

Dynamin mediates caveolar sequestration of muscarinic cholinergic receptors and alteration in NO signaling

Chantal Dessy, Ralph A. Kelly¹,
Jean-Luc Balligand² and Olivier Feron

Department of Medicine, Unit of Pharmacology and Therapeutics, FATH 53.49, Université Catholique de Louvain, Avenue E. Mounier, 53, B-1200 Brussels, Belgium and ¹Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA

²Corresponding author
e-mail: balligand@mint.ucl.ac.be

In cardiac myocytes, agonist binding to muscarinic acetylcholine receptors (mAChRs) leads to the targeting of stimulated receptors to plasmalemmal microdomains termed caveolae. Here, we examined whether this translocation leads to mAChR internalization and alteration in downstream NO signaling. Differential binding of membrane-permeant and -impermeant mAChR radioligands on caveolae-enriched membranes revealed that carbachol stimulation of cardiac myocytes induces sequestration of mAChRs through caveolae fission. GTP but not its non-hydrolyzable analog GTP γ S drove the further detachment of caveolae from myocyte sarcolemma. Also, incubation of extracts of carbachol-stimulated myocytes with recombinant GTPase dynamin induced mAChR sequestration in budded caveolae, while dominant-negative K44A dynamin inhibited it. These data were confirmed by immunofluorescence microscopy on m2 mAChR-expressing COS cells. Finally, repeated carbachol stimulations of mAChRs co-expressed in COS cells with endothelial nitric oxide synthase (eNOS) and wild-type, but not mutant, dynamin led to a progressive increase in mAChR sequestration and a concurrent stabilization of the inhibitory eNOS-caveolin complex. These findings emphasize the role of caveolae in mAChR trafficking and NO signaling, and suggest that caveolae fission may contribute to G-protein-coupled receptor desensitization.

Keywords: caveolae/caveolin/dynamin/eNOS/mAChR

Introduction

Muscarinic acetylcholine receptors (mAChRs) belong to the superfamily of G-protein-coupled receptors (GPCRs) that couple extracellular stimuli to plasmalemmal and intracellular effectors including ion channels and enzymes such as adenylate cyclase and phospholipases. Agonist stimulation of GPCRs also leads to adaptive changes that serve to facilitate responsiveness of the cell to successive stimulations. This process is initiated by receptor desensitization, which involves receptor phosphorylation by GPCR kinases (GRKs) and its sequestration in specialized cell compartments (Lefkowitz, 1998). Accordingly, phos-

phorylation of the agonist-stimulated receptor increases its affinity for arrestin, which sterically prevents further coupling with G-proteins, whereas sequestration reduces the access for membrane-impermeable agonists.

Several authors have addressed the existence of a link between GRK-dependent phosphorylation and internalization of the m2 subtype of mAChR in heterologous expression systems (Tsuga *et al.*, 1994; Pals-Rylandsdam *et al.*, 1995; Schlador and Nathanson, 1997). Although the extent of overexpression of each signal-transducing component of the sequestration pathways may confound the interpretation of some of these studies, discrepancies in the results also suggested the existence of cell type-specific mechanisms for receptor internalization. Evidence for distinct sequestration pathways was indeed provided recently by the finding that the m2 mAChR could be internalized by both arrestin/clathrin-dependent and -independent pathways (Pals-Rylandsdam *et al.*, 1997; Vögler *et al.*, 1998). These data underscore the plasticity of the mechanisms of m2 mAChR trafficking and justify the growing interest in the processes of mAChR sequestration/desensitization in primary cells such as cardiac myocytes where the m2 subtype is the predominant isoform.

We have reported previously that in rat cardiac myocytes, a large fraction of sarcolemmal m2 mAChR is targeted, upon agonist stimulation, to non-clathrin vesicles termed caveolae (Feron *et al.*, 1997a). Caveolae are small flask-shaped invaginations of the plasma membrane characterized by high levels of cholesterol, glycosphingolipids and lipid-anchored proteins (Anderson, 1998). These specialized lipid microdomains ensure the compartmentation of a number of signaling proteins including the endothelial nitric oxide synthase (eNOS), various kinases and G-proteins (Anderson, 1998; Okamoto *et al.*, 1998). In addition, a number of GPCRs including the B2 bradykinin, β -adrenergic, cholecystokinin, endothelin, angiotensin II and calcium-sensing receptors have been shown to be located within caveolae in native conditions or upon agonist stimulation (for references see Anderson, 1998; Okamoto *et al.*, 1998). However, these observations do not address the issue of whether the caveolar location of a specific GPCR is required to initiate its downstream signaling cascade or to mediate the receptor desensitization/resensitization process, or both. Recently, the latter hypothesis gained some ground from the finding that dynamin, which is known to play a crucial regulatory role in the processes of endocytosis through clathrin-coated pits, is largely enriched in caveolae and promotes cholera toxin B chain internalization through budded caveolae (Henley *et al.*, 1998; Oh *et al.*, 1998).

The inotropic and chronotropic state of the heart is closely regulated by the parasympathetic system (in part through the activation of eNOS; Balligand *et al.*, 1993;

Feron *et al.*, 1998b), and the cardiac function is therefore highly dependent on the responsiveness to mAChR stimulation, which varies according to the degree of mAChR sequestration. In this study, we examined the subcellular mechanisms contributing to the sequestration of the m2 mAChR through the caveolar pathway in freshly isolated cardiac myocytes. We took advantage of the existence of membrane-permeant and -impermeant mAChR radioligands and of the distinct buoyant density of caveolar vesicles to demonstrate the GTP-driven budding of caveolae in cardiac myocytes exposed to the mAChR agonist carbachol. We also established the role of dynamin in promoting caveolar fission and mAChR sequestration. Finally, using transfected COS-7 cells, we observed that agonist-induced dynamin-dependent sequestration of m2 mAChR diminishes the extent of activatability of eNOS, a downstream signaling target.

Results

Time-dependent mAChR sequestration in cardiac myocyte caveolae

We first examined the potential sequestration of mAChRs targeted to cardiac myocyte caveolae (upon agonist stimulation) by comparing the binding pattern of membrane-permeant and -impermeant mAChR radioligands. Following exposure to the muscarinic cholinergic agonist carbachol (100 μ M) for 0–60 min, adult rat cardiac myocytes were washed extensively and the intact cells were then exposed for 3 h at 4°C to the radiolabeled lipophilic and hydrophilic mAChR antagonists [³H]QNB (quinuclidinyl benzylate) and [³H]NMS (*N*-methyl-scopolamine), respectively. Figure 1A shows that, whereas the [³H]QNB-specific binding did not reveal changes in total mAChR density, the fraction of receptors accessible to [³H]NMS progressively decreased with the time of agonist exposure; a maximum of 26 \pm 3% of mAChRs appeared sequestered after a 1 h exposure to carbachol. It is of note that in experiments performed on corresponding myocyte lysates in the presence of saponin (used to disrupt vesicle membranes), we could completely restore the specific binding of [³H]NMS (not shown), suggesting that 1 h after carbachol exposure, sequestered mAChRs were not degraded and remained susceptible to be recycled.

