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Heterooligomers of the muscarinic receptor and G proteins purified from porcine atria

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ABSTRACT

Muscarinic receptor extracted from porcine atria in digitonin–cholate copurified with $G\alpha_o$, $G\alpha_{i1-3}$, and caveolins. The presence of complexes was confirmed by coimmunoprecipitation of the receptor, α-subunits, and caveolins in various combinations. Homooligomers of α_{i2} were detected on Western blots, and heterooligomers of α_{i2} and α_o were identified by coimmunoprecipitation; thus, a complex may contain at least two α-subunits. Other combinations of α-subunit were not detected. The ratio of total α-subunit to receptor was near 1, as measured by [^{35}S]GTPγS and the antagonist [^{3}H]quinuclidinylbenzilate, and the binding of [^{35}S]GTPγS was manifestly biphasic. The ratio of α_o to $\alpha_{i1,2}$ also was near 1, as determined from the intensity of Western blots. Cardiac muscarinic receptors therefore can be purified as a mixture of complexes that contain caveolins and oligomers of α-subunit, some of which are heteromeric. Each complex would appear to contain equal numbers of α-subunit and the receptor.

The M_2 muscarinic receptor can be purified from porcine atria as the receptor alone and as a complex with G proteins, designated M2G [1]. The complex was functional, in that binding of the agonist oxotremorine-M was sensitive to guanylylimidodiphosphate (GMP-PNP); moreover, the state favored by GMP-PNP bound the agonist in an apparently cooperative manner. Cooperativity implies oligomers, and multimeric forms have been reported for both the M_2 receptor [2] and the α-subunit of G proteins [3]. Evidence for oligomers and cooperativity is consistent with the notion that G protein-mediated signaling occurs via cooperative effects within a complex that comprises multiple equivalents of both receptor and holo-G protein [1,4,5].

Receptors, G proteins, and effectors all have been shown to localize in caveolin-rich domains [6]. Caveolins bind to and organize signaling proteins that contain a caveolin-binding motif, but several G protein-coupled receptors (GPCRs) have been shown to colocalize with caveolins despite lacking such a motif in regions presumably accessible to caveolins [7,8]. Caveolins have been implicated in the internalization, translocation, and regulation of G proteins and other components of the signaling process [9].

Cardiac muscarinic receptors copurify with at least two different members of the $G_{i/o}$ family [1]. The receptor appears to be multimeric [1,10], but the oligomeric status of the receptor-coupled G proteins is unknown. The composition of the purified preparation therefore has been examined further to reveal a mixture of complexes that contain the M_2 receptor, α_o, α_{i1-3}, and caveolins. The overall ratio of receptor to α-subunits is approximately 1:1, and the latter appear to occur at least partly as oligomers that can be homo- or heteromeric. If the receptor is a tetramer, as suggested previously [1,4,5,10], each receptor-G protein complex would include a tetramer of G proteins.

Materials and methods

Materials. (−)-[^{3}H]Quinuclidinylbenzilate (lot 3467373, 39 Ci/mmol; lot 3467877, 42 Ci/mmol) and [^{35}S]guanosine-5′-3-*O*-(thio)triphosphate ([^{35}S]GTPγS) (lots 0403, 0903, 1203, and 0204, 1250 Ci/mmol) were purchased from Perkin-Elmer Life Sciences. *N*-Methylscopolamine hydrobromide, carbamoylcholine chloride (carbachol) and the tetralithium salt of GTPγS were purchased from Sigma–Aldrich. To account for adsorbed water, the composition of GTPγS-Li_4 was estimated by elemental analysis (Canadian Microanalytical Service, Ltd., Delta, British Columbia). The results from two different batches are as follows: C-19.26%, H-3.39%, N-10.58%; C-17.13%, H-4.18%, N-8.94%. Based on the formula GTPγS-Li_4·nH_20, the values correspond to 5 and 8–9 equivalents of water, respectively.

