RhoA-Sensitive Trafficking of Muscarinic Acetylcholine Receptors

OLIVER VÖGLER, PATRICK KRUMLMENERL, MARTINA SCHMIDT, KARL H. JAKOBS and CHRIS J. VAN KOPPEN

Institut für Pharmakologie, Universitätsklinikum Essen, Essen, Germany

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ABSTRACT

The clathrin-mediated sequestration pathway is used by non-G protein-coupled receptors (e.g., transferrin receptors) and a large number of G protein-coupled receptors, including beta-2 adrenoceptors and various muscarinic acetylcholine receptor (mACHR) subtypes. Recently, the ubiquitously expressed small GTPase RhoA has been implicated as a negative regulator of transferrin receptor internalization. Because mACHRs and other G protein-coupled receptors are able to activate RhoA, we investigated in HEK-293 cells whether RhoA regulates the sequestration of m1 and m2 mAChRs, which internalize via clathrin-coated and nonclathrin-coated vesicles in HEK-293 cells, respectively. Overexpression of wild-type RhoA inhibited agonist-induced sequestration of both m1 and m2 mAChRs by as much as 70%. Inhibition could be reversed by coexpression of Clostridium botulinum C3 transferase, which inactivates RhoA by ADP-ribosylation. Overexpression of C3 transferase alone had no effect on m1 and m2 mAChR sequestration. In addition, overexpression of RhoA inhibited m1 and m2 mAChR transport to the plasma membrane by 60 and 31%, respectively, which was blocked by coexpression of C3 transferase. We conclude that RhoA is not an endogenous regulator of mAChR sequestration, but when overexpressed, strongly inhibits mAChR trafficking (i.e., sequestration and transport to the plasma membrane) in HEK-293 cells.

In most cellular systems, G protein-coupled receptors (GPCRs) desensitize due to prolonged agonist stimulation. An important mechanism of GPCR desensitization is receptor phosphorylation of the agonist-bound receptor by specific G protein-coupled receptor kinases, and the binding of the inhibitory protein, beta-arrestin to the phosphorylated receptor, which in turn blocks the interaction of receptor with G proteins. The binding of agonists to GPCRs often also initiates the internalization of receptors into the cell interior (Bogatkevitsch et al., 1996). The prevailing view is that beta-arrestin, bound to the receptor, binds with high affinity to clathrin, and immobilizes the receptors in clathrin-coated pits, which subsequently bud from the plasma membrane (Zhang et al., 1996, Goodman et al., 1996). The budding of the clathrin-coated pits is controlled by the monomeric GTPase dynamin (Zhang et al., 1996). Other plasma membrane receptors, which do not couple to G proteins such as transferrin receptors and low-density lipoprotein receptors, can also internalize in clathrin-coated vesicles (Van der Bliek et al., 1993). In addition to clathrin-mediated sequestration, GPCRs can sequester via alternative sequestration pathways (Pals-Rylaarsdam et al., 1997, de Weerd and Leeb-Lundberg, 1997, Vögl er et al., 1998).

Recently, the monomeric GTPase RhoA has been reported to regulate clathrin-mediated sequestration of transferrin receptors. Overexpression of constitutively active RhoA, but not of wild-type RhoA, significantly inhibited the sequestration of transferrin receptors in HeLa cells, whereas RhoGDI, the GDP dissociation inhibitor of Rho, as well as Clostridium botulinum C3 transferase, which inactivates RhoA by ADP-ribosylation, enhanced transferrin receptor endocytosis in permeabilized A431 cells (Lamaze et al., 1996). Because muscarinic acetylcholine receptors (mAChRs) and other GPCRs rapidly stimulate the translocation of RhoA from the cytosol to the plasma membrane and thereby allow activation of RhoA by GDP/GTP exchange (Fleming et al., 1996, Keller et al., 1997), we investigated whether sequestration of GPCRs is regulated by RhoA. Recently, we and others have demonstrated that m1, m3, and m4 mAChRs, transfected in HEK-293 cells, sequester via dynamin-dependent clathrin-coated vesicles, and recycle back to the plasma membrane (Vögl er et al., 1998, Tolbert and Lameh, 1996). In contrast, endocytosis of mAChRs of the m2 subtype in HEK-293 cells is fully dynamin-independent and almost completely irreversible, indicating that m2 mAChRs sequester by nonclathrin-coated vesicles (Pals-Rylaarsdam et al., 1997, Vögl er et al., 1998).