In a subsequent series of experiments, following exposure to carbachol (0–60 min), myocytes were lysed in a carbonate-containing buffer and fractionated by isopycnic centrifugation into caveolar and non-caveolar membrane fractions (see Materials and methods). The isolation of caveolar membranes from the remaining sarcolemmal and internal membranes was validated by using subcellular compartment-specific markers. Figure 1B shows that caveolin-3 and >90% of eNOS are concentrated in the low density CAV fraction; conversely, the plasma membrane protein (Na⁺,K⁺)-ATPase and the Golgi enzyme mannosidase were nearly completely (>95%) detected in non-caveolar membrane fractions (not shown). The distribution of agonist-stimulated mAChRs between the caveolar and non-caveolar membrane fractions was then examined following incubations with [³H]QNB and [³H]NMS, as detailed in Materials and methods. The data presented in Figure 1C reveal a time-dependent enrichment of mAChRs in caveolae with a maximum of 25–30%

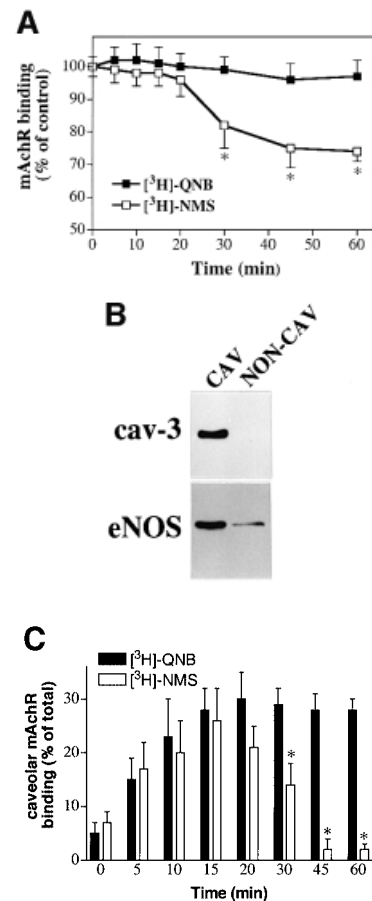


Fig. 1. Carbachol-induced targeting and sequestration of mAChRs in cardiac myocyte caveolae. (A) Time course of [³H]NMS (open squares) and [³H]QNB (filled squares) binding to intact cardiac myocytes exposed to 100 μ M carbachol. The data are expressed as a percentage (means \pm SEM, $n = 3$) of the total specific binding determined at time 0 ($*P < 0.01$ versus the corresponding [³H]QNB binding); the actual density of [³H]QNB-binding sites is 178 \pm 12 fmol/mg protein ($n = 3$). (B) Fractionation of cardiac myocytes. The distribution of caveolin-3 (cav-3) and eNOS between caveolae (CAV) and non-caveolae (NON-CAV) membrane-enriched fractions is shown; these data represent the result of a typical fractionation experiment from resting cardiac myocytes, as described in Materials and methods. (C) [³H]NMS (open columns) and [³H]QNB (black columns) binding to the caveolae-enriched fraction obtained from cardiac myocytes exposed to 100 μ M carbachol for the indicated time. The data are expressed as a percentage (means \pm SEM, $n = 3$) of the total amount of [³H]NMS- or [³H]QNB-specific binding determined in the CAV + NON-CAV fractions at time 0 ($*P < 0.01$ versus the corresponding [³H]QNB binding).

of total cell receptors after 15 min of carbachol exposure; this effect was observed independently of the radioligand used, suggesting that all mAChRs were still easily accessible at that time. However, while the proportion of caveolar [³H]QNB-bound mAChRs remained unchanged in myocytes exposed to carbachol for longer periods of time, [³H]NMS-specific binding rapidly decreased in the caveolar fraction and was almost undetectable after 45–60 min exposure to the agonist (Figure 1C).

Thus, the targeting of stimulated mAChRs to caveolae appeared to lead progressively to the caveolae pinching off and the consecutive sequestration of the translocated receptors. By convention, we will use the term