Digitonin used for solubilization and purification was purchased from Wako Bioproducts at purity near 100%. Digitonin used

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in the buffers for binding assays was from Calbiochem. Cholic acid and Lubrol-PX (polyoxyethylene-9-lauryl ether, polidocanol) were from Sigma–Aldrich, and *n*-dodecyl-β-D-maltoside was from Calbiochem. Protease inhibitors were from Sigma–Aldrich. Suppliers of other chemicals have been listed previously [5].

Monoclonal (mouse) and polyclonal (rabbit) antibodies to the M_2 muscarinic receptor were purchased from Affinity Bioreagents and Chemicon International, respectively. Monoclonal antibodies to $G\alpha_o$, $G\alpha_{i1}$, and $G\alpha_{i2}$ also were from Chemicon. Polyclonal antibodies to $G\alpha_o$ and $G\alpha_{i1,2}$ were from Santa Cruz Biotechnology, and the polyclonal antibody to $G\alpha_{i3}$ was from Wako. A polyclonal antibody to caveolins-1 and -2 was from BD Biosciences. Anti-IgGs conjugated to horseradish peroxidase were purchased from Amersham Biosciences.

Cardiac muscarinic receptor. Sarcolemmal membranes were prepared from porcine atria obtained at the time of death (Toronto Abattoirs Ltd.), and the M_2 muscarinic cholinergic receptor was extracted in various detergents (0.8% digitonin plus 0.16% cholate, 0.08% cholate plus 0.5 M NaCl, 1.1% Lubrol-PX, or 1.1% *n*-dodecyl-β-D-maltoside). Receptor solubilized in digitonin–cholate was purified as a complex with G proteins (designated M2G) and as the receptor alone (designated M2). Further details regarding the solubilization and purification have been described previously [1].

Immunoprecipitation, electrophoresis, Western blotting, and densitometry. Aliquots of detergent-solubilized extract were mixed with the immunoprecipitating antibody in 300 µl of buffer A (20 mM KH_2PO_4, 20 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulphonylfluoride, pH 7.60). For M2G, buffer A was supplemented with 0.1% digitonin and 0.02% cholate. The mixture was shaken for 1 h at 4 °C, supplemented with 20 µl of agarose-conjugated Protein G (Santa Cruz), and shaken overnight at 4 °C. Immunoadsorbed receptor was collected and washed [11], and the entire precipitate was applied to the gel.

Samples for electrophoresis were heated for 5 min at either 65 or 100 °C [12]. Unless otherwise indicated, the nitrocellulose membranes in Western blots were treated with the primary antibody at a dilution of 1:1000 and with IgG at a dilution of 1:3000. Proteins were visualized by chemiluminescence (ECL™ and Hyperfilm MP, Amersham Biosciences). Molecular weight standards (Bio-Rad) were processed in parallel, and the relative molecular mass has been divided by 1000 throughout. Further details regarding these procedures have been described previously [11,12].

The amounts of α_o and α_{i1} plus α_{i2} in M2G were estimated from the intensities of the bands revealed by the anti-α_o and anti-$\alpha_{i1,2}$ antibodies. Standard curves for α_o and $\alpha_{i1,2}$ were prepared from a mixture of purified, functional G proteins (Calbiochem). Quantities applied to the gel were based on the contents as reported by the manufacturer, taking the mean of the stated range.

Binding assays. Aliquots of M2G were mixed with [^{3}H]quinuclidinylbenzilate or [^{35}S]GTPγS and incubated at 30 °C for 2 h or 2.5 h, respectively. Non-specific binding was taken as total binding in the presence of 1 mM *N*-methylscopolamine or 0.1 mM unlabeled GTPγS. The data were analyzed according to Eq. (1), in which B_{max} represents the total concentration of either receptor or α-subunits in *m* classes of sites, and B_{sp} is the total specific binding of the radioligand at total concentration $[P]_t$. The parameter F_j represents the fraction of sites with affinity K_j and Hill coefficient $n_{H(j)}$. The parameter NS represents the fraction of unbound radioligand that appears as non-specific binding.

$$B_{obsd} = B_{max}\sum_{j=1}^{m}\frac{F_j([P]_t - B_{sp})^{n_{H(j)}}}{K_j^{n_{H(j)}} + ([P]_t - B_{sp})^{n_{H(j)}}} + NS([P]_t - B_{sp}) \quad (1)$$

Further details regarding the binding assays and subsequent analyses have been described previously [5,10,13].

