

Internalization of the M₂ muscarinic acetylcholine receptor proceeds through an atypical pathway in HEK293 cells that is independent of clathrin and caveolae

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SUMMARY

The M₂ muscarinic acetylcholine receptor is a G-protein coupled receptor that undergoes agonist-induced internalization through an unidentified pathway that exhibits an atypical dependence on dynamin function in HEK293 cells. In this report we utilized several independent approaches to reveal that the internalization of the M₂ muscarinic acetylcholine receptor did not utilize clathrin-coated pits or caveolae. However, we did observe that treatment with hypertonic sucrose, which is widely reported to specifically inhibit endocytosis through

clathrin-coated pits, completely inhibited internalization of the M₂ muscarinic acetylcholine receptor. Thus, the pathway that mediates the internalization of the M₂ muscarinic acetylcholine receptor appears to be atypical in that it exhibits an unusual sensitivity to dynamin and is inhibited by hypertonic sucrose but lacks the involvement of clathrin and caveolae.

Key words: Muscarinic receptor, Internalization, Caveolae, Clathrin, Sucrose

INTRODUCTION

Agonist stimulation of G-protein coupled receptors (GPCR) leads to a number of cellular events that regulate the GPCRs in order to control the level of the response within the cell. The removal of the GPCRs from the cell surface by a process known as receptor endocytosis or internalization is an important level of regulation of GPCRs (for reviews see Bunemann et al., 1999; Krupnick and Benovic, 1998; Lefkowitz, 1998). However, agonist-mediated internalization of GPCRs is a complex process that is not well understood. Multiple mechanisms for the agonist-induced internalization of GPCRs have been suggested. Internalization of the β_2 -adrenergic receptor (β_2 AR) by clathrin-coated pits (CCP) is probably the best studied pathway of internalization of GPCRs (Krupnick and Benovic, 1998; Lefkowitz, 1998). This pathway is dependent on the GTPase dynamin (Herskovits et al., 1993) and has been demonstrated to be utilized by a number of other GPCRs (Krupnick and Benovic, 1998). However, additional pathways also exist for the internalization of other GPCRs.

The M₂ muscarinic acetylcholine receptor (M₂ mAChR) is a member of the G-protein coupled receptor (GPCR) superfamily that plays an important role in the regulation of various physiological processes. The M₂ mAChR has been demonstrated to be regulated differently than most GPCRs that have been studied. When expressed in HEK293 cells the M₂ mAChR undergoes internalization via an atypical pathway that has yet to be identified. It was demonstrated that, when expressed in HEK293 cells, internalization of the M₂ mAChR was

independent of arrestin proteins and was insensitive to the dominant-negative K44A dynamin mutant (Pals-Rylaarsdam et al., 1997; Vogler et al., 1998). However, it was recently demonstrated that other mutant isoforms of dynamin were able to inhibit internalization of the M₂ mAChR (Werbonat et al., 2000). Both a mutant dynamin isoform lacking all three GTP binding domains, the N272 mutant, and a mutant dynamin isoform unable to be stimulated by phosphatidyl 4,5-bisphosphate, the K535M mutant, were able to inhibit internalization of the M₂ mAChR in HEK293 cells (Werbonat et al., 2000). Thus, the atypical pathway of internalization that is utilized by the M₂ mAChR in HEK293 cells appears to show a distinct sensitivity to dynamin. It is interesting that the K44A dynamin mutant, which is able to inhibit internalization of other GPCRs, such as the β_2 AR (Pals-Rylaarsdam et al., 1997) was unable to inhibit internalization of the M₂ mAChR (Pals-Rylaarsdam et al., 1997; Vogler et al., 1998). This suggests that the atypical pathway of internalization of the M₂ mAChR is distinct from those utilized by other GPCRs. In these studies, we have examined various dynamin-dependent endocytic pathways for their possible role in the internalization of the M₂ mAChR in an attempt to gain more information about the poorly understood pathway by which these receptors internalize.

MATERIALS AND METHODS

Materials

Cell culture reagents were purchased from Mediatech, Inc (Herndon,

VA). ^3H -labeled *N*-methyl scopolamine (^3H]NMS) was from Dupont NEN (Boston, MA). Nystatin was purchased from ICN Biomedicals, Inc. (Aurora, OH). The rat anti-M₂ mAChR antibody was purchased from Chemicon International, Inc (Temecula, CA) and the rabbit anti-caveolin antibody, which recognizes all known caveolin isoforms, was purchased from Transduction Laboratories (Lexington, KY). The mouse anti-clathrin X22 antibody was purchased from Affinity Bioreagents, Inc. (Golden, CO). The TRITC-conjugated goat anti-rat and the FITC-conjugated goat anti-rabbit antibodies were from Kirkegaard & Perry Laboratories (Gaithersburg, MD). The anti-T7 tag antibody (to recognize the clathrin hub mutant) was from Novagen, Inc. (Madison, WI). The enhanced chemiluminescence reagents were from Pierce (Rockford, IL). The B/C domain of amphiphysin and the rabbit anti-amphiphysin antibody were generous gifts from Pietro De Camilli (Yale University). The pcDM8T7 Hub construct (a dominant negative clathrin mutant) was a generous gift from F. M. Brodsky (University of California, San Francisco). Other reagents were purchased from Sigma (St Louis, MO).