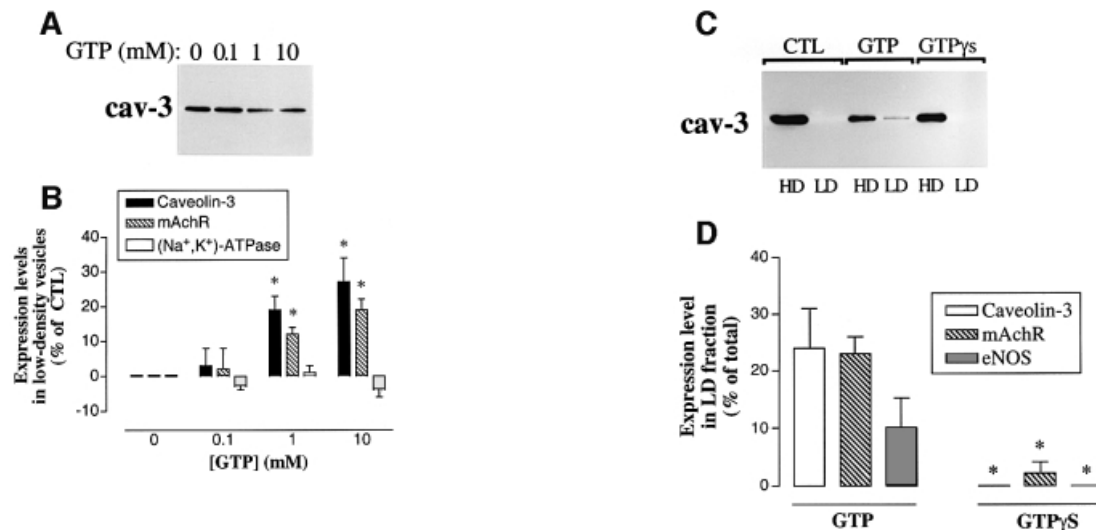


Fig. 2. GTP-mediated caveolar fission and mAChR internalization in carbachol-stimulated cardiac myocytes. **(A)** A representative caveolin-3 immunoblotting from plasma-membrane-enriched fractions obtained from carbachol-stimulated myocytes and exposed for 1 h to the indicated concentration of GTP. Note that when myocytes were first exposed to the mAChR antagonist atropine (instead of carbachol), no change in plasma-membrane caveolin-3 was observed (not shown). **(B)** Bar graph illustrating the amounts of caveolin-3 (black columns) translocated from the plasma membrane (in low density pinched-off vesicles) following GTP exposure [derived from the densitometric analysis of the caveolin-3 immunoblots shown in (A)] as well as the amounts of mAChRs (hatched columns) (estimated by [³H]QNB-specific binding) and (Na⁺,K⁺)-ATPase (open columns) (estimated by [³H]ouabain-specific binding). Data are shown as the means of three independent experiments and are expressed as the calculated percentage of the immunoblotting signal or of specific binding, respectively, determined in GTP-untreated plasma-membrane-enriched fractions (**P* < 0.01 versus no-GTP conditions). **(C)** The caveolin-3 immunoblotting from low (LD) and high density (HD) fractions obtained by isopycnic centrifugation from carbachol-stimulated, reversibly permeabilized cardiac myocytes. Myocytes were loaded, as indicated, with GTP, GTP γ S or control vehicle alone (CTL). **(D)** Bar graph illustrating the abundance of caveolin-3 (open columns), mAChRs (hatched columns) and eNOS (shaded columns) in LD fractions obtained from GTP- or GTP γ S-loaded myocytes. The data are expressed as percentages (means \pm SEM, *n* = 3) of the total amount of [³H]QNB-specific binding (mAChRs) or immunoblotting signal (caveolin-3, eNOS) determined in LD + HD fractions for each condition (**P* < 0.01 versus the corresponding GTP condition).

'sequestration' to refer to mAChRs that are inaccessible to the hydrophilic ligand [³H]NMS, i.e. receptors that are not only translocated to caveolae but trapped in 'closed' caveolar vesicles. It is of note that neither translocation nor sequestration was altered by treatments known to disrupt coated pit formation such as acidification or incubation in hypertonic sucrose (not shown).

GTP-dependent mAChR sequestration through caveolae budding and internalization

We next examined the GTP dependence of the agonist-induced sequestration of mAChRs in cardiac myocyte caveolae by using a cell-free assay for caveolar vesicle fission. Accordingly, myocytes were first exposed to carbachol for 15 min in order to initiate the translocation, but not the sequestration, of mAChRs to caveolae (see Figure 1C). Plasma-membrane-enriched extracts were then isolated from myocyte lysates by low speed centrifugation (as described in Materials and methods) and exposed for 1 h at 37°C to increasing concentrations of GTP. These membrane extracts were subsequently submitted to a centrifugation on a sucrose gradient in order to eliminate low density fractions containing GTP-driven pinched-off caveolae, i.e. to concentrate plasma membranes with undetached caveolae in the heavy fractions. These high density membrane fractions were then processed for caveolin-3 immunoblotting and quantification of mAChR density. Figure 2A shows that the level of caveolin-3 was decreased significantly in these fractions in proportion to the concentration of GTP. From this experiment, we could

estimate that the extent of caveolin-3 lost into low density fractions amounted to 19 and 27% at 1 and 10 mM GTP, respectively (see Figure 2B). We also used [³H]QNB and [³H]ouabain radioligands to detect any associated GTP-evoked changes in mAChR and (Na⁺,K⁺)-ATPase density, respectively. As shown in Figure 2B, the difference in specific binding between total lysates and collected high density fractions revealed the shift of a significant amount of mAChRs from GTP-exposed plasma-membrane fraction to budded vesicles, whereas no change in the subcellular location of the typical plasma-membrane (Na⁺,K⁺)-ATPase protein (Figure 2B) was observed following GTP treatment.

Since the integrity of the cell is likely to be required for efficient caveolar pinching off, in another set of experiments, intact myocytes were used to study the GTP dependence of the fission phenomenon. Accordingly, myocytes were exposed to carbachol for 15 min and subsequently permeabilized at low temperature to introduce excess amounts of GTP or GTP γ S [guanosine 5'-O-(3-thiotriphosphate)]; sham-permeabilized myocytes were used as controls. Following a further 15 min incubation at 37°C, loaded intact myocytes were lysed in the absence of carbonate (and without sonication) to avoid physicochemical detachment of caveolae. Crude homogenates were submitted to isopycnic centrifugation to collect low and high density fractions, as described in Materials and methods. Both fractions were then analyzed for caveolin-3 expression and [³H]QNB-specific binding. The immunoblotting presented in Figure 2C shows that caveolin-3 is

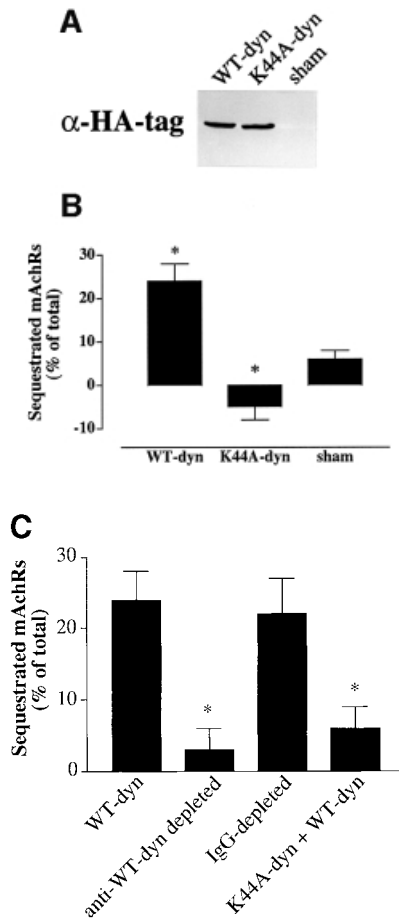


Fig. 3. Dynamin-mediated sequestration of mAChRs in carbachol-stimulated cardiac myocytes. **(A)** Immunoblot analysis (using anti-HA antibodies) of dynamin levels in the cytosolic extracts of COS cells transfected with expression vectors for either wild-type or K44A mutant dynamin; sham-transfected COS cell cytosol was used as control. **(B)** Bar graph illustrating the proportion of sequestered mAChRs in lysates from carbachol-stimulated myocytes after incubation with cytosolic extracts from sham-transfected COS cells, and COS cells expressing either wild-type or K44A mutant dynamin. The extent of sequestration is derived from the proportion of lost [3 H]NMS binding expressed as a percentage (means \pm SEM, $n = 3$) of the amount of total specific [3 H]NMS binding determined in myocyte extracts not exposed to COS cell cytosol ($*P < 0.05$ versus sequestered receptors in sham-transfected condition). **(C)** Bar graph illustrating the extent of mAChR sequestration in lysates of carbachol-stimulated myocytes incubated in the presence of cytosolic extracts from wild-type dynamin-expressing COS cells. In some experiments, recombinant dynamin was immunodepleted from the cytosol using anti-HA antibody immunoprecipitation; control cytosol was obtained following a non-specific IgG immunoprecipitation. In other experiments, K44A mutant dynamin-containing cytosol was pre-incubated with myocyte extracts before the exposure to wild-type dynamin. The data are expressed in percentages (means \pm SEM, $n = 3$) as in **(B)** ($*P < 0.01$ versus wild-type dynamin cytosol).