To achieve the required concentrations of [^{35}S]GTPγS, the specific radioactivity was reduced by diluting the radioligand with the unlabeled analogue. The capacities of M2G for undiluted and diluted [^{35}S]GTPγS were compared in concomitant assays, and the ratios from two such experiments are 1.37 and 1.40 if the specific radioactivity of the diluted material is based on the formula weight of GTPγS-Li_4 (i.e., 562.97 g/mol). The mean ratio of capacities is reduced to 1.19 if the specific radioactivity of the diluted material is based on the formula weight of GTPγS-$Li_4 \cdot 5H_2O$, as inferred from one elemental analysis; the mean ratio is 1.08 if the composition is taken as GTPγS-$Li_4 \cdot 9H_2O$.

Results

Bands corresponding to monomers of α_o, α_{i1}, α_{i2}, and α_{i3} were detected on Western blots of M2G (Fig. 1A) and of unprocessed extracts prepared in digitonin–cholate, Lubrol-PX, cholate–NaCl, and *n*-dodecyl-β-D-maltoside. The presence of receptor-α complexes in M2G was confirmed by coprecipitation of the receptor with both α_o (Fig. 1B–C, lanes 3) and α_{i2}. Carbachol had no effect on the association between the receptor and α_o (Fig. 1C, lane 4).

Some of the α-subunits in M2G occur as heterooligomers, as indicated by the reciprocal coimmunoprecipitation of α_o and α_{i2} (Fig. 2A–B, lanes 3). Although the anti-α_o antibody cross-reacted

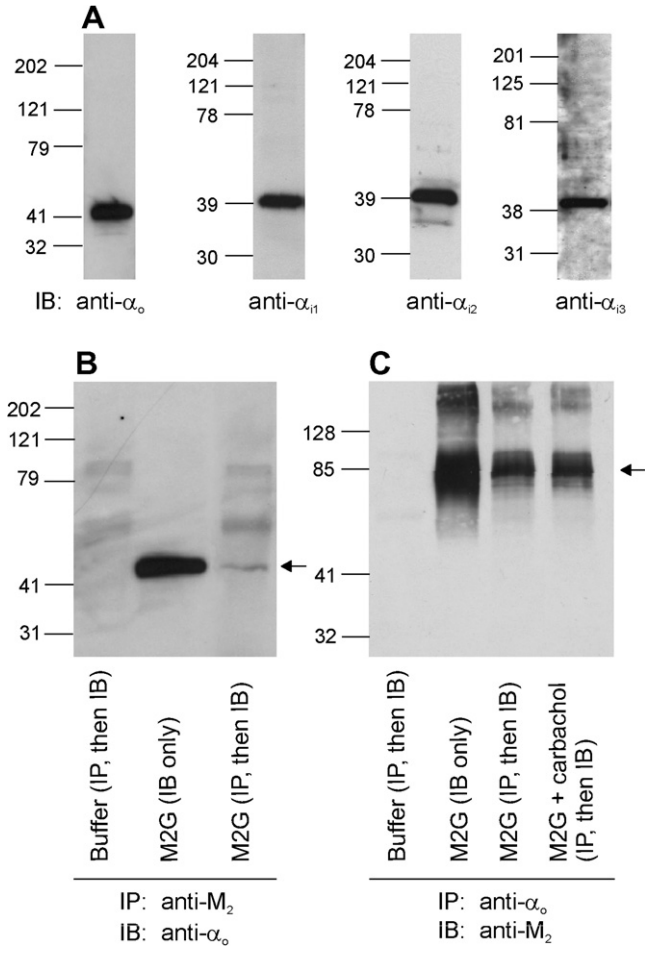

Fig. 1. Gα-subunits that copurify with the cardiac muscarinic receptor. (A) Samples of M2G were probed with antibodies to different α-subunits. (B and C) Samples of M2G were applied directly to the gel (lane 2) or examined for coimmunoprecipitation of the M_2 receptor and α_o (lane 3). Immunoprecipitation with the anti-α_o antibody (C) was performed in parallel on samples preincubated for 1 h at 30 °C in the absence (lane 3) and presence (lane 4) of 1 mM carbachol. Samples lacking receptor were processed in parallel (lane 1). Similar results were obtained with antibodies to the receptor and α_{i2}. *Abbreviations:* IP, immunoprecipitation; IB, immunoblot.