Cell culture and transfection

HEK293 cells stably expressing the M₂ mAChR were previously described (Pals-Rylaarsdam and Hosey, 1997). Cell surface receptor expression levels for the M₂ mAChR were 200-300 fmol/mg protein. Feline cardiac atrial myocytes were prepared as described (Follmer et al., 1987). Transient transfections were performed using HEK-tsA201 cells (hereafter referred to as tsA201 cells), a clone of HEK293 cells stably expressing simian virus 40 large T antigen (Margolskee et al., 1993) to increase cellular expression of the target proteins. tsA201 cells were transfected on 100 mm plates using the calcium phosphate precipitation method as described (Pals-Rylaarsdam et al., 1995). Cell surface receptor levels for the M₂ mAChR were 200 fmol/mg protein to 2.7 pmol/mg protein. The amount of DNA used per transfection was as follows: pcMV M₂ mAChR, 3-5 μg ; pcDNA3 B/C amphiphysin, 10 μg ; pcDM8T7 Hub, 10 μg .

Receptor internalization assay

Internalization assays were performed by assessing the observed change in the number of M₂ mAChRs located at the cell surface as a function of time in the presence of the agonist carbachol (CCh) as described previously (Roseberry and Hosey, 1999).

Immunoblot analysis of cellular lysates

Expression of the clathrin hub domain (Liu et al., 1998) and the B/C domain of amphiphysin (Slepnev et al., 1998) were analyzed by western blotting as described previously (Lee et al., 1998). Primary antibodies were diluted as follows: anti-amphiphysin, 1:200; anti-T7 tag (to detect the clathrin hub), 1:10000.

Immunofluorescent staining of cells

HEK293 cells stably expressing the M₂ mAChR, or freshly isolated cardiac atrial myocytes, were seeded onto poly-L-lysine-coated coverslips and incubated at 37°C overnight prior to fixation and staining as described previously (Roseberry and Hosey, 1999). For experiments utilizing plasma membrane patches, the patches were isolated prior to fixation as described below. Primary antibodies were diluted as follows: anti-M₂ mAChR, 1:200 (5 $\mu\text{g}/\text{ml}$ final); anti-caveolin, 1:250 (4 $\mu\text{g}/\text{ml}$ final); anti-clathrin, 1:10000 (0.6 $\mu\text{g}/\text{ml}$ final). For double labeling experiments, two primary antibodies were used simultaneously.

Preparation of isolated plasma membrane patches

Plasma membrane patches were prepared using a technique (Cao et al., 1998) adapted from that of Sanan and Anderson (Sanan and Anderson, 1991). Coverslips containing treated cells were chilled in ice-cold PBS at 4°C on ice for 10 minutes. The coverslips were then inverted onto another chilled, poly-L-lysine coated coverslip. Slight pressure was applied using a rubber stopper, and excess fluid forced

from between the coverslips was aspirated. The top coverslip was ripped away, leaving the bottom coverslip with the isolated plasma membrane fractions. The plasma membrane fraction containing coverslips were then fixed and stained with antibodies as described above.

Internalization of cholera toxin

Internalization of cholera toxin was performed as described previously (Henley et al., 1998; Oh et al., 1998). Cells were plated to coverslips as described above and treated with or without nystatin for 3 hours at 37°C. Cells were refed with ice-cold DMEM containing 5 $\mu\text{g}/\text{ml}$ FITC-labeled cholera toxin and incubated at 4°C for 45-60 minutes to allow for binding of the toxin. Cells were washed with ice-cold DMEM to remove unbound toxin, refed with fresh DMEM, and incubated at either 4°C or 37°C for 45-60 minutes. Cells were then washed with PBS, fixed with ice-cold methanol, washed again with PBS, and mounted to slides for viewing on a confocal microscope.

RESULTS AND DISCUSSION

Internalization of the M₂ mAChR is sensitive to hypertonic sucrose

While the internalization of the M₂ mAChR is not inhibited by the K44A dynamin mutant, other mutant dynamin isoforms were able to inhibit internalization of the M₂ mAChR (Werbonat et al., 2000). Since internalization of the M₂ mAChR is dependent on dynamin function it was important to clarify if there was any role for clathrin-mediated endocytosis in the internalization of the M₂ mAChR in HEK293 and tsA201 cells. Treatment of cells with hypertonic sucrose is generally reported to specifically inhibit endocytosis through clathrin-coated pits (CCP) (Hansen et al., 1993). Treatment of stably transfected HEK293 cells with CCh led to a rapid and extensive internalization of the M₂ mAChRs (Fig. 1) as previously reported (Pals-Rylaarsdam and Hosey, 1997; Roseberry and Hosey, 1999). Interestingly, treatment with hypertonic sucrose prior to internalization of the M₂ mAChRs caused total ablation of internalization (Fig. 1). If sucrose was truly a selective inhibitor of clathrin-mediated endocytosis, this interesting finding suggested a potential role for clathrin-mediated endocytosis in the atypical internalization of the M₂ mAChR. Thus, we decided to more thoroughly examine a role for clathrin-mediated endocytosis in the internalization of the M₂ mAChR.

