Dynamic Targeting of the Agonist-stimulated m2 Muscarinic Acetylcholine Receptor to Caveolae in Cardiac Myocytes*

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In cardiac myocytes, as well as specialized conductance and pacemaker cells, agonist binding to muscarinic acetylcholine receptors (mAchRs) results in the activation of several signal transduction cascades including the endothelial isoform of nitric-oxide synthase (eNOS) expressed in these cells. Recent evidence indicates that, in endothelial cells, eNOS in cardiac myocytes is localized to plasmalemma caveolae, specialized lipid microdomains that contain caveolin-3, a muscle-specific isoform of the scaffolding protein caveolin. In this report, using a detergent-free method for isolation of sarcolemmal caveolae from primary cultures of adult rat ventricular myocytes, we demonstrated that the muscarinic cholinergic agonist carbachol promotes the translocation of mAchR into low density gradient fractions containing most myocyte caveolin-3 and eNOS. Following isopycnic centrifugation, the different gradient fractions were exposed to the muscarinic radioligand [3H]quinuclidinyl benzilate (QNB), and binding was determined after membrane filtration or immunoprecipitation. In a direct radioligand binding assay, we found that [3H]QNB binding can be detected in caveolin-enriched fractions only when cardiac myocytes have been previously exposed to carbachol. Furthermore, most of this [3H]QNB binding can be specifically immunoprecipitated by an antibody to the mAchR, indicating that the translocation of this receptor subtype is responsible for the [3H]QNB binding detected in the low density fractions. Moreover, the [3H]QNB binding could be quantitatively immunoprecipitated from the light membrane fractions with a caveolin-3 antibody (but not a control IgG1 antibody), confirming that the m2 mAchR is targeted to caveolae after carbachol treatment. Importantly, atropine, a muscarinic cholinergic antagonist, did not induce translocation of m2 mAchR to caveolae and prevented receptor translocation in response to the agonist carbachol. Thus, dynamic targeting of sarcolemmal m2 mAchR to caveolae following agonist binding may be essential to initiate specific downstream signaling cascades in these cells.

The activation of a muscarinic acetylcholine receptor (mAChR) triggers a number of signal transduction pathways that, in the heart, may elicit both positively and negatively inotropic and chronotropic effects (1, 2). Recent studies have shown that, of the five mAChR subtypes identified to date, only the m1 and m2 subtypes are expressed in adult mammalian cardiac tissues (3, 4). According to these reports, the m2 mAchR, which is expressed at a much higher level than the m1 mAchR, triggers the inhibitory response while m1 receptor activation elicits, when stimulated by higher concentrations of agonist, a compensatory excitatory effect on heart function. Therefore, distinct downstream signaling cascades must be involved following m1 and m2 mAchR activation. Both m1 and m2 receptor subtypes also have been reported to undergo translocation into specific subcompartments derived from the plasma membrane (5–10), a characteristic of many G protein-coupled receptors (GPR) following agonist binding. To date, two major pathways for GPR clustering and sequestration have been reported, which involve plasma membrane modifications that lead to the formation of either clathrin-coated or non-coated vesicles (11). While the human muscarinic cholinergic receptor Hm1 has been shown to internalize via clathrin-coated vesicles (10), mAChR have also been shown to be internalized through non-clathrin-coated vesicles in human fibroblasts, although the identity of these vesicular structures has not been defined (6).

Recently, a clathrin-independent sequestration pathway has received attention with the characterization of a population of plasmalemmal vesicles termed caveolae. Caveolae are small flask-shaped invaginations of the plasma membrane characterized by high levels of cholesterol and glycosphingolipids (12), the principal scaffolding protein of which are the caveolins, 20–24 kDa integral membrane proteins that undergo homooligomerization (13). These specialized lipid microdomains have been shown to play a role in the compartmentation of a number of plasma membrane-linked signal transduction pathways, including those mediated by receptor tyrosine kinases (14, 15). In addition, a recent report by Parton et al. (16) provides additional evidence that coalescence and fission of caveolae may be essential for the development of the T-tubular system that is essential for normal intracellular calcium homeostasis and excitation-contraction coupling in cardiac and skeletal muscle. The specific mechanisms involved in receptor sequestration may differ among distinct cellular phenotypes. For example, several reports have proposed the involvement of