with immobilized anti-α_{i2} to yield a band coincident with that of α_o (Fig. 2A, lane 1), the signal from M2G was consistently more intense than that from the control. A single molecule or oligomer of the M_2 receptor therefore can associate with both α_o and α_{i2} within a single complex. No coimmunoprecipitation was detected for α_o and either α_{i1} or α_{i3}, or for α_{i2} and α_{i3}. The possibility of complexes containing both α_{i1} and α_{i2} was not examined owing to cross-reactivity between those antibodies.

Heterooligomers of α_o and α_{i2} also were identified in detergent-solubilized extracts (e.g., Fig. 2C–D, lanes 3); other combinations of α-subunits were not detected, as in the case of M2G. Bands arising from coimmunoprecipitation of α_o and α_{i2} generally were of weaker intensity with M2G than with unprocessed extracts, despite the lower concentration of M_2 receptor in the latter. Heteromers of α_o and α_{i2} therefore may be associated with other GPCRs in the heart.

Bands corresponding to homooligomers of α_{i2} were detected in unprocessed extracts (e.g., Fig. 2E, lane 3) and in some preparations of M2G (e.g., Fig. 2E, lane 1). Antibodies to α_{i2} identified not only the monomer (M_r = 43.8 ± 0.2, n = 156), but also an apparent dimer (M_r = 74.1 ± 1.0, n = 49), trimer (M_r = 130.6 ± 2.0, n = 48), and tetramer (M_r = 164.4 ± 8.7, n = 13). The multimeric forms were not detected by antibodies to the other α-subunits (Fig. 2E) and therefore appear to represent homooligomers of α_{i2} rather than heteromers of α_{i2} plus α_o, α_{i1}, or α_{i3}. They were equally prevalent in samples that had been heated at different temperatures (i.e., 65, 75, 85, 95, and 100 °C), suggesting that they were not a consequence of heat-induced aggregation [12].

In binding assays on M2G, the Hill coefficient was near 1 for the muscarinic antagonist [^{3}H]quinuclidinylbenzilate (n_H = 0.91 ± 0.08, n = 5) and markedly lower for [^{35}S]GTPγS (n_H = 0.40 ± 0.04, n = 9) (Eq. (1), m = 1). If $n_{H(j)}$ is taken as 1 throughout (Eq. (1)), two components are required to describe the data for both the receptor (P = 0.002) and the α-subunits (P < 0.00001) (Fig. 3). In the case of [^{3}H]quinuclidinylbenzilate, the values of K_j and F_2 were consistent among different preparations. In the case of [^{35}S]GTPγS, there was little variability in K_j; in contrast, the values of F_2 differed among preparations but appeared to cluster around two means: 0.80 ± 0.03 (n = 5) and 0.54 ± 0.07 (n = 4).

The capacities for [^{35}S]GTPγS and [^{3}H]quinuclidinylbenzilate were measured in parallel to obtain the ratio of total α-subunits to total receptor ($[G]_t/[R]_t$). The mean value of $[G]_t/[R]_t$ is 0.80 ± 0.06 (n = 9) if unlabeled GTPγS bore 9 waters of hydration; it is 0.98 ± 0.08 if unlabeled GTPγS was anhydrous. Since there was little variation in $[G]_t/[R]_t$ among different experiments, the different values of F_2 appear to reflect the distribution of α-subunits between two states within a population of defined size. The complement of α-subunits within M2G was examined by means of densitometry performed on Western blots, and the ratio of α_o to α_{i1} plus α_{i2} in two experiments was 0.89:1 and 1.07:1.