Are clathrin-coated pits involved in the internalization of the M₂ mAChR?

We sought to directly examine the role of clathrin in the internalization of the M₂ mAChR through the use of two dominant-negative inhibitors of clathrin-mediated endocytosis. We expressed the M₂ mAChR with the clathrin hub mutant (Liu et al., 1998) or the B/C domain of amphiphysin (Slepnev et al., 1998) in tsA201 cells and examined the effects on the internalization of the M₂ mAChR. While there appeared to be a tendency for a slight inhibition of internalization at each time point in the presence of the clathrin hub mutant, there was no significant effect on internalization of the M₂ mAChR (Fig. 2A). Similarly, the amphiphysin construct had no effect on internalization of the M₂ mAChR except after a 60 minute treatment with the muscarinic agonist CCh (Fig. 2B). Previous studies have demonstrated that the clathrin hub mutant

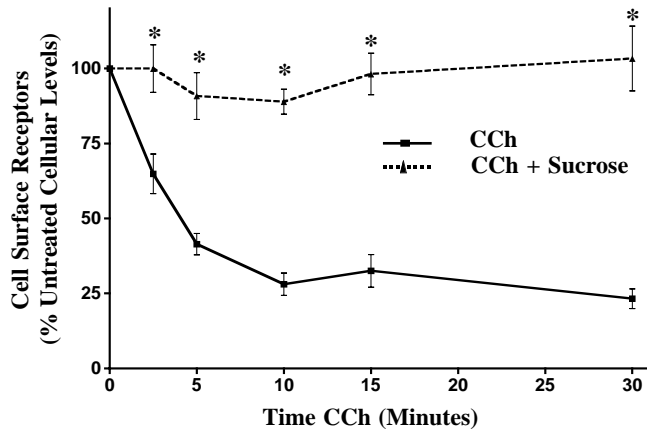


Fig. 1. Internalization of M₂ mAChRs with hypertonic sucrose. HEK293 cells stably expressing M₂ mAChRs were untreated or treated with 400 mM hypertonic sucrose prior to the addition of CCh for the times indicated. Cell surface receptor levels were measured using the hydrophilic antagonist [³H]NMS (1.0–2.0 nM). Results shown are means ± s.e.m. of 5 independent experiments. **P* < 0.05 vs control.

inhibited internalization of the M₁, M₃ and M₄ mAChRs after a 60 minute treatment with CCh in tsA201 cells, while the internalization of the M₂ mAChR was unaffected under identical conditions (Vogler et al., 1999). In this study, the effects of the clathrin hub mutant on the internalization of mAChR subtypes were only tested at a single time point following CCh treatment. Here, we more closely examined the effects of the hub mutant at multiple time points following CCh treatment. We have also examined the effects of expression of the B/C domain of amphiphysin on internalization of the M₂ mAChR at various time points. While the B/C domain of amphiphysin had no effect on the internalization of the M₂ mAChR, preliminary experiments suggested that the B/C domain of amphiphysin was able to inhibit internalization of the M₃ mAChR in tsA201 cells (data not shown). Since neither the B/C domain of amphiphysin nor the clathrin hub mutant was able to inhibit internalization of the M₂ mAChR (Fig. 2), while both constructs were able to inhibit internalization of other mAChR subtypes (Vogler et al., 1999; and data not shown), it appears that the internalization of the M₂ mAChR is mediated by a different pathway than that utilized by other mAChR subtypes. However, the experiments in Fig. 2 did raise the possibility that a small proportion of M₂ mAChRs may internalize via CCPs.

Based on the above results and a recent report demonstrating that distinct populations of CCPs mediate the internalization of different proteins (Cao et al., 1998), we explored the possibility that if the M₂ mAChR was associating with only a small population of CCPs, this might be detected by the use of immunofluorescent microscopy. We examined the immunofluorescent staining of the M₂ mAChR and clathrin in intact cells, where sections were taken at the very bottom of cells, in order to examine the staining patterns of the receptors and clathrin near the plasma membrane (Gaidarov et al., 1999). In addition, the distribution of M₂ mAChRs and clathrin were examined in isolated patches of the plasma membrane, through techniques that have previously been utilized to examine internalization of the β₂AR (Cao et al., 1998). In untreated cells,