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1 The abbreviations used are: mAChR, muscarinic acetylcholine receptor(s); GPR, G protein-coupled receptor; β-AR, β-adrenergic receptor; eNOS, endothelial isoform of nitric-oxide synthase; NO, nitric oxide; ARVM, adult rat ventricular myocytes; QNB, 1-quinuclidinyl benzilate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; MBS, Mes-buffered saline; PVDF, polyvinylidene difluoride; TBST, Tris-buffered saline with Tween 20; PAGE, polyacrylamide gel electrophoresis.
clathrin-coated pits in the mechanism of internalization of β-adrenergic receptors (β-AR) (17), and yet a recent report indicated that in epidermoid A431 cells, β-AR are clustered within caveoleae in response to agonist stimulation (18).

The recent development of antibodies directed against different tissue-specific isoforms of caveolin has permitted a better characterization of caveolar microdomains. Using these antibodies in immunoprecipitation experiments, we have recently shown that eNOS, the constitutively expressed isoform of nNOS, is targeted to sarcolemmal caveoleae in cardiac myocytes and endothelial cells (19). Interestingly, reports from our laboratory and by others have shown that the generation of nitric oxide (NO) is an obligate intermediate step in the signal transduction cascade involved in the m2 mACHR-mediated inhibitory responses of the heart, particularly following β-adrenergic stimulation (20–23). Caveoleae may, therefore, constitute the structural framework within which this signaling cascade operates. Thus, the dynamic targeting of agonist-stimulated muscarinic cholinergic receptors to caveoleae in cardiac myocytes could facilitate the activation of eNOS, which we have shown to be quantitatively and specifically associated with caveolin-3, the muscle-specific isoform of caveolin (19, 24–26). The co-localization in caveoleae of this Ca2⁺/calmodulin-dependent NOS isoform with proteins known to regulate Ca2⁺ homeostasis, including a Ca2⁺-ATPase and InsP3 receptor-like proteins (27), as well as with heterotrimERIC G proteins (12, 26, 28), suggest that these plasmalemmal microdomains may constitute a platform for the recruitment and regulation of the signaling proteins involved in the NO-mediated muscarinic cholinergic pathway in heart muscle.

In this report, we describe experiments designed to explore the hypothesis that m2 mACHR are targeted to plasmalemmal caveoleae upon agonist stimulation in adult rat ventricular myocytes. Using a detergent-free method for caveoleae isolation followed by isopycnic centrifugation, we provide evidence that the m2 mACHR, after agonist stimulation, co-purifies with caveolin-3 and eNOS. Furthermore, we show that the radioligand m2 mACHR can be specifically immunoprecipitated from these caveolin-enriched fractions using antibodies directed against caveolin-3.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Lysate Preparation, and Subfractionation—** Purified adult rat ventricular myocyte (ARVM) primary cultures were plated on laminin and cultured for 24 h in a defined medium as reported previously (19). ARVM were incubated either with or without carbachol (100 μM, 15 min), lysed, and fractionated on sucrose gradients; in some experiments (see “Results and Discussion”), myocytes were preincubated in the presence of 1 μM atropine (15 min) or 5 mM acetic acid (5 min) before carbachol treatment. Before harvesting, cells were washed extensively with ice-cold phosphate-buffered saline to ensure complete removal of drugs. This was validated by the lack of any detectable difference in specific ³H]quinuclidinyl benzylate (QNB) binding levels (see below) in total lysates of ARVM, whether treated or not with a muscarinic agonist or antagonist.

ARVM were scraped in a freshly prepared solution of 200 mM Na₂CO₃ and lysed by sonication (three 5-s bursts, minimal output power) using a Branson sonifier 450 (Branson Ultrasonic Corp., Danbury, CT), according to a method modified from Song et al. (26). The cell lysate was then adjusted to 45% sucrose by addition of a sucrose stock solution prepared in MBS (25 mM Mes, pH 6.5, 150 mM NaCl) and placed at the bottom of a 5–15–25–35% discontinuous sucrose gradient (in MBS containing 100 mM Na₂CO₃) for an overnight ultracentrifugation (150,000 g). The gradient was fractionated in nine fractions corresponding to sucrose concentrations 5, 15, 25, 35, and 45%, and the four intermediate interfaces. Each fraction was neutralized with HCl before further analysis.