Various GPCRs have been shown to localize within caveolin-rich domains [8], and bands corresponding to caveolins were detected both in M2G and in unprocessed extracts (Fig. 4A). Their presence as part of a stable complex was confirmed by their coimmunoprecipitation with α_o (Fig. 4B) and with the M_2 receptor (Fig. 4C). Similar data were obtained with α_{i1} and α_{i2}. Although the anti-M_2 antibody detected a band with the mobility expected of caveolin in the M2G-free control (Fig. 4C, lane 1), that seen with the immunoprecipitate from M2G was consistently darker. Caveolins were absent from preparation M2 (Fig. 4A, lane 2).

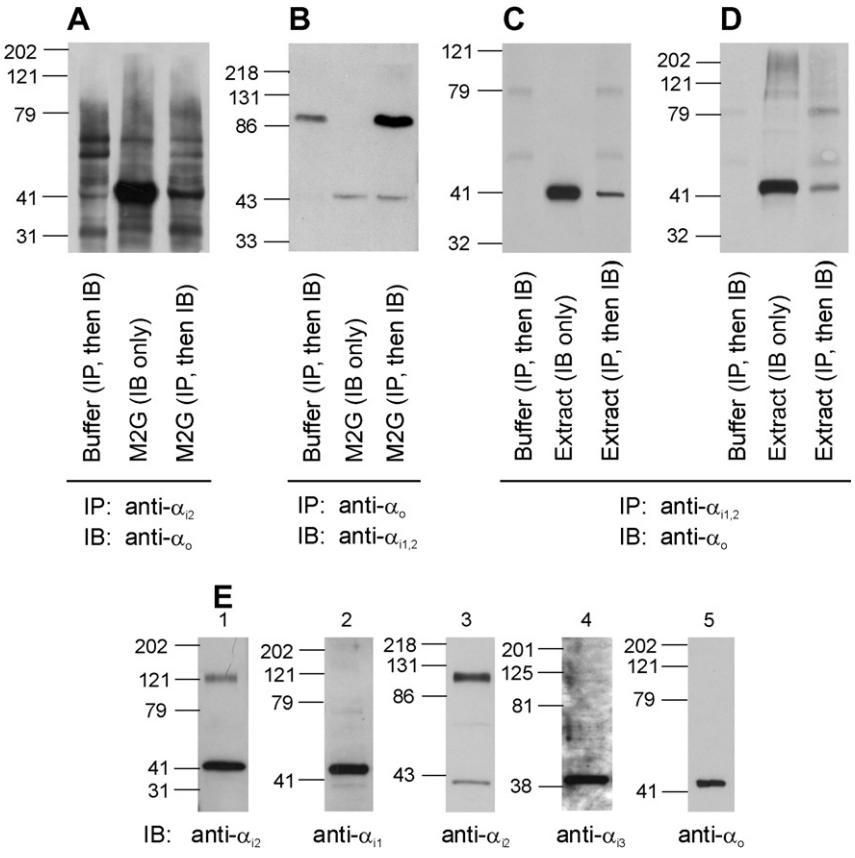

Fig. 2. Multimeric forms of α-subunits. (A–D) Samples of M2G (A and B) or the unprocessed extract in digitonin–cholate (C), Lubrol-PX (D), or *n*-dodecyl-β-D-maltoside (not shown) were immunoprecipitated and blotted as shown. Samples lacking receptor were taken as controls (lanes 1). (E) Samples of M2G (lane 1) or the extract in digitonin–cholate (lanes 2–5) were applied to the gel and blotted as shown. Similar results were obtained with extracts in the other detergents.