detected exclusively in the high density fraction from the GTP γ S- and sham-permeabilized myocytes. In contrast, in myocytes loaded with GTP, we detected caveolin-3 in both the low and high density membrane fractions (Figure 2C). While caveolin-3 signal was reproducibly below the detection level in the immunoblottings corresponding to GTP γ S- and sham-permeabilized myocytes,

three independent experiments led to the recovery of 17, 18 and 38% of caveolin-3 in the low density fraction from GTP-loaded myocytes (see Figure 2D). Similarly, while [3 H]QNB binding was detected almost exclusively in the heavy fraction from GTP γ S-loaded and control myocytes, a significant fraction ($23 \pm 3\%$, $P < 0.01$, $n = 3$) of radiolabeled receptors was found in the low density fractions (Figure 2D); the total specific [3 H]QNB binding was not different in the three groups (not shown). As shown in Figure 2D, we also detected eNOS expression in the low density fraction obtained from GTP-loaded myocytes. It should be noted that the extent of eNOS compartmentation in these free (vesicular) caveolae, although observed consistently, was lower ($10 \pm 5\%$, $n = 3$) than the degree of mAChR trapping in GTP-driven budded caveolae.

Together, these findings demonstrate that in two variants of a controlled *in vitro* caveolae fission assay, i.e. in the absence of any mechanical stress or detergent action used to purify caveolae, caveolar vesicles detach from cardiac myocyte sarcolemma in a GTP-dependent manner. This process led to the sequestration of 25–30% mAChRs, i.e. the same proportion of receptors that targeted to caveolae upon agonist stimulation (see Figure 1C), where they were shown to be associated with caveolin-3 (Feron *et al.*, 1997a).

Dynamin-dependent caveolae fission induced by mAChR stimulation

To examine whether the GTP-dependent caveolar budding leading to mAChR sequestration in cardiac myocytes was mediated by the GTPase dynamin, we used another *in vitro* assay of caveolae fission derived from a protocol established by Oh *et al.* (1998). Briefly, myocyte lysates were exposed to cytosol extracts from COS-7 cells expressing hemagglutinin (HA)-tagged wild-type dynamin or dominant-negative K44A dynamin mutant. We then took advantage of the non-permeant properties of the hydrophilic [3 H]NMS radioligand to probe the extent of caveolae budding. Transfected COS-7 cells were collected, lysed and submitted to a 100 000 g ultracentrifugation for 1 h in order to eliminate the membrane pellet. HA-tagged dynamin expression in the centrifugation supernatant was verified by immunoblotting. Figure 3A shows that similar amounts of wild-type and K44A dynamin are detected by anti-HA antibody. Before exposure to COS cell cytosols, myocytes were exposed to carbachol for 15 min, washed and lysed (in the absence of detergent). Dynamin-containing or sham-transfected COS cell cytosol was added to myocyte extracts, and the mixture was submitted to gentle stirring at room temperature for 1 h; membranes were then exposed to [3 H]NMS, as described in Materials and methods, to measure sequestered mAChR density. Incubation with cytosolic extracts from sham-transfected COS cells led to only a slight sequestration of mAChRs, as determined by the proportion of caveolae-sequestered receptors, i.e. inaccessible to the hydrophilic radioligand [3 H]NMS (Figure 3B). In contrast, incubation with wild-type dynamin-containing cytosol led to a significantly higher level of caveolae budding since sequestration of mAChRs amounted to $24 \pm 4\%$ of total receptors. Conversely, incubation of myocyte extracts with COS cytosol containing GTPase-inactive K44A

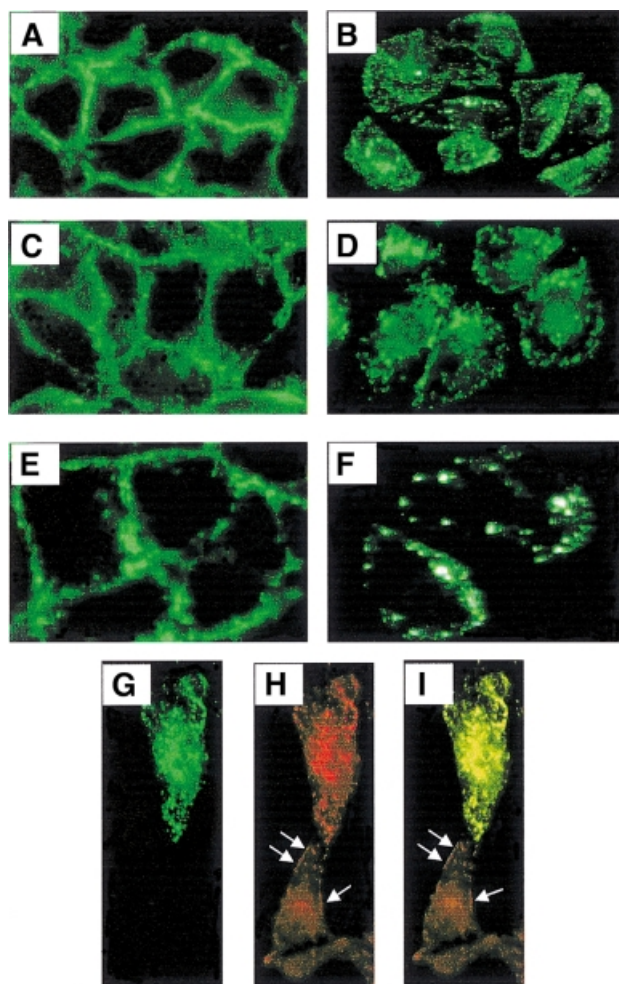


Fig. 4. Carbachol-stimulated m2 AChRs are redistributed with caveolin through a dynamin-mediated pathway insensitive to inhibitor of clathrin-coated pits. After fixation and permeabilization, m2AChR-expressing COS cells were stained with rat anti-m2 AChR antibody followed by FITC-conjugated anti-rat antibody (A–F). Images were visualized by conventional fluorescence microscopy. Except in (A), where cells were not pre-stimulated with an agonist, in all other panels (B–I) cells were pre-exposed for 15 min to carbachol. (B) No additional drug treatment; (C) + filipin; (D) + chlorpromazine; (E) + recombinant co-expression of the K44A dynamin mutant; (F) + recombinant co-expression of wild-type dynamin. In (G), carbachol-exposed cells were stained with the anti-m2 AChR antibody; note that in contrast to the upper cell, the lower cell barely expressed the receptor. (H) The same two cells as in (G) immunostained with rabbit anti-caveolin antibody followed by TRITC-conjugated anti-rabbit antibody. (I) Merged image from (G) and (H) showing co-localization of m2 AChRs and caveolin in the upper cell; arrows indicate the membrane staining for caveolin in the lower cell.