ABBREVIATIONS: DME, Dulbecco’s modified Eagle’s; mAChR, muscarinic acetylcholine receptor; G protein, guanine nucleotide-binding protein; GPCR, G protein-coupled receptor; NMS, N-methylscopolamine; PBS, phosphate-buffered saline; QNB, quinuclidinyl-benzilate; SDS, sodium dodecyl sulfate.
We therefore used m1 and m2 mAChRs expressed in HEK-293 cells as model systems of GPCRs possessing different sequestration pathways of GPCRs to analyze the role of RhoA in GPCR sequestration.

Materials and Methods

Materials. [3H]Quinuclidinyl benzilate ([3H]QNB, specific activity 43 Ci/mmole) and [3H]methylscopolamine ([3H]NMS, specific activity 84 Ci/mmole) were purchased from New England Nuclear (Boston, MA). Anti-RhoA monoclonal antibody (26C4) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Plasmid Construction. All recombinant DNA procedures were carried out following standard protocols. DNA encoding murine m1 mAChR (Shapiro et al., 1988) and porcine m2 mAChR (Peralta et al., 1987) were subcloned into pCD-PS containing RhoA wild-type DNA, myc-tagged C. botulinum with 0.2 g/ml horseradish peroxidase-conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany).  

Cell Culture and Transfection. HEK-293 tsA201 cells were grown in Dulbecco’s modified Eagle’s (DME/F-12 medium supplemented with 10% fetal calf serum, penicillin G (100 U/ml), and streptomycin (100 g/ml) in an atmosphere of 5% CO2. Cells on 150-mm plates were transiently transfected with either 12.5 or 25 pg of pCD-PS containing m1 or m2 mAChR DNA, together with 50 pg of pRK5 containing RhoA wild-type DNA, myc-tagged C. botulinum C3 transferase DNA, or 50 pg of pRK5 (Van Koppen and Nathanson, 1990). Transfection efficiency was 10 to 20% as measured by [3H]NMS ([3H]QNB, specific activity 43 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Anti-RhoA monoclonal antibody (26C4) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Plasmid Construction. All recombinant DNA procedures were carried out following standard protocols. DNA encoding murine m1 mAChR (Shapiro et al., 1988) and porcine m2 mAChR (Peralta et al., 1987) was subcloned into pRK5, and myc-tagged C. botulinum C3 transferase DNA, or 50 pg of pRK5 (Van Koppen and Nathanson, 1990). Transfection efficiency was 10 to 20% as measured by [3H]NMS ([3H]QNB, specific activity 43 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Anti-RhoA monoclonal antibody (26C4) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).  

Immunoblot Analysis of RhoA Expression. A total of 48 to 60 h after DNA transfection, cells on 150-mm plates were washed twice with phosphate-buffered saline (PBS; 150 mM NaCl, 6.5 mM Na2HPO4, 2.7 mM KCl, pH 7.4) and lysed by the addition of 1.0 ml of HEPES-buffered saline at 4°C to measure nonspecific binding. After a 4-h incubation, cells were washed with ice-cold HEPES-buffered saline, solubilized in 1% Triton X-100, scraped, and transferred to scintillation vials, which received 3.5 ml of scintillation fluid before radioactivity counting. Sequestration is expressed as (1 - quotient of cell surface receptors of carbachol-treated and untreated cells) x 100%. For determination of the equilibrium dissociation constant Kd of [3H]NMS, intact cells were incubated at 4°C for 12 h instead of 4 h (to attain binding equilibrium at low radioligand concentrations) with 10 to 1000 pM [3H]NMS in the absence and presence of 3 µM atropine. For monitoring dissociation of [3H]NMS from m1 mAChRs, intact transfected cells on 24-well plates were first incubated with 1 nM [3H]NMS in the presence and absence of 3 µM atropine for 4 h. Cells were then washed with ice-cold HEPES-buffered saline and adding 500 µl ice-cold HEPES-buffered saline containing 3 µM atropine to prevent reassociation of [3H]NMS. Total mAChR number was determined by binding of the membrane-permeable muscarinic antagonist [3H]QNB to crude cell homogenates at receptor-saturating concentrations of 600 pM (Van Koppen and Nathanson, 1990). Untransfected HEK-293 tsA201 cells do not express detectable levels of mAChRs as measured by [3H]QNB binding to total cell homogenates.  

