unlabeled agonists. Experiments employing 10 nM labeled oxo-M as an agonist confirmed that within 10 min of the addition of 0.1 mM GTP, there was no detectable agonist bound to receptors for up to 3 h when the GTP was added during membrane labeling (data not shown) or for at least 1 h when added subsequent to membrane solubilization (Figs. 1 and 2). Similarly, high affinity agonist binding, measured by competition of carbachol for [3H]NMS binding, was absent for up to 3 h when 0.1 mM GTP was added to the membranes (Fig. 5). The above results were

3 A study identical to that shown in Fig. 8 was performed with rabbit antiserum to the peptide Cys-Aep-Pro-Val-Gly-Arg-Ile-Gln-Met-Arg-Arg-Arg-Arg-Leu conjugated to keyhole limpet hemocyanin. This antiserum recognizes the 36- and 35-kDa G, subunits equally well. The described experiment also failed to confirm the presence of G, subunits in Western blots of agonist-labeled material.
reproduced in similar experiments using Gpp(NH)p and GDP (not shown). These data confirm that added guanine nucleotides completely inhibit or reverse the high affinity component of agonist binding to membranes bound to muscarinic heart mAChRs. However, G protein subunits co-purified with mAChRs initially labeled with low concentrations of either oxo-M or carbachol in the absence or presence of 0.1 mM GTP, 0.1 mM GDP, or 1 mM GDP under the same conditions. Thus, formation of mAChR-G protein complexes can occur in the apparent absence of high affinity agonist binding, suggesting that following the agonist-induced formation of mAChR-G protein complexes, the complexes do not require the continuing presence of bound agonist for stability. The agonist is nonetheless required for mAChR-G protein complex formation, as no detectable G protein subunits co-purified with mAChRs in the absence of added agonist or in the presence of antagonist. Because Gpp(NH)p and only high concentrations (1 mM) of GTP could inhibit or reverse formation of the mAChR-G protein complex in membranes, we conclude that a GTPase activity present in the membranes hydrolyzes GTP after dissociation of the agonist from the receptor but before mAChR-G protein dissociation, when the GTP is present at lower concentrations. The relative abilities of GTP, GDP, and Gpp(NH)p to inhibit mAChR-G protein interactions therefore are unrelated to the presence or absence of measurable high affinity agonist-binding activity. Low (10 nM) concentrations of oxo-M or carbachol permit mAChR-G protein complex formation in membranes, even in the presence of GTP at concentrations that reverse high affinity agonist binding. Therefore a transient high affinity state for agonist binding must exist in the presence of GTP and be experimentally unmeasurable because of rapid agonist dissociation from the mAChR subsequent to mAChR-G protein complex formation. In membranes, in the presence of both agonist and GTP, an equilibrium may be reached such that GTP would appear to have no effect on mAChR-G protein complex formation. This could be possible due to (a) continued presence of agonist and formation of mAChR-G protein complexes; and (b) rapid GTP hydrolysis before dissociation of the mAChR-G protein complexes can occur. These results are supported by the observation that removal of agonist from the membranes prior to GTP addition allows reversal of mAChR-G protein complexes by concentrations of GTP which are ineffective in the presence of agonist. By adding GTP at all steps during membrane isolation and solubilization (the latter in the absence of agonist), following solubilization and throughout mAChR purification, or by use of high concentrations of GTP, the mAChR-G protein dissociation event can be observed. This suggests that under our experimental conditions, the sequelae of agonist-mAChR and mAChR-G protein dissociation events can be temporally resolved. Gpp(NH)p effectively reverses mAChR-G protein complexes in membranes but not in solubilized preparations, whereas agonist binding was reversed by Gpp(NH)p under either condition. Thus, it appears that the conditions of solubilization may alter the relative efficacy of the particular guanine nucleotide to promote mAChR-G protein dissociation. The cardiac mAChR was chosen for this study as it represents a model receptor system (probably a unique mAChR coded for by the m2 mAChR gene (Bonner et al., 1987) known to be linked to the pathway for inhibition of adenylyl cyclase (Bonner et al., 1987; Peralta et al., 1988). Other well characterized responses of the heart mAChR include coupling to inwardly rectifying K* channels and the M current (Nathanson, 1987). These interactions are possibly all mediated by G proteins. For example, any of the known G, like proteins can support mAChR effects on K* channels in patch clamp studies (see, e.g. Yatani et al., 1989). Based on the historical evidence that the a subunit of the G protein associated with inhibition of adenylyl cyclase (G,) is expected to have a molecular mass of 40–41 kDa, we anticipated that the cardiac mAChR would be associated with a G protein whose a subunit molecular mass was 40–41 kDa. The results we obtained using whole heart, based solely on relative molecular mass estimation by SDS-PAGE, established an interaction of the presumed unique m2 mAChR with both 40–41- and 39–39 kDa subunits at high carbachol concentrations (1 mM). Additionally, as shown in Fig. 9, in atrium, mAChRs appear to interact selectively with a 39-kDa G,, subunit more similar to G,.. In ventricle, two G subunits of 39 and 40–41 kDa were found to interact with mAChRs. Our further studies in this regard are in progress and are based on the use of antibodies with more selectivity to identify G-like and G-like a subunits in addition to the anti-G, (common) antibodies used in the present study. These results provide additional evidence to support a role for coupling of the m2 mAChR to a G-like protein in atrium and to both G, and G-like proteins in ventricle.

In conclusion, our data demonstrate that the rat cardiac mAChR, in the presence of agonist, forms a stable complex with at least the a subunit of a G protein or G proteins and that these complexes can be immunoaffinity purified to determine their molecular components and regulation. We describe a technique that may be amenable to study, in other receptor systems, those G proteins which may interact with a given receptor in situ, and such studies should contribute to a better understanding of the factors governing interactions of receptors with G proteins.

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REFERENCES


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