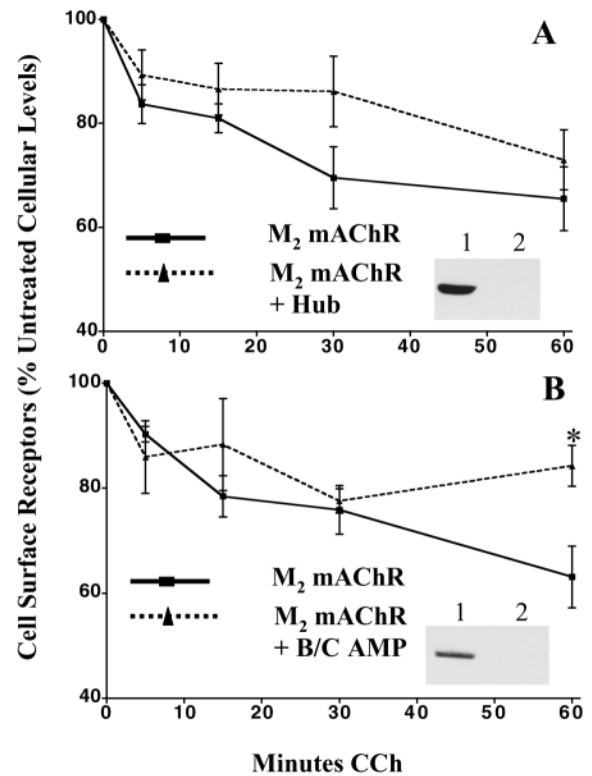


Fig. 2. Internalization of M₂ mAChRs with inhibitors of clathrin-mediated endocytosis. tsA201 cells were transfected with the M₂ mAChR ± dominant negative inhibitors of clathrin-mediated endocytosis, and internalization was examined. (A) Internalization of the M₂ mAChR ± the dominant negative clathrin hub mutant. tsA201 cells were transfected with the M₂ mAChR + empty pcDNA3 or pcDM8T7 Hub followed by treatment with CCh for the indicated times and measurement of cell surface receptors. Results shown are means ± s.e.m. of 4–5 independent experiments. No statistically significant differences were observed for internalization ± the clathrin hub mutant. Inset: western blot of cellular lysates with anti-T7 antibodies. Lane 1: M₂ mAChR + pcDM8T7 Hub; Lane 2: M₂ mAChR + pcDNA3. (B) Internalization of the M₂ mAChR ± dominant negative amphiphysin. tsA201 cells were transfected with the M₂ mAChR + empty pcDNA3 or the B/C domain of amphiphysin followed by treatment with CCh for the indicated times and measurement of cell surface receptors. Inset: western blot of cellular lysates with anti-amphiphysin antibodies. Lane 1: M₂ mAChR + B/C amphiphysin; Lane 2: M₂ mAChR + pcDNA3. Results shown are means ± s.e.m. of 4–5 independent experiments. **P* < 0.05 vs control.

there was extensive staining of both the M₂ mAChR and clathrin in intact cells (Fig. 3A–C) and in isolated plasma membrane patches (Fig. 4A–C). While there also appeared to be some colocalization of the two proteins, as indicated by the yellow color in Figs 3C and 4C, this was difficult to determine at this level due to the high amount of clathrin staining present in the cells. Following treatment with CCh for 5 minutes, there was a strong loss of M₂ mAChR staining in the sections examined from intact cells (Fig. 3D) and from isolated plasma membrane patches (Fig. 4D), presumably due to internalization and removal of the M₂ mAChRs from the cell surface. Concomitantly there was virtually a total loss of colocalization with clathrin (Figs 3F and 4F; note the arrowheads showing M₂ mAChR staining in the absence of clathrin staining). This

Fig. 3. M₂ mAChR and clathrin staining in HEK293 cells. HEK293 cells stably expressing M₂ mAChRs were either untreated (A-C) or treated with CCh for 5 minutes at 37°C (D-F) prior to fixation. Cells were stained with anti-M₂ mAChR antibodies (A,D) and anti-clathrin antibodies (B,E) and visualized with TRITC-conjugated anti-rat antibodies and FITC-conjugated anti-mouse antibodies on a confocal laser scanning microscope. (C and F) Overlays of the corresponding M₂ mAChR and clathrin images, and yellow color indicates colocalization. Sections were taken at the very bottom of whole cells to isolate the staining of M₂ mAChRs and clathrin in the plasma membrane. Images are representative of an entire population of cells. Arrows indicate staining of M₂ mAChRs that are in the process of internalizing and do not colocalize with clathrin staining.