To determine m1 mAChR-mediated [3H]NMS uptake, cells were incubated at 37°C with 1 nM [3H]NMS in 25 mM HEPES-buffered DME/F12 medium, pH 7.4, in the absence and presence of 3 µM atropine for 0 to 120 min. Then, to remove plasma membrane-bound [3H]NMS, cells were washed twice with ice-cold 50 mM glycine buffer, pH 3.0, containing 150 mM NaCl and incubated in the same buffer at 4°C for 60 min, followed by two washes in the same buffer at 4°C. After this procedure, cells were lysed with 0.5 ml 1% Triton X-100 and transferred into scintillation vials for radioactivity counting.  

This procedure (i.e., washing twice in glycine buffer, subsequent incubation for 60 min in glycine buffer and two final washes with glycine buffer) reduced plasma membrane binding of [3H]NMS by 90%. In parallel, total specific [3H]NMS binding to plasma membrane receptors was measured by incubation of intact cells with 1 nM [3H]NMS ([3H]NMS (±3 µM atropine) in HEPES-buffered saline 4 h at 4°C. Binding of [3H]NMS at 37°C to untransfected intact HEK-293 cells (i.e., plasma membrane + intracellular components) was nonspecific and less than 5% of total specific [3H]NMS binding to m1 mAChRs expressing HEK-293 cells as measured after 1 h of incubation at 37°C. Phalloidin Staining of Actin Cytoskeleton. Twenty-four hours after transfection with green fluorescent protein (GFP)-encoding pEGFP-C1 (Clontech, Palo Alto, CA) together with RhoA pRK5 or control pRK5 (50 pg/150-mm plate each), HEK-293 tsA201 cells were replated and grown overnight on poly-L-lysine-coated 18-mm glass coverslips. The cells were incubated in 25 mM HEPES-buffered DME/F12 medium (pH 7.4) containing 5 µg/ml cytochalasin B (Sigma) or vehicle (dimethyl sulfoxide, final concentration of 0.2%) at 37°C. Cells were rinsed twice with PBS and fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. Then cells were washed twice with PBS for 5 min each and permeabilized in 0.05% Triton X-100 in PBS for 2 min. After washing three times for 5 min with PBS, cells were incubated for 15 min in 10 µg/ml tetramethylrhodamine isothiocyanate-conjugated phalloidin (Sigma) in PBS. After three washes with PBS, stained cells were mounted with Moviol (Calbiochem, San Diego, CA) and viewed by fluorescence microscopy using a Zeiss Axiosvert microscope. Transfected cells were identified by green fluorescence of expressed GFP, and actin by tetramethylrhodamine isothiocyanate-phalloidin fluorescence using standard wavelength settings. Results