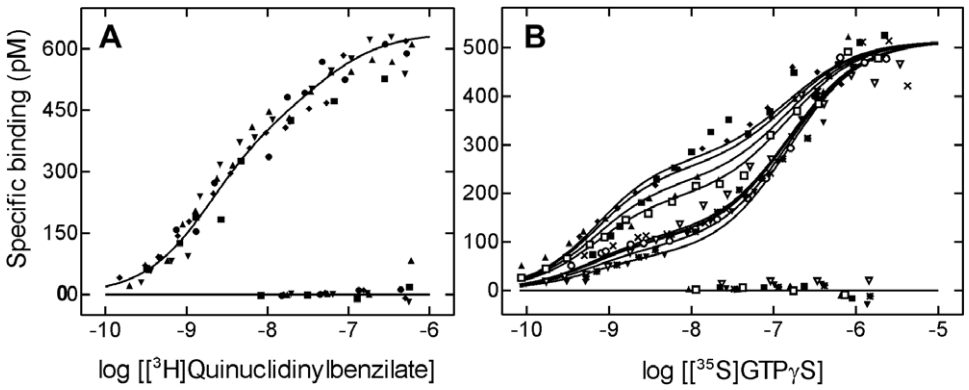

Fig. 3. Binding of [^{3}H]quinuclidinylbenzilate and [^{35}S]GTPγS to the M_2 receptor and G proteins in M2G. Estimates of total binding were analyzed according to Eq. (1) ($m = 2$, $n_{H(j)} = 1$). Single values of log K_j were common to all of the data in each panel. A single value of F_j was common to all of the data in panel A, and separate values were assigned to the data from individual experiments in panel B. Values plotted on the y-axis were normalized to the mean value of B_{max} for [^{3}H]quinuclidinylbenzilate (640 ± 80 pM, $n = 5$) (A) or to that value times the mean ratio of capacities for the two radioligands, assuming unlabeled GTPγS bore 9 waters of hydration (0.80 ± 0.06, $n = 9$) (B). The lines depict the fitted curves, and the parametric values are as follows: (A) log $K_1 = -8.78 \pm 0.07$, log $K_2 = -7.35 \pm 0.32$, $F_2 = 0.33 \pm 0.07$; (B) log $K_1 = -9.23 \pm 0.05$, log $K_2 = -6.82 \pm 0.05$, $0.47 < F_2 < 0.86$.