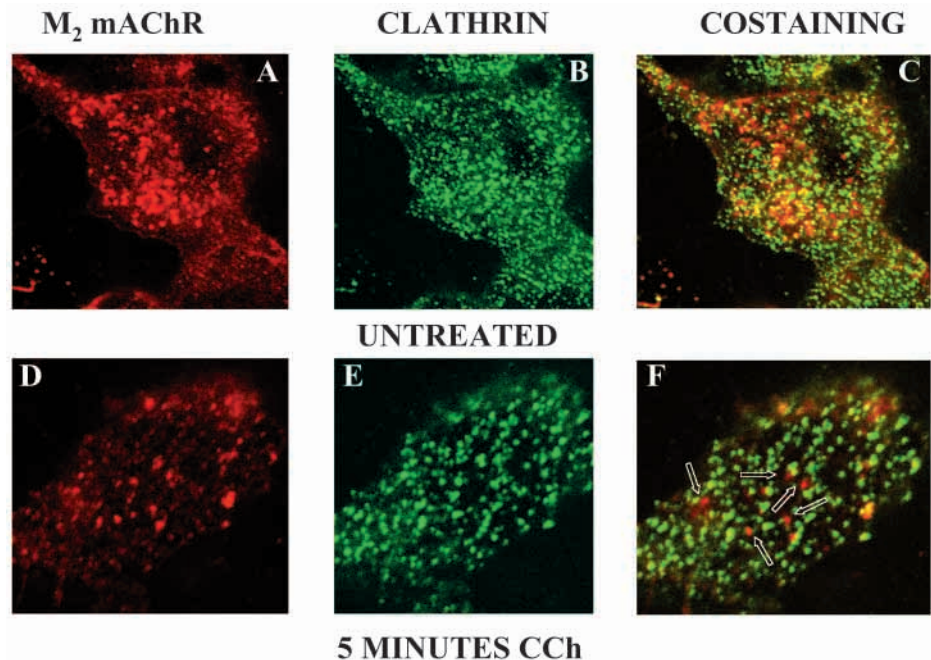
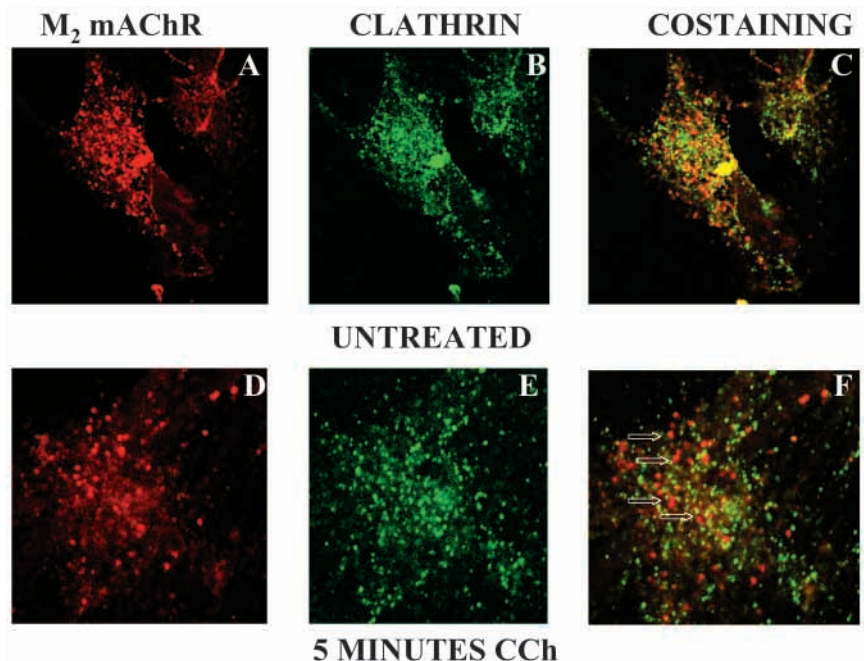


Fig. 4. M₂ mAChR and clathrin staining in isolated plasma membrane patches from HEK293 cells. HEK293 cells stably expressing M₂ mAChRs were either untreated (A-C) or treated with CCh for 5 minutes at 37°C (D-F), and isolated plasma membrane fractions were prepared prior to fixation. Cells were stained with anti-M₂ mAChR antibodies (A,D) and anti-clathrin antibodies (B,E) and visualized with TRITC-conjugated anti-rat antibodies and FITC-conjugated anti-mouse antibodies on a confocal laser scanning microscope. (C and F) Overlays of the corresponding M₂ mAChR and clathrin images, and yellow color indicates colocalization. Images are representative of an entire population of cells. Arrows indicate staining of M₂ mAChRs that are in the process of internalizing and do not colocalize with clathrin staining.



suggested that M₂ mAChRs were not primarily internalized via CCPs in HEK293 cells. Based on the lack of colocalization of the M₂ mAChR with clathrin after agonist treatment in both whole cell preparations and isolated plasma membrane patches, as well as the results from the experiments utilizing the dominant-negative inhibitors of clathrin-mediated endocytosis, we concluded that the principal pathway used for internalization of the M₂ mAChRs did not utilize clathrin-dependent events.

Since the M₂ mAChR does not appear to utilize CCPs for its internalization, it was interesting that sucrose was able to inhibit internalization of the M₂ mAChR. While sucrose is generally reported to specifically inhibit clathrin-mediated internalization, these results provide a strong argument that the

effect of sucrose on endocytosis is not specific for clathrin-mediated endocytosis, and demonstrate that sucrose can also inhibit other pathways of endocytosis.

Is there a role for caveolae in the internalization of M₂ mAChRs in HEK293 cells?

As the internalization of the M₂ mAChR is dependent on dynamin function, but does not appear to utilize clathrin-coated pits, we next examined other dynamin dependent pathways of internalization for their possible role in internalization of the M₂ mAChR. The identification of M₂ mAChRs in caveolae-enriched fractions isolated from atrial myocytes (Feron et al., 1997) and the dynamin dependence of caveolae internalization (Henley et