Inhibition of m1 and m2 mAChR Sequestration by RhoA. To test a regulatory role of RhoA in GPCR sequestration, we transiently coexpressed wild-type RhoA with...
mACHRs in HEK-293 tsA201 cells. Transfection with RhoA DNA (50 μg/150-mm plate) resulted in approximately 20- to 40-fold overexpression of RhoA as measured by densitometry and corrected for a transfection efficiency of 10 to 20% in HEK-293 tsA201 cells (Vogler et al., 1998) (Fig. 1A). Sequestration of m1 mACHRs in HEK-293 tsA201 cells was inhibited dose dependently by transfecting cells with increasing amounts of RhoA DNA (Fig. 1B). Transfection with 5.0 μg of pRK5 RhoA DNA per 150-mm tissue culture plate reduced m1 mACHR sequestration in response to incubation with 1 mM carbachol for 60 min from 53 ± 3 to 36 ± 4%, and transfection with 50 and 150 μg pRK5 RhoA DNA diminished m1 mACHR sequestration to 21 ± 4 and 21 ± 6%, respectively. A time course of m1 mACHR internalization in control and RhoA-overexpressing cells is shown in Fig. 2 (left panel). Inhibition of m1 mACHR sequestration by RhoA was apparent at low concentrations of carbachol as well. For example, m1 mACHR sequestration induced by incubation with 10^{-5} M carbachol for 60 min was reduced from 10 ± 5% in control cells to 2 ± 3% in RhoA-overexpressing cells (data not shown). To investigate whether RhoA also interferes with clathrin-independent GPCR sequestration, the effect on m2 mACHR sequestration was determined. As shown in Fig. 2 (right panel), transfection of HEK-293 cells with RhoA DNA reduced m2 mACHR sequestration in response to treatment with 1 mM carbachol for 60 min from 79 ± 3% to 29 ± 3%. Similar to m1 mACHR sequestration, RhoA overexpression reduced m2 mACHR sequestration at low carbachol concentrations as well. The m2 mACHR sequestration induced by incubation with 10^{-5} M carbachol for 60 min was reduced from 65 ± 2% in control cells to 17 ± 3% in RhoA overexpressing cells (data not shown).

To ascertain that functional RhoA is required for the inhibition of mACHR sequestration, the influence of C3 transferase expression on receptor sequestration was studied (Fig. 3). As expected, C3 transferase fully reversed the inhibitory effect of RhoA overexpression on m1 and m2 mAChR sequestration. However, expression of C3 transferase alone did not alter the magnitude of m1 and m2 mAChR sequestration in HEK-293 cells (Fig. 3). Also, time course and carbachol concentration dependence of m1 and m2 mAChR sequestration were not changed (data not shown). Because RhoA is involved in mAChR-mediated activation of phospholipase D (Schmidt et al., 1996), and phospholipase D is implicated in vesicular trafficking (Roth and Sternweis, 1997), we determined whether the inhibitory action of RhoA on mAChR sequestration is mediated by activation of phospholipase D. For this, we pretreated the cells with 2% ethanol for 15 min and determined m1 mAChR sequestration after 60 min of incubation with 1 mM carbachol in the continuing presence of 2% ethanol. This pretreatment, which inhibits the formation of phosphatidic acid from phosphatidylethanolamine by phospholipase D by 80 to 90% in HEK-293 cells (M. Schmidt, unpublished observations), did not alter m1 mAChR sequestration in RhoA-overexpressing cells (or in control cells), as determined in three sets of independent transfection experiments. Thus, the inhibition of mAChR sequestration in RhoA-overexpressing cells is most likely not caused by RhoA-mediated activation of phospholipase D. As overexpression of RhoA can induce actin cytoskeleton reorganization and thereby potentially affect mAChR trafficking, we tested whether cytochalasin B, which causes depolymerization of actin filaments was effective in reversing the RhoA effect. As shown in Fig. 4, pretreatment of the cells with cytochalasin B (5 μg/ml) for 10 min before receptor challenge, effectively depolymerized the actin cytoskeleton in both control cells (Fig. 4A and B) as well as in RhoA-overexpressing cells (Fig. 4C and D). However, cytochalasin B treatment did not block the inhibitory effect of RhoA on either m1 or m2 mAChR sequestration (Table 1), indicating the RhoA effect is not due to assembly of actin filaments.