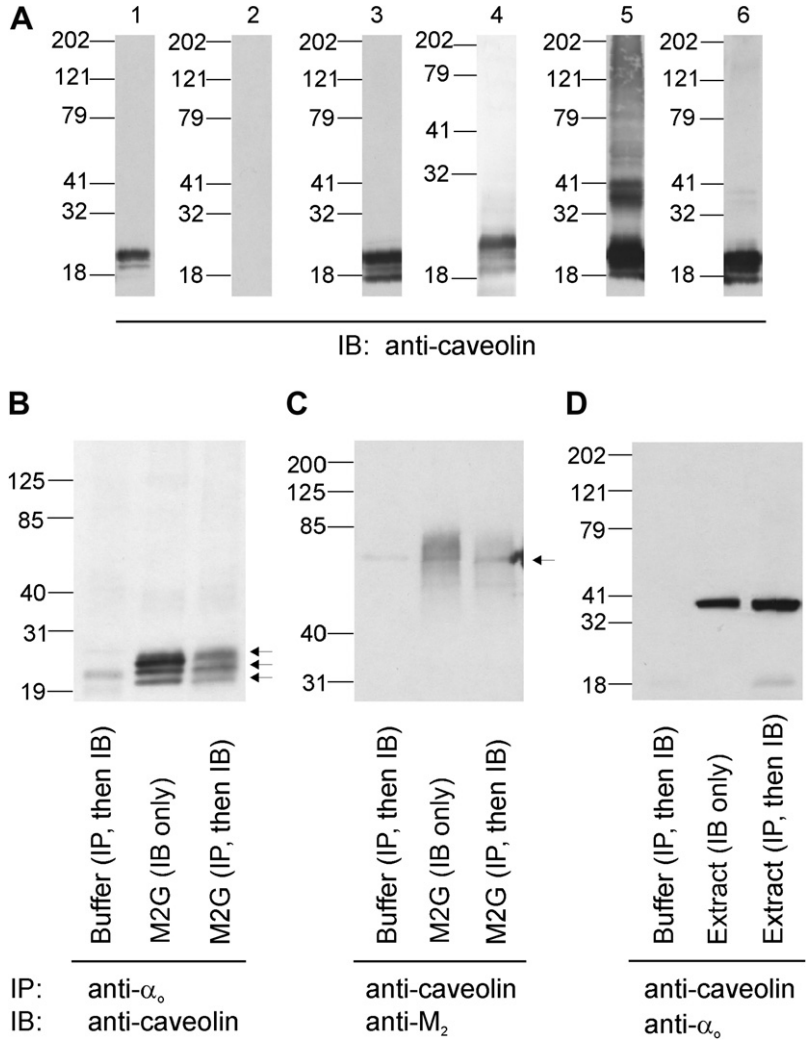

Fig. 4. Identification of caveolins. (A) Samples of M2G (lane 1), M2 (lane 2), or extracts in digitonin–cholate, cholate–NaCl, Lubrol-PX, or n-dodecyl-β-D-maltoside (lanes 3–6) were applied to a 4–15% gradient gel, and the transferred material was blotted with the anti-caveolin antibody (1:5000). (B–D) Samples of M2G (B–C) or the extract in digitonin–cholate (D) were immunoprecipitated and blotted as shown. Samples lacking receptor were taken as controls (lane 1).

Following immunoprecipitation by the anti-caveolin antibody, the fraction of receptor remaining in the supernatant was 0.41 ± 0.12 ($n = 3$) as determined from the binding of 200 nM [^{3}H]quinuclidinylbenzilate; it was 0.50 ± 0.04 ($n = 17$) as determined from the density of the band detected by the anti-M_2 antibody. The fraction of caveolin remaining in the supernatant was

0.44 ± 0.03 (n = 10) as determined from the density of bands detected by the anti-caveolin antibody. The anti-caveolin antibody therefore removed similar fractions of caveolin and the M_2 receptor from preparations of M2G, as expected for a stable complex.

Caveolins and each of the four α-subunits also could be coimmunoprecipitated from unprocessed extracts in digitonin–cholate, Lubrol-PX, and n-dodecyl-β-D-maltoside (e.g., Fig. 4D). The intensity of the immunoprecipitated bands was greater than that obtained with M2G, although the quantity of receptor applied to the gel was greater in the latter. Caveolins therefore may associate with α-subunits that are coupled to other receptors found in the heart.

Discussion

The cardiac muscarinic receptor can be purified from porcine atria as a complex or a mixture of complexes containing α_o, α_{i1}, α_{i2}, α_{i3}, and caveolins. Since the purified receptor devoid of G proteins does not interact with purified G proteins in solution [5], the presence of α-subunits in M2G is not an artifact of solubilization. As shown previously [1], G proteins that copurify with the receptor retain their ability to mediate allosteric interactions between agonists and guanyl nucleotides.

The muscarinic receptor consistently copurified with a near-equimolar quantity of α-subunits, an observation that also has been reported for the μ-opioid receptor and $\alpha_{i/o}$ from rat brain [14]. Since the amounts of α_o and $\alpha_{i1/2}$ in M2G were essentially the same, neither α-subunit seems to be present as a contaminant. These observations point to a specific complex or complexes with a defined composition, although the possibility of fortuitous agreement cannot be ruled out.

GPCRs are known to exist at least partly as oligomers [15], and the M_2 receptor appears to be at least tetrameric in M2G [1], cardiac membranes [4], detergent-solubilized extracts [10,16], and reconstituted vesicles [5]. The occurrence of α_{i2} as a homodimer and as a heterooligomer with α_o indicates that the receptor or an oligomer thereof can be associated with at least two equivalents of α-subunit. The overall ratio of one α-subunit per receptor suggests that a purified complex contains equal numbers of both proteins.

Not all complexes in M2G are identical, as indicated by the failure to observe coimmunoprecipitation of α_o with either α_{i1} or α_{i3}, or of α_{i2} with α_{i3}. It follows that M2G is a mixture of complexes, some of which contain heterooligomers of α_{i2} and α_o; the balance presumably contain homooligomers of α_{i2}, and perhaps of α_{i1}, α_{i3}, or α_o. Although α_o, α_{i1}, and α_{i3} migrated exclusively as monomers, complexes containing homooligomers of those subtypes may not survive the conditions of electrophoresis. Such a dissociation would account for the failure to observe a heterooligomer of α_o and α_{i2} on Western blots despite the coimmunoprecipitation of those subunits.