**SDS-PAGE and Immunoblotting—** Heat-denatured proteins were loaded and separated on 12% SDS-polyacrylamide gels (Mini Protein II, Bio-Rad) and transferred to a PVDF membrane (Bio-Rad). After blocking with 5% non-fat dry milk in Tris-buffered saline with 0.1% (v/v) Tween 20 (TBST), membranes were incubated with the specified primary antibody (Transduction Labs) for 1 h in TBST containing 1% non-fat dry milk. After six washes (10 min each), the membranes were incubated for 1 h with a horseradish peroxidase-labeled goat anti-mouse immunoglobulin secondary antibody (Jackson Immunoresearch Labs) at a 1:10,000 dilution in TBST containing 1% non-fat dry milk. After five additional washes, the membranes were rinsed once in TBST, incubated with a chemiluminescent reagent according to the manufacturer protocols (Renaissance, NEN Life Science Products), and exposed to x-ray film.

**Membrane Markers—** Mannosidase II activity was determined by hydrolysis of p-nitrophenyl-o-mannopyranoside (Sigma) with volume-reduced to facilitate a 98-well dilution in 30 μl TBST containing 1% non-fat dry milk. A 100,000 dilution in TBST containing 1% non-fat dry milk. After five additional washes, the membranes were rinsed once in TBST, incubated with a chemiluminescent reagent according to the manufacturer protocols (Renaissance, NEN Life Science Products), and exposed to x-ray film.

**Radioligand Experiments and Immunoprecipitation—** The gradient fractions (buffered at pH 7.4) were adjusted to 5 mM MgCl₂, 1 mM EDTA, 0.1 mg/ml leupeptin, 1 μM pepstatin, and 1 mM ethylenediaminetetraacetic acid (EDTA) and aliquots of the different fractions were incubated with 2 μM ³H]QNB (NEN Life Science Products) at 30 °C for 60 min; nonspecific binding was determined in the presence of 1 μM atropine. Assays were performed in triplicate and terminated by rapid filtration on Whatman GF/B filters or followed by an immunoprecipitation protocol (adapted from those in Refs. 31 and 29). For these immunoprecipitation experiments, the binding buffer also contained 1% digitonin and 0.2% CHAPS; nonspecific ³H]QNB binding was determined by performing all the steps of the immunoprecipitation protocol in the presence of 1 μM atropine. After sequential incubations of the ³H]QNB-bound receptors with an antibody directed against the m2 mACHR (4 h, 4 °C, Chemicon) and agarose-conjugated protein-G (1–2 h, 4 °C), immuno complexes were precipitated by centrifugation, washed four times with 25 mM Mes buffer containing 1% digitonin and 0.2% CHAPS, and resuspended in 1% SDS. A similar protocol was used for the immunoprecipitation with the caveolin-3 antibody (Transduction Labs) except that binding and washing buffers did not contain digitonin. The isofrom specificity and lack of cross-reactivity of the caveolin (19, 24–26) and muscarinic (22) antibodies have been established previously. Moreover, the specificity of the caveolin-3 immuno precipitation was optimized by comparing the ³H]QNB binding detected from immunoprecipitates performed using an non-immune idotype-specific purified mouse myeloma IgG1 (Zymed). In all the experiments described here above, samples were transferred in counting vials containing 10 ml of scintillant, and the radioactivity was determined in a liquid scintillation counter.