**Inhibition of m1 and m2 mAChR Cell Surface Targetting by RhoA Overexpression.** Rho GTPases have also been implicated in the intracellular movement of organelles (Murphy et al., 1996). We therefore investigated whether RhoA affects trafficking of receptors to the plasma membrane. As shown in Fig. 5, RhoA overexpression decreased the expression of m1 and m2 mAChRs on the cell surface by 60% and 31%, respectively, as determined by [3H]QNB binding to intact cells and [3H]QNB binding to cell homogenates in parallel (P < .01, t test). Whereas in control cells, 82 ± 13% of m1 and 80 ± 6% of m2 receptors were present at the cell surface, in RhoA-overexpressing cells, only 33 ± 5% of m1
and 55 ± 6% of m2 receptors were on the cell surface, respectively. In contrast, RhoA overexpression did hardly reduce total m1 and m2 receptor number (see legend of Fig. 5). Control experiments demonstrated that RhoA overexpression did not change 1) the equilibrium dissociation constant of [3H]NMS \( K_d \) values of 126 ± 614 and 164 ± 30 PM in cells transfected with pRK5 RhoA and control pRK5, respectively (mean ± S.D., \( n = 2 \) independent transfection experiments) or 2) the dissociation rate constant of [3H]NMS (k values of 0.03 ± 0.01 and 0.04 ± 0.01 min\(^{-1}\), respectively (mean ± S.D., \( n = 2 \) independent experiments). Thus, the reduced number of cell surface receptors in RhoA-overexpressing cells is not caused by reduced binding affinity of the receptor, or increased dissociation of [3H]NMS from the receptor during washing at the end of the [3H]NMS binding assay. Cotransfection with C3 transerase DNA blocked the effect of RhoA on m1 and m2 mAcHR cell surface expression completely. Overexpression of C3 transerase alone did not change the subcellular distribution of m1 and m2 mAcHRs in HEK-293 cells.

The generation and maintenance of the plasma membrane of cells require a continuous supply of newly synthesized components, including GPCRs. The expression of GPCRs at the cell surface is therefore a dynamic equilibrium between constitutive receptor transport to the plasma membrane (i.e., as a result of de novo receptor synthesis in the endoplasmic reticulum or delivery from other internal stores) and constitutive receptor transport from the plasma membrane into the cell interior (i.e., into lysosomes or other internal stores) (Koenig and Edwardson, 1994, 1996). We therefore investigated whether the intracellular accumulation of mAcHRs in RhoA-overexpressing cells was caused by enhanced receptor internalization or reduced receptor transport to the plasma membrane. For this, we took advantage of a recent observation in our laboratory indicating that there is m1 mAcHR-mediated uptake of [3H]NMS in HEK-293 cells at 37°C. We therefore determined receptor-mediated intracellular accumulation of [3H]NMS in cells coexpressing m1 mAcHRs and RhoA and in cells expressing m1 mAcHRs alone. In both cell types, incubation with 1 nM [3H]NMS at
creased, but on the contrary, reduced [3H]NMS uptake as
However, RhoA-overexpressing cells did not display in-
uptake, while [3H]NMS uptake in RhoA-overexpressing cells
was 12
6
recycling to the plasma membrane.

mAChRs is caused by inhibition of receptor transport or
clude that RhoA-induced intracellular accumulation of m1
mAChR sequestration, but when overexpressed, signifi-
cation of wild-type RhoA strongly inhibited agonist-induced
sequestration of both m1 and m2 mAChRs, and that this
effect was prevented by coexpression of C3 transerase. How-
ever, overexpression of C3 transerase alone had no influence
on m1 and m2 mAChR sequestration. We therefore conclude
that RhoA at physiological concentrations is not a regulator
of mAChR sequestration, but when overexpressed, signifi-
antly inhibits clathrin-dependent and -independent mAChR
sequestration.