Binding of [^{35}S]GTPγS revealed a biphasic pattern that is not detected at the comparatively low concentrations of radioligand typically used in such assays (e.g., [14]). The two states may be due in part to the heterogeneous nature of M2G, but they appear to arise primarily from interactions within each complex. The measured affinities differ by 260-fold (i.e., K_2/K_1, Eq. (1)), whereas the affinity of [^{35}S]GTPγS for purified α_o and α_i differs by less than 10-fold (e.g., [17]). It therefore appears that heterogeneity has been induced in a population of G proteins that otherwise is functionally homogeneous, an effect that may arise from asymmetry within the complex, cooperativity in the binding of [^{35}S]GTPγS, or both. Such an arrangement within a tetramer of α-subunits is consistent with the observation that the distribution of sites between the states of high and low affinity can approximate 50:50 or 25:75 (Fig. 3B).

Heterogeneity among the G proteins associated with cardiac muscarinic receptors is evident in the multiphasic inhibitory effect of unlabeled guanyl nucleotides, even at comparatively low concentrations of [^{35}S]GTPγS. Moreover, the binding of guanyl nucleotides to receptor-coupled G proteins is affected by agonists in a manner that mirrors the effect of guanyl nucleotides on the multiphasic binding of agonists to GPCRs (e.g., [13]). Such a reciprocal interaction suggests that each presumed oligomer of receptors is associated with an oligomer of α-subunits; it argues against the alternative possibility that each equivalent of α-subunit is linked to a receptor but is functionally independent of other α-subunits. Although the molecular basis of the interaction between agonists and guanyl nucleotides remains a subject of discussion [4,5], the reciprocal nature of the effect suggests that heterogeneity in the binding of guanyl nucleotides is an important element of signaling.

An overall ratio of 1 receptor per α-subunit is consistent with reports that GPCRs can function as monomers (e.g., [12,18]). In contrast, other studies have shown that dimerization of the receptor is required for interaction with the G protein and signaling [19]. Also, attempts to model the complex between rhodopsin and transducin favor a ratio of 2 receptors per α-subunit [20]. The ratio of 1:1 in M2G could be reconciled with a ratio of 2:1 if one G protein were linked to the receptor via another.

Caveolins also form a stable complex with the M_2 receptor, either directly or via a third component such as a G protein. Although the receptor contains two caveolin-binding motifs (i.e., **Φ**XXXX**Φ**XX**Φ**: positions 75–83, **F**SMNL**Y**TL**Y;** positions 422–430, **W**TIGY**W**LC**Y**) [21], both segments lie in transmembrane and extracellular regions that appear to be inaccessible to caveolins. Other GPCRs have revealed a similar arrangement wherein a caveolin-specific motif is present but appears not to be directly responsible for an interaction with caveolins [7,22].

The similarities between unprocessed extracts and M2G suggest that stable complexes of receptor, G protein, and caveolins are not unique to the M_2 muscarinic receptor. The specific homomer of α_{i2} and the heteromer of α_o and α_{i2} that copurified with the M_2 receptor also were found in appreciable amounts in unprocessed extracts. Other oligomeric species of α-subunit were not detected. The prevalence and specificity of oligomers containing α_o and α_{i2} suggest that they may associate with other receptors found in the heart.

The heteromeric structures identified in M2G are consistent with the notion that signaling occurs via cooperative interactions among agonists and guanyl nucleotides within stable oligomers that comprise multiple equivalents of receptor and G protein [4,13]. Such aggregates appear to be assembled early in the biosynthetic process and to contain other signaling components such as effectors [23] and scaffolding elements such as caveolins [24]. It follows that they may be common among GPCRs and serve to host all of the events that constitute the pathway whereby signals traverse the cellular membrane.

Acknowledgments

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