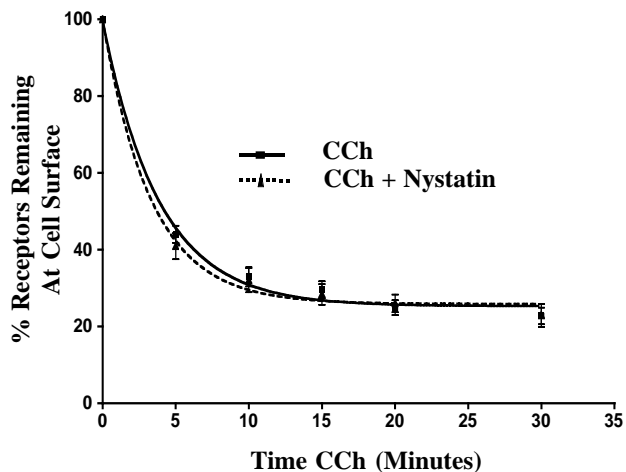


Fig. 5. Internalization of M₂ mAChRs following nystatin pretreatment. HEK293 cells stably expressing M₂ mAChRs were treated with or without 50 µg/ml nystatin for 3 hours prior to the addition of CCh for the times indicated. Cell surface receptor levels were measured using the hydrophilic antagonist [³H]NMS (1.0-2.0 nM). Results shown are means ± s.e.m. of 5 independent experiments.

al., 1998; Oh et al., 1998) led us to examine whether caveolae were involved in the internalization of M₂ mAChRs in HEK293 cells. Cholesterol-sequestering agents, such as nystatin and filipin, have been shown to disrupt caveolae formation (Rothberg et al., 1992; Rothberg et al., 1990). Pretreatment with nystatin had no effect on the internalization of M₂ mAChRs (Fig. 5). In order to determine whether caveolae were disrupted following nystatin treatment, we observed the effects of nystatin on the staining of the caveolae marker protein, caveolin. Nystatin treatment resulted in redistribution of the punctate staining of caveolin at or near the cell surface (Fig. 6A) to intracellular

locales (Fig. 6B), demonstrating that disruption of caveolae occurred following nystatin treatment. We also examined the effects of nystatin treatment on the internalization of cholera toxin, which has previously been demonstrated to occur via caveolae (Henley et al., 1998; Oh et al., 1998). As expected, nystatin treatment inhibited the internalization of cholera toxin (Fig. 6C-E). Incubation of HEK293 cells with FITC-labeled cholera toxin at 4°C resulted in a staining pattern that was restricted to the plasma membrane (Fig. 6C). Internalization of cholera toxin, which was evident by the redistribution of the staining from the cell surface to an intracellular location (Fig. 6D), then proceeded following a shift of the incubation temperature to 37°C. However, nystatin pretreatment was able to inhibit internalization of cholera toxin at 37°C, as demonstrated by the plasma membrane delimited staining (Fig. 6E). These results demonstrated that caveolae were totally disrupted and that their internalization was inhibited following nystatin treatment (Fig. 6), while the internalization of M₂ mAChRs was unaffected following disruption of caveolae (Fig. 5).

We further examined a possible role for caveolae in the internalization of M₂ mAChRs through immunohistochemical analyses of the cellular distribution of the receptors and caveolin. In untreated cells caveolin was located primarily at the cell surface with some intracellular staining (Fig. 7B), but in contrast to the continuous staining of the M₂ mAChR at the cell surface (Fig. 7A), caveolin was located in very distinct punctate sites at the cell surface. While there may have been some colocalization of M₂ mAChR and caveolin staining in untreated cells (Fig. 7C), this could have been due to the continuous staining of the M₂ mAChR at the cell surface. Thus, it was not possible to discern any true colocalization of the receptors with caveolae in untreated cells at this level of detection. Treatment of cells with CCh led to a vast redistribution of M₂ mAChR staining (Fig. 7D) to a perinuclear site. In contrast, the caveolin staining was not altered following CCh treatment (Fig. 7E), and there was no colocalization of