In addition to inhibition of mAChR sequestration, we noted
that RhoA overexpression also leads to intracellular accumu-
lation of m1 mAChRs and m2 mAChRs, without significantly
affecting total receptor expression, as determined by
[3H]QNB binding to cell homogenates. As the intracellularly
accumulated mAChRs retained their capacity to bind
[3H]QNB, we conclude that they are probably correctly folded
in intracellular vesicles. As the intracellular concentration of
receptors can be considered to be an equilibrium between
constitutive receptor internalization and transport of intra-
cellular receptors to the plasma membrane, the intracellular
accumulation of mAChRs could be the result of either en-
hanced constitutive receptor internalization or decreased
transport of intracellular receptors to the plasma membrane.
We observed that constitutive internalization of [3H]NMS-
occupied m1 mAChRs was significantly inhibited by RhoA
overexpression, suggesting that RhoA overexpression most
likely inhibits transport of mAChRs to the plasma membrane
rather than increases constitutive receptor internalization.

The mechanism by which RhoA inhibits agonist-induced
mAChR sequestration in HEK-293 cells has yet to be identi-
ied. The decreased agonist-induced internalization in RhoA-
overexpressing cells could be due to a diminished number of
receptors at the plasma membrane. This explanation, how-
ever, seems very unlikely. The percentage of internalized m1
or m2 receptors as a result of agonist exposure is larger in
HEK-293 tsA201 cells expressing low numbers than high
numbers of cell surface receptors per cell (our unpublished
observations; Pals-Ryläärsdam and Hosey, 1997). A more
plausible (and unifying) mechanism is that RhoA inhibits
mAChR internalization primarily by inhibiting receptor re-
cycling to the plasma membrane, resulting in a loss of cell
surface receptors and saturation of intracellular receptor
pools, which in turn inhibits the flow of internalized recep-
tors from the plasma membrane. However, we cannot ex-
clude that RhoA overexpression inhibits receptor internaliza-
 tion as well as transport to the plasma membrane indepen
dently from each other.

The molecular mechanisms by which RhoA interferes with
receptor trafficking remain to be elucidated. It is possible
that overexpressed RhoA competitively inhibits one or more,
as yet unknown, monomeric GTP-binding proteins, which
regulate particular mAChR transport pathways. It is also
possible that overexpressed RhoA acts via downstream effec-
tor enzymes, which change the phospholipid composition of
the plasma membrane or vesicles, thereby altering the mo-
tility of receptors. RhoA is able to stimulate various lipid
kinases, including phosphatidylinositol 4-phosphate 5-ki-
nase, an enzyme that is essential for the production of
phosphatidylinositol-4,5-bisphosphate, and phosphatidylinositol
3-kinase, producing phosphatidylinositol-3,4-bisphosphate,
and phosphatidylinositol-3,4,5-trisphosphate (Machesky and
Hall, 1996). By changing the phospholipid composition, RhoA
may also indirectly regulate the activity of key proteins like
dynamin, clathrin adaptor AP2 subunits and other proteins
(such as those required for dynamin-independent GPCR se-
questation), which are involved in receptor trafficking in-
cluding sequestration and which activity is regulated by neg-
atively charged phospholipids (Lin and Gilman, 1996,
Gaidarov et al., 1996). Several lines of evidence suggest that
phosphoinositides in specific intracellular locations can sig-
nal the recruitment or activation of proteins essential for
vesicular transport (De Camilli et al., 1996). It is therefore
also possible that overexpression of RhoA may have induced

Discussion

Evidence implicating RhoA in the regulation of clathrin-
mediated sequestration pathway has recently been presented
by Schmid and coworkers (Lamaze et al., 1996). Constitu-
tively active RhoA impedes sequestration of transferrin re-
cptors, whereas inhibitors of RhoA activation (RhoGDI and
C3 transerase) stimulate transferrin receptor internaliza-
tion. In the present study, we demonstrated that overexpres-
sion of wild-type RhoA strongly inhibited agonist-induced
sequestration of both m1 and m2 mAChRs, and that this
effect was prevented by coexpression of C3 transerase. How-
ever, overexpression of C3 transerase alone had no influence
on m1 and m2 mAChR sequestration. We therefore conclude
that RhoA at physiological concentrations is not a regulator
of mAChR sequestration, but when overexpressed, signifi-
cantly inhibits clathrin-dependent and -independent mAChR
sequestration.