**RESULTS AND DISCUSSION**

**Caveoleae Isolation by Subcellular Fractionation of Cardiac Myocytes—** Caveolin-enriched membranes have been historically isolated on the basis of their insolubility in Triton due to their specialized lipid composition (12, 33). However, it has been reported recently that the inclusion of detergent can result in the loss of proteins normally associated with caveoleae (26, 34), as well as in apparent redistribution of mitochondrial and endoplasmic reticulum proteins into caveoleae (35). Therefore, for isolating caveoleae from cardiac myocytes, we have optimized a detergent-free purification method based on the resistance to extraction of caveolin complexes by sodium carbonate and on the fine disruption of cellular membrane by sonication (18, 26). Thus, after homogenization of ARVM in a sodium carbonate buffer, the lysate was adjusted to 45% sucrose and placed at the bottom of a 5–15–25–35% discontinuous gradient for an overnight ultracentrifugation (150,000 g). Aliquots of the different fractions collected were separated by SDS-PAGE, transferred onto PVDF membranes, and immunoblotted with anti-caveolin-3 or anti-eNOS antibodies. The immunoblots presented in Fig. 1 show that the majority of caveolin-3 and...
myocytes were lysed and submitted to isopycnic centrifugation on a sucrose gradient. Aliquots of the different fractions obtained were incubated with [3H]QNB, a muscarinic antagonist radioligand, at 30 °C for 60 min. In a first set of experiments, membranes were directly filtered on Whatman GF/B glass filters. As shown in Fig. 2A, in lysates prepared from untreated myocytes, the binding of [3H]QNB is only detected in the high-density fractions. In contrast, following carbachol treatment, 27.4 ± 3.3% of the [3H]QNB binding (n = 6) can be recovered in the low-density fractions 2 and 3, which correspond to the caveolin-enriched membranes (Fig. 1A). The rest of the [3H]QNB binding remains concentrated in fractions 7–9 and likely represents binding to non-caveolar sarcomembranul muscarinic receptors.

In a second series of experiments, we used a complementary approach to explore the carbachol-induced shift in [3H]QNB binding. The different fractions collected after isopycnic centrifugation were immunoprecipitated using an m2 mAchR antibody, and the amount of specific [3H]QNB binding in each immunoprecipitate was determined. As shown in Fig. 2B, the pattern of distribution of m2 mAchR is similar to that directly deduced from the [3H]QNB binding to each fraction (Fig. 2A) with, however, a more accentuated shift of [3H]QNB bound m2 mAchR toward the low density fractions when myocytes have been exposed to carbachol. 34.6 ± 3.9% (n = 6) of the [3H]QNB binding is now detected in the caveolar fractions 2 and 3. Importantly, when ARVM are preincubated with 1 μM atropine before carbachol treatment (Fig. 2C), the enrichment of the m2 mAchR in fractions 2 and 3 is no longer observed, thereby indicating the specificity of the agonist-mediated clustering process. Interestingly, in a previous study, Raposo et al. (6) reported that treatment of human fibroblasts, either with a muscarinic cholinergic agonist or with the muscarinic cholinergic antagonist atropine, triggered the redistribution of the Hm1 mAchR into specific regions of the plasma membrane, presumably caveolae, and that only longer exposures with the agonist lead to the receptor endocytosis. Furthermore, Tolbert and Lameh (10) showed, using immunofluorescence confocal microscopy, that the Hm1 mAchR, after agonist stimulation, are internalized via clathrin-coated vesicles in HEK cells stably transfected with the epitope-tagged Hm1 receptors. Together with the data reported here, these results suggest that the extent and the mode of receptor compartmentation in response to agonist stimulation may be governed by both the receptor subtype and the cell type in which it is expressed.

In our experimental conditions, it is unlikely that clustering of the m2 mAchR into coated pits can explain the shift in mAchR into lower density sucrose gradients. Indeed, evidence from the literature indicates that the equilibrium density of clathrin-coated pits is higher than that of caveolae (38) and therefore would not match the pattern of distribution of carbachol-stimulated muscarinic receptors obtained in Fig. 2, A and B. Furthermore, when myocytes are pre-incubated with 5 mM acetic acid, pH 5.0, a treatment known to disrupt clathrin-mediated endocytosis (39), a movement of m2 mAchR in caveolin-enriched fractions is still detected (Fig. 2C).

Immunoprecipitation by Caveolin-3 Antibody of Agonist-stimulated Muscarinic Cholinergic Receptors—To confirm the dynamic targeting of muscarinic receptors to caveolae in cardiac myocytes, we used a caveolin-3 antibody to immunoprecipitate caveolar membranes and identify the m2 mAchR by radioligand binding assays. In these studies, cardiac myocytes preincubated either with or without carbachol were lysed and fractionated on sucrose gradients, and the fractions corresponding to caveolae were pooled and incubated with [3H]QNB. After subsequent incubation with either an anti-caveolin-3 an-