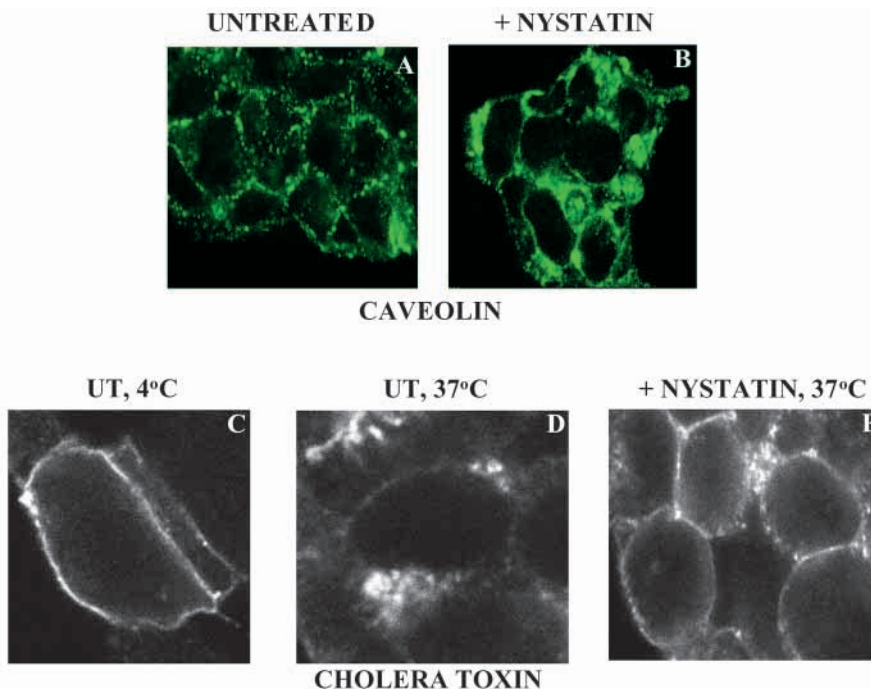


Fig. 6. Caveolin staining and Internalization of Cholera Toxin in HEK293 cells treated with nystatin. (A,B) Staining of caveolin following nystatin treatment. Untreated cells (A) or nystatin treated cells (B) were fixed and stained with affinity purified polyclonal rabbit anti-caveolin antibodies that recognize all caveolin isoforms, and visualized with FITC-conjugated anti-rabbit antibodies. (C-E) Internalization of cholera toxin following nystatin treatment. Untreated cells (C,D) or nystatin treated cells (E) were labeled with FITC-cholera toxin at 4°C, followed by fixation (C) or incubation at 37°C for 45-60 minutes prior to fixation (D,E). Images were visualized on a confocal laser scanning microscope, and are representative of an entire population of cells.

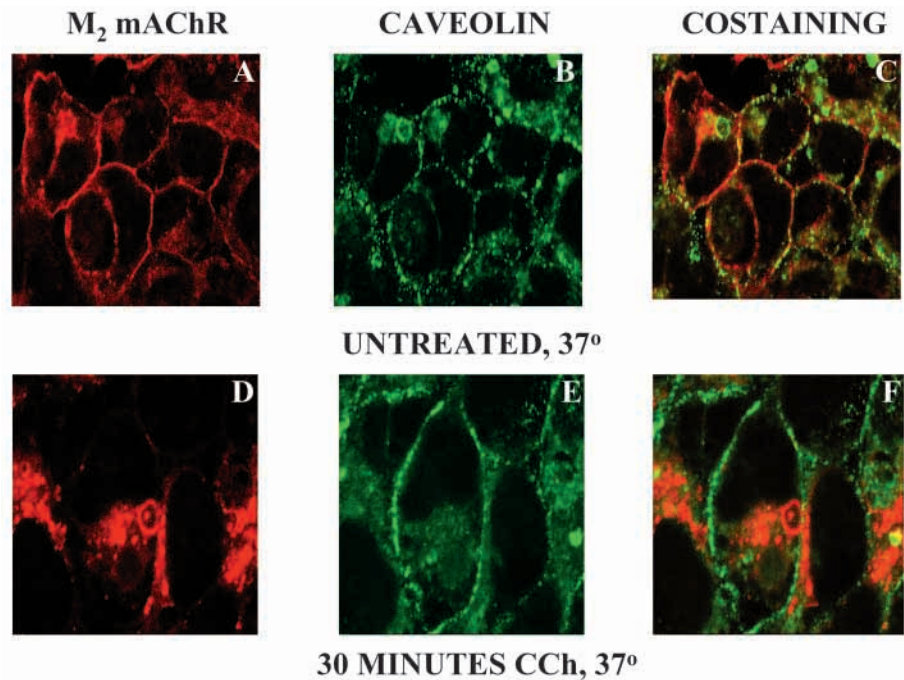


Fig. 7. M₂ mAChR and caveolin staining in HEK293 cells. HEK293 cells stably expressing M₂ mAChRs were either untreated (A-C) or treated with CCh for 30 minutes at 37°C (D-F) prior to fixation. Cells were stained with anti-M₂ mAChR antibodies (A,D) and anti-caveolin antibodies (B,E) and visualized with TRITC-conjugated anti-rat antibodies and FITC-conjugated anti-rabbit antibodies. (C and F) Overlays of the corresponding M₂ mAChR and caveolin images, and yellow color indicates colocalization.