In addition to inhibition of mAChR sequestration, we noted
that RhoA overexpression also leads to intracellular accumu-

![Image](https://via.placeholder.com/150)
whereas about 14 and 22% of antagonist-occupied receptors mAChRs are endocytosed within 30 and 60 min, respectively, receptors. For example, 41 and 50% of agonist-activated m1 receptors was much slower than that of agonist-occupied interior. However, sequestration of the antagonist-occupied 293 cells. This suggests that not only agonist-activated but occupied m1 mAChRs sequester into the cell interior of HEK-B or vehicle. After washing, cell surface mAChR was determined by specific [3H]NMS binding to intact cells. Each experiment represents an independent set of transfections (0.2% dimethyl sulfoxide) for 10 min at 37°C. Thereafter, cells were incubated with or without 1 mM carbachol for 60 min at 37°C in the continuing presence of cytochalasin B or vehicle. After washing, cell surface mAChR was determined by specific [3H]NMS binding to intact cells. Each experiment represents an independent set of transfections with pRK5 RhoA and control pRK5 DNA with the binding experiments done in duplicate.

Table 1

<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>Experiment</th>
<th>pRK5</th>
<th>Sequestration</th>
<th>RhoA</th>
<th>Dimethyl sulfoxide</th>
<th>Cytochalasin B</th>
<th>Dimethyl sulfoxide</th>
<th>Cytochalasin B</th>
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<tr>
<td>m1</td>
<td>1</td>
<td>44%</td>
<td>(113)</td>
<td>45%</td>
<td>(125)</td>
<td>26%</td>
<td>(32)</td>
<td>22%</td>
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<tr>
<td></td>
<td>2</td>
<td>46%</td>
<td>(103)</td>
<td>44%</td>
<td>(138)</td>
<td>24%</td>
<td>(26)</td>
<td>22%</td>
</tr>
<tr>
<td>m2</td>
<td>3</td>
<td>69%</td>
<td>(68)</td>
<td>73%</td>
<td>(80)</td>
<td>26%</td>
<td>(19)</td>
<td>31%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>73%</td>
<td>(82)</td>
<td>74%</td>
<td>(88)</td>
<td>20%</td>
<td>(24)</td>
<td>30%</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>74%</td>
<td>(92)</td>
<td>74%</td>
<td>(89)</td>
<td>36%</td>
<td>(20)</td>
<td>41%</td>
</tr>
</tbody>
</table>

HEK-293 cells transiently transfected with m1 or m2 mAChR pCD-PS, along with pRK5 RhoA or control pRK5 were pretreated with 5 µg/ml cytochalasin B or vehicle (0.2% dimethyl sulfoxide) for 10 min at 37°C. Thereafter, cells were incubated with or without 1 mM carbachol for 60 min at 37°C in the continuing presence of cytochalasin B or vehicle. After washing, cell surface mAChR was determined by specific [3H]NMS binding to intact cells. Each experiment represents an independent set of transfections with pRK5 RhoA and control pRK5 DNA with the binding experiments done in duplicate.

Values in parentheses, cell surface receptor number in untreated cells expressed in fmol/mg protein.