mutant dynamin blocked the caveolar fission process since it did not promote the sequestration of mAChRs but even decreased it when compared with the effects of cytosolic extracts from sham-transfected COS cells (Figure 3B); the difference probably reflects the contribution of native dynamin to the budding process. Importantly, total amounts of mAChRs were not different following exposure to wild-type or K44A mutant dynamin-containing cytosol since the [³H]QNB-specific binding amounted to 99 ± 3 and $103 \pm 3\%$, respectively, of the value obtained by exposing the myocyte homogenates to control COS cell

cytosol (not shown). As a control for the specific role of recombinant dynamin in the caveolae fission process, we repeated these experiments after depleting dynamin from the cytosolic extracts with anti-HA antibodies. As shown in Figure 3C, sequestration of mAChRs (as probed by the proportion of lost [³H]NMS-specific binding) was almost completely abrogated when dynamin-immunodepleted cytosol was used; control experiments using an irrelevant antibody failed to prevent sequestration of [³H]NMS-binding sites. In addition, pre-incubation of cardiac myocyte extracts with an excess of the dominant-negative K44A mutant dynamin abrogated the effects of wild-type dynamin exposure on mAChR sequestration (see Figure 3C).

It is of note that in the absence of carbachol pre-stimulation, dynamin was unable to induce mAChR sequestration from myocyte extracts. Thus, the co-incubation with recombinant wild-type dynamin but not the dominant-negative K44A dynamin promoted the fission of caveolae from membranes of carbachol-exposed myocytes, indicating that under the conditions of this assay, active dynamin was essential for closing mAChR-containing plasmalemmal caveolae.

Morphological analysis of the dynamin-dependent mAChR sequestration in a heterologous expression system

We have shown previously that, as in cardiac myocytes, agonist-stimulated m2 mAChRs recombinantly expressed in COS cells can be recovered in the caveolin-enriched low density fraction obtained by isopycnic centrifugation (Feron *et al.*, 1997b). Therefore, we used a specific anti-m2 antibody (exclusively recognizing recombinant m2 receptor) to visualize the subcellular localization of the m2 AChRs in transfected COS cells. As shown in Figure 4, while m2 mAChRs were distributed uniformly at the periphery of the cell before agonist stimulation (Figure 4A), exposure to carbachol led to a dramatic concentration of the receptors in focal areas (Figure 4B). To characterize this redistribution better, we repeated these experiments in the presence of filipin, an agent known to disrupt the internalization through caveolae, and chlorpromazine that blocks endocytosis of clathrin-coated pits (Orlandi and Fishman, 1998). As shown in Figure 4D, the pattern of carbachol-evoked mAChR redistribution was not influenced by chlorpromazine whereas it was dramatically inhibited by filipin treatment (Figure 4C); neither of these treatments altered mAChR distribution in unstimulated cells (not shown). We next used COS cells co-transfected with plasmids encoding the m2 mAChR and either wild-type or K44A mutant dynamin. As shown in Figure 4E, treatment with carbachol failed to induce m2 mAChR redistribution in cells expressing the dominant-negative K44A mutant dynamin; conversely, the effect of carbachol was more prominent in cells expressing an excess of recombinant wild-type dynamin (compare intense patchy staining in Figure 4F with B).

Finally, to obtain further evidence of mAChR internalization via caveolae, we used double immunostaining to examine whether mAChR redistribution paralleled the translocation of caveolin-1. COS cells express significant amounts of endogenous caveolin-1, which is located in both the periphery and intracellular areas in the absence of

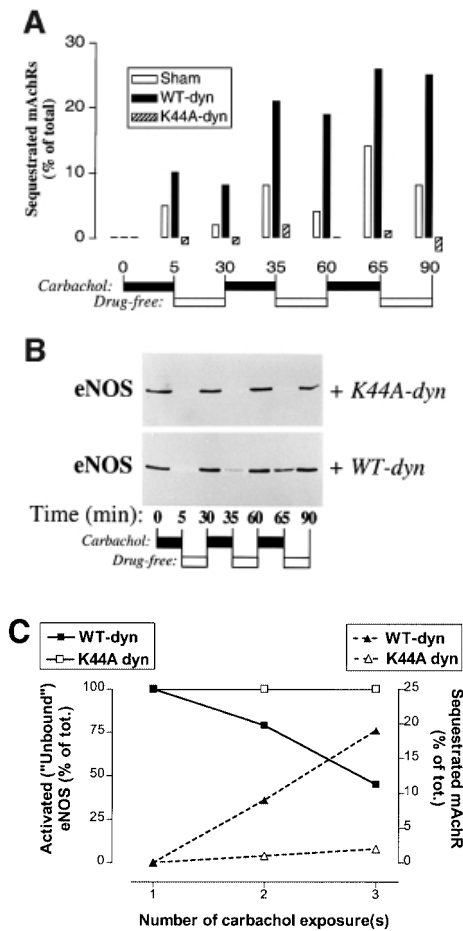


Fig. 5. Repeated stimulation of the m2 mAChR leads to dynamin-mediated attenuation of the muscarinic cholinergic coupling to the eNOS pathway. COS cells were transfected, as detailed in Materials and methods, with plasmids encoding the m2 mAChR, eNOS and either wild-type or K44A mutant dynamin. Cells grown to confluence in 6-well dishes were then exposed to carbachol during the following periods 0–5, 30–35 and 60–65 min and to drug-free medium in the intervals (5–30, 35–60 and 65–90 min). At each time point of this protocol, cells were washed and engaged in either radioligand binding (A) or caveolin immunoprecipitation experiments (B). (A) A bar graph illustrates the effects of wild-type (black columns) or K44A dynamin (hatched columns) expression on the m2 mAChR sequestration process. COS cells expressing m2 mAChR and eNOS but not recombinant dynamin were used as controls (open columns). [^3H]NMS-specific binding was used to quantify the amounts of sequestered m2 mAChR. Data (means \pm SEM) are expressed as the percentage of total m2 mAChR, calculated according to the formula: $[1 - (\text{[}^3\text{H}\text{]NMS-specific binding at the indicated time} / \text{[}^3\text{H}\text{]NMS-specific binding at time 0})] \times 100\%$. (B) eNOS immunoblots following caveolin immunoprecipitations performed on extracts of transfected cells exposed to the same protocol of carbachol pulse stimulations as used above. These experiments were performed twice with similar results. (C) A graph illustrating the inverse relationship between the proportion of activated, caveolin-free eNOS (left y-axis, solid lines) and the density of sequestered mAChRs (right y-axis, dotted lines) as a function of the number of rounds of carbachol exposure. Data are derived from the values presented in (A) (at time 0, 30 and 60 min), and from the densitometric analysis of immunoblots presented in (B).