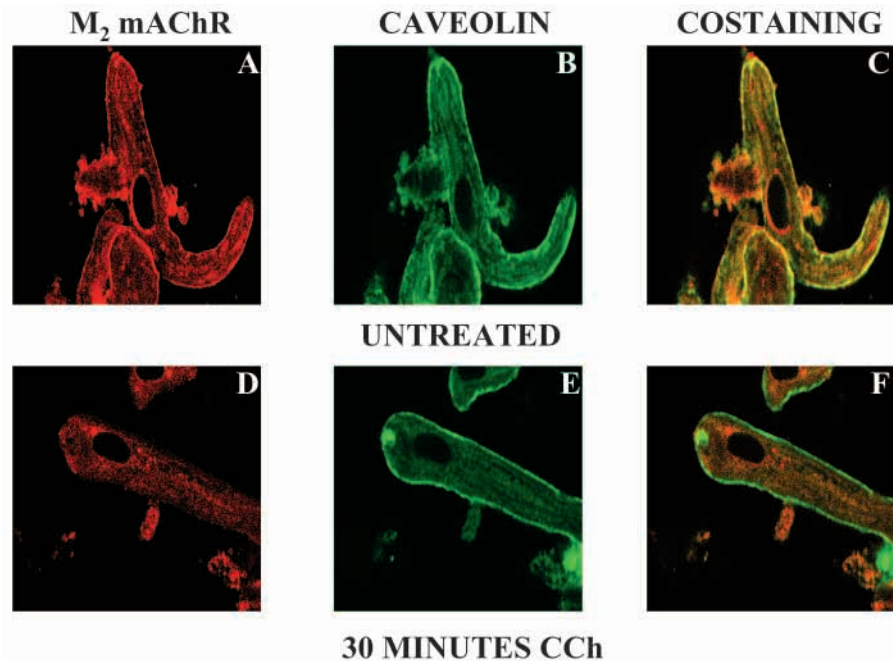


Fig. 8. M₂ mAChR and caveolin staining in atrial myocytes. Feline atrial myocytes were either untreated (A-C) or treated with CCh for 30 minutes at 37°C (D-F) prior to fixation. Cells were stained with anti-M₂ mAChR antibodies (A,D) and anti-caveolin antibodies (B,E) and visualized with TRITC-conjugated anti-rat antibodies and FITC-conjugated anti-rabbit antibodies. (C and F) Overlays of the corresponding M₂ mAChR and caveolin images, and yellow color indicates colocalization.

M₂ mAChR and caveolin staining observed following internalization of M₂ mAChRs (Fig. 7F). Similar results were observed for cells treated with CCh for 5 and 15 minutes (data not shown). Furthermore, in experiments performed at 15°C, which results in a decrease in the rate of internalization, we also observed punctate cytosolic staining of M₂ mAChRs that were in the process of being internalized and showed no colocalization with caveolin (data not shown).

Does the M₂ mAChR colocalize with caveolae following agonist treatment in atrial myocytes?

As M₂ mAChRs were identified previously in caveolae-

enriched fractions isolated from atrial myocytes (Feron et al., 1997), we performed immunohistochemistry on atrial myocytes isolated from feline heart to assess a potential role for caveolae in internalization in a native system. Both the M₂ mAChR (Fig. 8A) and caveolin (Fig. 8B) were stained intensely at the cell surface. Consequently, there appeared to be colocalization of the two proteins (Fig. 8C). However, as with the staining seen in HEK293 cells, we cannot conclude that M₂ mAChRs were located in caveolae, as the apparent colocalization could be due to the extremely heavy labeling of both M₂ mAChRs and caveolin at the plasma membrane. Treatment of atrial myocytes with CCh led to a total loss of

M₂ mAChR staining from the cell surface (Fig. 8D), while there was no change in caveolin staining (Fig. 8E). Importantly, after agonist treatment no colocalization of the receptors and caveolin was observed (Fig. 8F). Thus, it appears that caveolae were not responsible for the internalization of M₂ mAChRs in atrial myocytes or HEK293 cells.

Our findings may seem to be in contrast to a previous report in which M₂ mAChRs were identified in caveolar fractions isolated from cardiac myocytes (Feron et al., 1997). There are a number of possible explanations for the differences in these observations. First, it is entirely possible that M₂ mAChRs may be located in caveolae in untreated cells. Caveolae have been widely reported to be microdomains for the localization of signaling proteins (Couet et al., 1997; Parton, 1996). It is also possible that M₂ mAChRs associated with caveolae before being internalized and recycled by a separate pathway, however this association cannot be critical for the eventual internalization of the receptors since disruption of caveolae had no effect on receptor internalization. Alternatively, it is possible that M₂ mAChRs do not reside in caveolae in HEK293 or cardiac cells. Although the technique used to demonstrate the presence of M₂ mAChRs in isolated caveolae fractions (Feron et al., 1997) has been widely used, it has been demonstrated that this fractionation technique also results in the isolation of other structures including detergent insoluble microdomains, or rafts (Oh and Schnitzer, 1999; Simons and Ikonen, 1997). Thus, it is possible that the M₂ mAChRs observed in caveolae-enriched fractions were associated with rafts, or another structure that was isolated by this technique.

Our results also appear to be in contrast to another recent report examining the role of caveolae in internalization of mAChRs (Dessy et al., 2000). In this report, the authors demonstrate that mAChRs sequester into caveolae following agonist stimulation in rat ventricular myocytes (Dessy et al., 2000). There are a number of possible explanations for the observed differences in our results. First, as demonstrated previously, cell type is a very important determinant for the pathway utilized for internalization of the M₂ mAChR (Pals-Rylandsdam et al., 1997). In the report by Dessy et al., rat ventricular myocytes were utilized, while in our studies HEK293 cell and feline atrial myocytes were used. This alone may account for the observed differences. Furthermore, it is possible that rat ventricular myocytes may contain other mAChR subtypes that utilize caveolae for their internalization and would be detected through the use of binding of the non-specific muscarinic ligand, [3H]NMS, while our studies utilized M₂ mAChR specific antibodies. Thus there are many possible explanations for the differences observed between our studies and those of Dessy et al. (Dessy et al., 2000).

In these studies we have examined various dynamin-dependent endocytic pathways for their possible role in the internalization of the M₂ mAChR in an attempt to gain more information about the poorly understood pathway by which these receptors internalize. Internalization of the M₂ mAChR in HEK293 cells is a very complex process that proceeds through an atypical pathway. This pathway is dependent on dynamin function, but is not inhibited by the K44A dynamin mutant (Werbonat et al., 2000). Furthermore, the M₂ mAChR does not utilize CCPs or caveolae for its internalization. Thus it appears that the M₂ mAChR utilizes an unidentified atypical pathway to internalize in HEK293 cells.

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