![Fig. 1. Fractionation of cardiac myocytes. A, distribution of caveolin-3 and eNOS proteins. After isopycnic centrifugation of adult rat ventricular myocytes on sucrose gradients as described in the text, aliquots of 1 ml fractions were resolved by SDS-PAGE (12.5% acrylamide), transferred onto PVDF membranes, and immunoblotted with an anti-caveolin-3 antibody or eNOS antibody. Fraction 1 refers to the top of the gradient. The data represent the results of a typical fractionation experiment. B, distribution of protein ( ), plasma membrane ( ), and Golgi ( ) markers along sucrose density gradient. The mannosidase II activity and the [3H]ouabain binding have been used as specific markers of the Golgi and the plasma membrane, respectively. Fraction 1 refers to the top of the gradient. The data represent the results of typical fractionation procedures with individual measurements performed in triplicate.](image-url)
tibody or a nonspecific IgG1 antibody and agarose-conjugated protein-G, immunocomplexes were collected by centrifugation, and radioactivity was determined in a scintillation counter. As summarized in Fig. 3, in the absence of carbachol treatment, there was no significant immunoprecipitation of [3H]QNB binding by caveolin-3 antibodies since the level of [3H]QNB binding was similar to that obtained when using the nonspecific IgG1 for the immunoprecipitation. In contrast, following agonist treatment, a substantial fraction of specific [3H]QNB binding can be immunoprecipitated by anti-caveolin-3 antibodies (Fig. 3); no change in caveolin-3 expression was observed after carbachol treatment (not shown). In fact, 73 ± 6% (n = 3) of the [3H]QNB binding originally present in pooled fractions 2 and 3 (determined by direct filtration on Whatman GF/B glass filters) could be recovered after anti-caveolin-3 immunoprecipitation. Similar experiments (not shown) performed on fractions 7–9, which correspond to the bulk of plasma membrane (80–95% of total protein when pooled together), did not reveal any specific [3H]QNB binding in the caveolin-3 immunoprecipitate, in agreement with the low abundance of caveolin-3 in these fractions (see Fig. 1A). Importantly, in myocytes incubated with carbachol in the presence of the muscarinic antagonist atropine, the [3H]QNB binding immunoprecipitated by anti-caveolin-3 antibodies remained at the level detected in a control immunoprecipitation performed with a nonspecific IgG1. This is in agreement with the data shown in Fig. 2C in which no significant binding was detected in the anti-m2 mAchR immunoprecipitates from caveolar fractions of myocytes incubated with carbachol in the presence of atropine. Taken together, these data establish that the m2 mAchR redistributes to plasmalemmal caveolae of cardiac myocytes following agonist binding.

The dynamic targeting of the m2 mAchR to caveolae has important implications for muscarinic receptor biology as well as for the regulation of eNOS activation. Although several laboratories have reported evidence for the translocation to low density gradient fractions of the muscarinic receptors upon...
agonist stimulation (5–7), there are, to our knowledge, no data in the literature that address the specific nature of these “light membranes.” The co-purification and co-immunoprecipitation (this study, and also see Refs. 19 and 36) of caveolin, eNOS, and the agonist-stimulated m2 mAChR in isopycnic centrifugation fractions, which together represent less than 5% of the total amount of protein, indicate that caveolae are the common structural platform for these proteins. Together with immuno-electron microscopy data showing that, in A431 cells, β-AR are sequestered within caveolae in response to agonist stimulation (18), our data indicate that clathrin-coated pit formation may exist in some cells.

While numerous studies present the sequestration of G protein-coupled receptors after agonist stimulation as a key event for initiating a process of desensitization (for review, see Ref. 40), the data in this manuscript support the hypothesis that, following stimulation by agonist, cardiac m2 mAChR translocation to caveolae may be necessary to initiate specific downstream signaling cascades. Interestingly, several recent studies have shown that internalization of the m2 and m4 mAChR is mediated by mechanisms distinct from the phosphorylation by the G protein-coupled receptor kinase (GRK) family known to lead to receptor desensitization (41, 42). The translocation of muscarinic receptors within caveolae should allow their interaction with the heterotrimeric G protein complexes known to be concentrated within these plasma-membrane microdomains (12, 26, 28) and lead, after recruitment of co-factors and intermediate effector proteins, to the activation of eNOS, a resident caveolar protein in cardiac myocytes. Analysis of caveolin-enriched fractions, which together represent less than 5% of the total membrane, in A431 cells, structural platform for these proteins. Together with immunoelectron microscopy data showing that, in A431 cells, β-AR are sequestered within caveolae in response to agonist stimulation (18), our data indicate that clathrin-coated pit formation may exist in some cells.

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