agonist stimulation (not shown). Among these cells, 25% were transfected efficiently so that at the resting level, they expressed recombinant m2 mAChRs at the periphery of the cells as shown in Figure 4A. Interestingly, upon carbachol stimulation, only those cells that expressed the m2 mAChR

(see upper cell, Figure 4G) displayed a patchy redistribution of caveolin-1 (Figure 4H, upper cell) that exactly paralleled that of m2 mAChR (see Figure 4I, upper cell). Conversely, caveolin-1 immunostaining remained consistently detectable at the periphery of the cells that did not express mAChRs (see arrows in Figure 4H).

Thus, our morphological analysis is consistent with the previous biochemical evidence of mAChR sequestration into caveolar vesicles and validates the key role of dynamin in receptor trafficking.

Dynamin-dependent mAChR sequestration alters the carbachol-induced dissociation of the eNOS–caveolin heterocomplex

In order to test how the dynamin-mediated sequestration of mAChRs influences the activation of eNOS, we co-transfected COS-7 cells with plasmids encoding the m2 mAChR, eNOS and either wild-type or K44A mutant dynamin. We had shown previously that co-expression of eNOS in this heterologous system quantitatively and qualitatively recreates the mAChR agonist-induced disruption of the eNOS–caveolin interaction seen in myocytes (Feron *et al.*, 1998c). Since the proportion of eNOS complexed to caveolin, as measured by co-immunoprecipitation, is inversely correlated to the extent of NO production (Garcia-Cardena *et al.*, 1997; Feron *et al.*, 1998a,b,c), we used this approach in transfected COS cells to address the effects of dynamin-mediated mAChR sequestration on carbachol-evoked activation of eNOS. Briefly, cells were transfected in two sequences, as detailed in Materials and methods, and grown to confluence in 6-well dishes. Cells were exposed repeatedly to carbachol for 5 min, washed and incubated in drug-free medium during 25 min intervals. At different stages of this protocol, cells were washed and engaged in either radioligand binding or co-immunoprecipitation experiments.

Incubation with [^3H]NMS was performed to quantify the levels of m2 mAChR sequestration after each of the three 5 min pulses of carbachol exposure. Figure 5A shows that in control transfected cells that recombinantly expressed m2 mAChR and eNOS, but not dynamin, sequestration of mAChRs reached 5, 9 and 13% after each of the three consecutive pulses of stimulation, respectively. Furthermore, the corresponding 25 min recovery periods allowed the sequestration process to be partially reversed. In transfected cells expressing wild-type dynamin, mAChR sequestration reached 10% after the first 5 min exposure to carbachol, and [^3H]NMS-binding sites were still inaccessible after the first 25 min recovery period. The second and third 5 min pulses of carbachol stimulation led to 21 and 26% mAChR sequestration, which appeared to be irreversible since extensive washing did not decrease the fraction of masked mAChRs. In contrast, in K44A mutant dynamin-expressing cells, we did not observe carbachol-induced mAChR sequestration following each of the repeated pulses of stimulation. In these different experiments, control binding experiments using the lipophilic [^3H]QNB did not reveal any significant difference in total mAChR density between the different stages of the repeated stimulation protocol (not shown).

In parallel with these binding experiments, transfected cells were collected before and after each carbachol pulse, and corresponding lysates were immunoprecipitated using

caveolin antibodies. The co-immunoprecipitation of eNOS was examined by western blotting and quantified by optical densitometry. Figure 5B shows that in cells co-expressing eNOS, m2 mAChR and the K44A mutant dynamin, the caveolin–eNOS heterocomplex was disrupted following each pulse of carbachol stimulation and re-formed in the next 25 min. In contrast, in the cells co-expressing wild-type dynamin instead of the K44A mutant, the extent of the eNOS–caveolin complex disruption (reflective of eNOS activation) progressively decreased following the repeated carbachol stimulation, as shown in Figure 5B by the increasing amounts of eNOS in the caveolin immunoprecipitate (0, 21 and 55% at 5, 35 and 65 min, respectively).

Figure 5C summarizes the data reported above and illustrates the inverse relationship between the proportion of activated, caveolin-free eNOS (see Figure 5B) and the extent of sequestered mAChRs (see Figure 5A) as a function of the number of rounds of agonist stimulation. Note that in K44A dynamin-expressing cells, the activation of eNOS is maximal for each round of agonist stimulation, in agreement with the lack of receptor internalization; in contrast, the progressive and irreversible sequestration of mAChRs in the presence of wild-type dynamin leads to the inability of the second and third rounds of agonist stimulation to activate caveolar eNOS completely.

Thus, dynamin-mediated sequestration of only 20–25% of mAChRs was associated with a 55% increase in inactivated (caveolin-associated) eNOS, thereby providing evidence that dynamin-driven mAChR sequestration may alter the coupling of mAChRs to the NO pathway.

Discussion

This study provides the first characterization of molecular mechanisms involved in the sequestration of a GPCR through caveolar fission. The evidence also suggests that this endocytic pathway may participate in the desensitization of receptor signaling. We took advantage of the differential binding pattern of membrane-permeant and -impermeant mAChR radioligands to establish that the receptor sequestration is first preceded by a stage of dynamic translocation of mAChRs in caveolae ‘open’ to the extracellular medium, and that caveolae are then pinched off from the sarcolemma in a GTP-dependent fashion, leading to the sequestration of ~25–30% of the mAChRs. Importantly, the budding of caveolae was associated with a loss of a significant amount of caveolin-3 from the sarcolemmal pool, and this was paralleled by its recovery in low density membranes isolated without the use of any detergent or carbonate treatment. These findings therefore document that caveolar budding is a physiologically relevant cellular process promoting mAChR internalization in cardiac myocytes. This endocytic pathway appears similar to the process of potocytosis initially named to describe the ability of caveolae to concentrate and internalize ligand-bound folate receptors and to release folate in the cytoplasm (Anderson *et al.*, 1992), and later extended to the ability of caveolae to concentrate and move other molecules into the cell (see Anderson, 1998).