Fig. 5. Inhibition of cell surface expression of m1 and m2 mAChRs by RhoA. HEK-293 tsA201 cells were transfected with either 25 µg m1 or m2 mAChR DNA in pCD-PS together with 100 µg of pRK5 (pRK5), 50 µg of pRK5 RhoA + 50 µg of pRK5 (RhoA), 50 µg of pEF C3 transferase + 50 µg of pRK5 (C3), or 50 µg of pRK5 RhoA + 50 µg of pEF C3 transferase (RhoA + C3) per 150-mm tissue culture plate. C3 transferase overexpression did not influence RhoA overexpression as verified by Western blotting. Cell surface receptor number was determined by specific [3H]NMS binding to intact cells on six-well plates. Total receptor number was measured by [3H]QNB binding to cell homogenates from six-well plates in parallel (m1 + pRK5: 883 ± 6; m1 + RhoA: 685 ± 256; m1 + C3: 411 ± 125; m1 + RhoA + C3: 596 ± 281; m2 + pRK5: 425 ± 34; m2 + RhoA: 340 ± 36; m2 + C3: 224 ± 18, and m2 + RhoA + C3: 240 ± 43 fmol/mg protein). The data represent the mean ± S.E.M. of 6 (m1) or 12 (m2) independent transfection experiments, except for “m2 + C3” and “m2 + RhoA + C3,” which are the mean ± S.E.M. of 5 experiments. Cell surface expression of m1 and m2 mAChRs was statistically larger in pRK5-transfected cells than in pRK5 RhoA-transfected cells (P < .01, two-tailed t test). Total expression of m1 and m2 mAChRs in pRK5-transfected cells (pRK5) was not statistically different from that in pRK5 RhoA-transfected (RhoA) (P = .55 and .10, respectively, two-tailed t test).

Of particular interest was the observation that [3H]NMS occupied m1 mAChRs sequester into the cell interior of HEK-293 cells. This suggests that not only agonist-activated but also antagonist-occupied mAChRs internalize into the cell interior. However, sequestration of the antagonist-occupied receptors was much slower than that of agonist-occupied receptors. For example, 41 and 50% of agonist-activated m1 mAChRs are endocytosed within 30 and 60 min, respectively, whereas about 14 and 22% of antagonist-occupied receptors sequester within the same time periods. These findings provide additional evidence for the recent notion that antagonist-occupied GPCRs, such as the cholecystokinin receptors (Roettger et al., 1997) and angiotensin II type 1 receptors (Conchon et al., 1994) can internalize. It is possible that m1 mAChR sequestration is stimulated by the binding of NMS in a similar way as by agonists. However, this is not likely because 1) NMS is an inverse antagonist which stabilizes the inactive state of m1 mAChRs, preventing it from interacting with G proteins (Jakubík et al., 1995) and 2) NMS does not induce receptor down-regulation, but on the contrary, may up-regulate receptor number during hours of antagonist incubation (Fukamauchi et al., 1993). The sequestration kinetics of NMS-occupied m1 mAChRs is remarkably similar to the kinetics of constitutive delivery of mAChRs to the surface of various cell types (Koenig and Edwardson, 1994, 1996). We therefore hold the view that [3H]NMS occupied m1 mAChRs sequester by a constitutive internalization process. In the absence of antagonist, this pathway is in equilibrium with constitutive delivery of intracellular mAChRs to the cell sur-
S.E.M. of four sets of experiments.

mediated uptake of [3H]NMS was determined by incubation of the cells, and membrane receptors in control and RhoA-overexpressing cells (260 to remove plasma membrane-bound [3H]NMS. Uptake of [3H]NMS is expressed as a percentage of total specific [3H]NMS binding to plasma membrane receptors in control and RhoA-overexpressing cells (260 ± 22 and 1 ± 9 fmol/mg protein, respectively). The data represent the mean ± S.E.M. of four sets of experiments.

face, i.e., as a result of de novo receptor synthesis in the endoplasmic reticulum or delivery from other internal stores), keeping receptor number at the plasma membrane at a steady-state level. After binding of NMS, receptor internalization is presumably slightly inhibited as prolonged incubation with NMS can result in receptor upregulation.

In summary, our study has demonstrated that RhoA is not a regulator of mACHR sequestration in HEK293 cells. However, when overexpressed, RhoA inhibits both clathrin-dependent and -independent mACHR sequestration, presumably by inhibiting recycling of mACHRs to the plasma membrane. The identification of the RhoA targets involved will provide new information on the mechanisms that regulate GPCR trafficking in HEK-293 cells.

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Send reprint requests to: Chris J. van Koppen, Ph.D., Institut für Pharmakologie, Universitätsklinikum Essen, D-45122 Essen, Germany. E-mail: van_koppen@uni-essen.de