We have identified dynamin as the GTPase involved in the process of mAChR sequestration in cardiac myocyte caveolae. Co-incubation of agonist-stimulated cardiac myocyte extracts and dynamin-containing COS cell cytosol was, indeed, sufficient to induce the *in vitro* fission of caveolae, as probed by a decrease in the binding of the membrane-impermeant mAChR ligand [³H]NMS. The internalization of the caveolar resident cholera toxin B chain recently was also documented to occur through a dynamin-dependent pathway (Henley *et al.*, 1998; Oh *et al.*, 1998). Here we have shown that the m2 mAChR, a non-related receptor type, also follows this mode of sequestration in primary cultures of cardiac myocytes and in transfected COS cells (although our data do not exclude the possibility of receptor sequestration through distinct vesicular pathways in other cell types). Interestingly, in the more tractable transfected COS cell model, we could document dynamin-mediated adaptive changes in response to repetitive agonist stimulation, which were characterized by increasing amounts of m2 mAChRs irreversibly sequestered in caveolae. In our experiments, the use of both the non-hydrolyzable GTP analog GTPγS and the GTPase-deficient K44A mutant dynamin allowed us to ascertain the requirement for active dynamin to promote the sequestration and internalization of m2 mAChR within budded caveolae. This observation is in apparent contrast to two recent studies (Pals-Rylaarsdam *et al.*, 1997; Vögler *et al.*, 1998) which concluded that the m2 mAChR displays a dynamin-independent sequestration pathway in transfected human embryonic kidney (HEK) cells. However, although these authors recently confirmed that this mode of sequestration distinguishes the m2 mAChR from the other mAChR subtypes in HEK cells (Lee *et al.*, 1998; Vögler *et al.*, 1999), they also acknowledged that their findings could not be reproduced in COS cells (Pals-Rylaarsdam *et al.*, 1997; Vögler *et al.*, 1998). This emphasizes the plasticity of the GPCR desensitization but more importantly underscores the need for studies on native tissues or primary isolates of specialized cells. Yet our identification of dynamin and GTP as essential mediators of mAChR sequestration through caveolae budding in cardiac myocytes does not preclude their implication in other signaling pathways contributing (or not) to the effects reported here. Moreover, it should be noted that in the absence of carbachol treatment, we never observed significant levels of mAChRs sequestration despite the presence of GTP, suggesting that the fission of caveolae requires additional mechanisms induced by the agonist binding to mAChR. This may explain why only some populations of caveolae are pinched off and a limited amount of plasmalemmal caveolin is internalized and recovered in low density vesicles. This is also consistent with the absence of correlation between total caveolin abundance and caveolae density as reported by Smart *et al.* (1994) or the existence of structurally different subpopulations of caveolae as proposed by Scherer *et al.* (1995).

Finally, this study also illustrates that the caveolar sequestration of agonist-stimulated mAChRs may alter the coupling of the muscarinic cholinergic receptor to eNOS activation. In cardiac myocytes (Feron *et al.*, 1998b), as well as in transfected COS cells co-expressing the m2 mAChR and eNOS (Feron *et al.*, 1998c; this study), stimulation with the Ca²⁺-mobilizing agonist carbachol

leads to the Ca²⁺/calmodulin-mediated disruption of the inhibitory eNOS–caveolin complex, thereby leading to the stimulation of NO production and subsequent generation of cGMP. We now report that by co-expressing wild-type dynamin in the heterologous model of transfected COS cells, repeated stimulations with carbachol progressively failed to induce the disruption of the eNOS–caveolin heterocomplex. The inaccessibility to extracellular agonists of mAChRs sequestered in budded caveolae (see above) certainly accounts for part of the decrease in eNOS activation following agonist pulse stimulations. However, the striking inhibition of the eNOS–caveolin complex disruption observed with only 25% of m2 mAChR internalized (see Figure 5C) suggests some dedication of this population of receptors to mediating downstream NO signaling, or that additional, as yet undetermined, mechanisms are at play.

We have reported previously that the caveolar compartmentation of eNOS was playing a paradoxical role, both tonically repressing basal eNOS activity by the enzyme's interactions with caveolin, and also ensuring the efficient activation of the enzyme upon agonist stimulation (Feron *et al.*, 1998b, 1999). Our data now suggest that caveolae may also promote further desensitization of an initially facilitated response. We have documented the existence of a dynamin-mediated process of mAChR internalization in caveolae that accounts, at least in part, for the negative feedback mechanism terminating the stimulated NO production in cardiac myocytes. Interestingly, Carman *et al.* (1999) have recently reported the enrichment of various GRKs in caveolae. Combined with our findings, this indicates that caveolae are likely to be involved, as clathrin-coated pits, in similar processes of GPCR sequestration and phosphorylation. Further investigations will be required to define the pathways regulating the apparent plasticity in GPCR endocytic processes.

Materials and methods

Materials

[³H]NMS and [³H]QNB were purchased from NEN Life Science Products. Anti-caveolin and anti-eNOS antibodies were obtained from Transduction Laboratories and anti-HA antibody from Boehringer Mannheim. The eukaryotic expression vectors encoding the following proteins were used: m2 mAChR in pcDNA3 (Bonner *et al.*, 1987) (gift from T.I. Bonner, National Institute of Mental Health, Bethesda, MD), caveolin-3 in pCB-7 (gift from M. Lisanti, Einstein College of Medicine, NY), HA-tagged human dynamin (wild type or K44A mutant) in pRK5 (gift from J.L. Benovic, Thomas Jefferson University, PA) and β-gal in pcDNA3 (Invitrogen, Carlsbad, CA).

Cardiac myocyte culture and subfractionation

Purified adult rat ventricular myocytes (ARVMs) were prepared as previously reported (Feron *et al.*, 1996), plated on laminin and cultured for 10–12 h in serum-free medium. ARVMs were then incubated at 37°C in HEPES-buffered Dulbecco's modified Eagle's medium (DMEM) with or without carbachol (100 μM) for the indicated period of time. After washing, myocytes were either incubated directly in the presence of radioligands (see below), or lysed and fractionated on sucrose gradient. In the latter case, ARVMs were scraped into a freshly prepared solution of 200 mM Na₂CO₃ and lysed by sonication (three 5 s bursts, minimal output power) using a Branson sonifier B-12 (Branson Ultrasonic Corp., Danbury, CT), as previously reported (Feron *et al.*, 1997a). 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 5–18–25–35% discontinuous sucrose gradient (in MBS containing 100 mM Na₂CO₃) for an overnight ultracentrifugation (150 000 g); in preliminary experiments, we determined that the 5–18%

sucrose interface is optimal to concentrate >95% of the caveolin-3 protein from cardiac myocytes. The gradient was fractionated into two fractions corresponding to sucrose concentrations 5–18% and 25–45% (including the 18–25% interface), which were named the caveolar (CAV) and non-caveolar (NON-CAV) membrane fractions, respectively.

mAChR binding experiments

To measure the changes in the density of mAChRs present on the cell surface following agonist exposure, the radiolabeled hydrophilic antagonist [³H]NMS and lipophilic antagonist [³H]QNB were used. Briefly, carbachol-stimulated cells were washed carefully with 5 × 10 ml of ice-cold phosphate-buffered saline (PBS), and incubated with 2 nM [³H]NMS or 1 nM [³H]QNB in HEPES-buffered DMEM for 3 h at 4°C; non-specific binding was defined in the presence of 1 μM atropine. Cells were then rinsed with 3 × 10 ml of ice-cold PBS, solubilized in 1% Triton X-100 and transferred into counting vials containing 10 ml of scintillant. Sequestration is expressed as (1 – ratio of cell surface receptors of carbachol-treated versus non-treated cells) × 100%; protein assays were performed to correct for any differences in cell number between plates.

In some experiments, binding experiments were carried out on gradient fractions, which were then buffered at pH 7.4 with 10 mM HEPES and adjusted to 5 mM MgCl₂, 1 mM EGTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride. Aliquots of membrane fractions were incubated with 2 nM [³H]QNB or 1 nM [³H]NMS at 30°C for 2 h; non-specific binding was determined in the presence of 3 μM atropine. Assays were performed in triplicate and terminated by rapid filtration on Whatman GF/B glass filters. Samples were transferred in counting vials containing 10 ml of scintillant, and the radioactivity was determined in a liquid scintillation counter.

COS-7 cells transfection and culture

COS-7 cells were transfected using Lipofectamine reagent (Life Technologies) as previously reported (Feron *et al.*, 1998a,c). When triple co-transfection was used (see text), COS-7 cells were first transfected with plasmids encoding the m2 mAChR and eNOS, cultured for 16 h and transfected again with expression vectors for either wild-type or K44A mutant dynamin; note that control COS cells were transfected with the same amount of a β-galactosidase-encoding plasmid. Cells were grown to confluence in 6-well dishes and collected in OG (octylglucoside) buffer containing 50 mM Tris–HCl pH 7.4, 60 mM OG, 125 mM NaCl, 2 mM dithiothreitol (DTT), 50 μM EGTA and various protease inhibitors, as previously described (Feron *et al.*, 1998a,c). For cytosolic extract isolation, transfected cells were lysed in PBS, and crude homogenates were submitted to 100 000 g centrifugation to collect the supernatant fraction.

Caveolae fission assays

Plasmalemma-enriched fractions from cardiac myocytes were prepared as previously reported (Belhassen *et al.*, 1997). Briefly, myocytes (exposed for 15 min to carbachol) were lysed in a buffer containing 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT and protease inhibitors as above, and then submitted to a 15 min centrifugation at low speed (8000 g, 4°C). A cell-free assay for caveolar fission was then carried out according to a protocol slightly modified from Schnitzer *et al.* (1996). Briefly, pelleted membranes were resuspended in a buffer containing 1 mM ATP, 10 U/ml creatine phosphokinase and 10 mM phosphocreatine, and were then exposed for 1 h at 37°C to 1 mM GTP or GTPγS. Membranes were finally mixed with ice-cold sucrose to achieve a final concentration of 25% (in the absence of carbonate treatment); the mixture was then topped with 10% sucrose and, after ultracentrifugation, the pelleted membranes were processed for immunoblotting.

In a second series of experiments, myocytes were permeabilized according to a method modified from a protocol that we recently developed for the introduction of synthetic peptides into neonatal myocytes (Feron *et al.*, 1998b). Briefly, following a 15 min exposure to carbachol, myocytes were slowly brought down in temperature by incubation with PBS for 30 min on ice. The PBS was discarded, and freshly prepared (ice-cold) permeabilization buffer (20 mM HEPES pH 7.4, 10 mM EGTA, 140 mM KCl, 5 mM Na₃, 5 mM oxalic acid dipotassium salt, 5 mM ATP, 50 U/ml creatine phosphokinase and 50 mM phosphocreatine) was added to the myocytes with 5 mM GTP or GTPγS for 15 min; this treatment spontaneously detached myocytes from their support. Myocytes were then collected by centrifugation and washed three times with PBS at room temperature. The suspension of myocytes was then incubated for 15 min at 37°C in PBS containing 1 mM Ca²⁺. Finally, the PBS was aspirated gently and the myocytes were fractionated

(in the absence of carbonate treatment) on a 5–18–25–35% discontinuous sucrose gradient to separate low and high density membranes as detailed above.

Caveolae fission was also examined by using a third protocol modeled on that of Oh *et al.* (1998). Briefly, myocytes (exposed for 15 min to carbachol) were lysed in the absence of detergent or sodium carbonate, and crude homogenates were exposed for 1 h to recombinant wild-type or K44A mutant dynamin present in cytosolic extracts (1 mg protein/ml) from transfected COS cells. Radioligand-binding experiments were then performed as described above.

Immunoprecipitation and immunoblotting

Heat-denatured proteins from cell lysates or caveolin immunoprecipitates were separated by SDS-PAGE, and electrotransferred for immunoblotting as previously described (Feron *et al.*, 1998a,c).

Immunofluorescence

Transfected COS cells cultured on glass coverslips were washed three times with PBS and fixed for 30 min with 4% paraformaldehyde in PBS. Fixed cells were rinsed with PBS, incubated for 5 min at room temperature with 0.1 mM glycine in 2% bovine serum albumin (BSA)-PBS and then with 0.5% Triton X-100 in PBS. Permeabilized cells were rinsed with PBS, incubated with 2% BSA-PBS for 30 min at 37°C in a humidified incubator, and then with primary antibodies diluted in 2% BSA-PBS for 90 min at 37°C, i.e. anti-m2 mAChR (Chemicon) or anti-caveolin-1 antibodies (Transduction Laboratories). After three washes with 2% BSA-PBS, cells were incubated with the secondary antibodies for 90 min at 37°C: fluorescein isothiocyanate (FITC)-conjugated anti-rat or tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit antibodies (Jackson Immunoresearch). Cells were washed three times with 2% BSA-PBS, and slides were mounted with anti-fade reagent (Vectashield) and examined by fluorescence microscopy.

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