Caveolin 2 Regulates Endocytosis and Trafficking of the M1 Muscarinic Receptor in MDCK Epithelial Cells^D

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Clathrin and caveolins are known for their involvement in the internalization of numerous receptors. Here we show that in polarized epithelial Madin-Darby canine kidney cells, both the clathrin machinery and caveolins are involved in the endocytosis and delivery to the plasma membrane (PM) of the M1 muscarinic acetylcholine receptor (mAChR). We initially localized this receptor to the lateral membrane, where it accumulates proximal to the tight junctions. From there it is internalized through the clathrin-mediated pathway. In addition, the receptor may associate on the PM with caveolin (cav) 2 or in intracellular compartments with either cav 2, or monomeric or oligomeric cav 1. Association of the PM M1 mAChR with cav 2 inhibits receptor endocytosis through the clathrin-mediated pathway or retains the receptor in an intracellular compartment. This intracellular association attenuates receptor trafficking. Expression of cav 1 with cav 2 rescues the latter's inhibitory effect. The caveolins stimulate M1 mAChR oligomerization thus maintaining a constant amount of monomeric receptor. These results provide evidence that caveolins play a role in the attenuation of the M1 muscarinic receptor's intracellular trafficking to and from the PM.

INTRODUCTION

Endocytosis and the events leading up to it are of prime importance as they enable cells to regulate receptor-dependent signaling pathways as well as a variety of cellular functions. Clathrin-mediated endocytosis (CME) is the most well-characterized pathway for the internalization of soluble macromolecules and integral membrane proteins from the plasma membrane (PM; Conner and Schmid, 2003). G-protein-coupled receptors (GPCRs), being the largest receptor group, resort to a unique set of events that couple signaling and endocytosis (von Zastrow, 2003). Within the GPCRs, the muscarinic acetylcholine receptors (mAChRs) mediate the acetylcholine-dependent stimulus and are, therefore, highly regulated by G-protein activation and receptor endocytosis. In mammals, five distinct mAChR subtypes (M1–M5) exist. The M1, M3, and M5 mAChRs are coupled to the Gαq/11 and Gαq13 G proteins, leading to, for example, activation of phospholipase C (PLC) and phospholipase D (PLD), which have been shown to affect endocytosis (Shen *et al.*, 2001); M2

and M4 mAChRs activate Gi proteins, leading to the inhibition of adenylyl cyclase (van Koppen and Kaiser, 2003). The different mAChR subtypes display unique, but not exclusive, expression patterns in the CNS and peripheral organs, such as the heart, epithelial exocrine glands, and smooth muscle tissue (Matsui *et al.*, 2004). Activation of mAChRs has been found to elicit pigment-granule dispersion in retinal pigment epithelium, which is blocked by antagonist specific to M1 and M3 mAChRs (Phatarpekar *et al.*, 2005). A study performed on human conjunctival epithelium revealed the expression of all five mAChRs. Specifically, M1 mAChRs were detected only intracellularly, but were mobilized to the PM when cholera toxin and hydrocortisone were omitted from the culture medium (Enriquez de Salamanca *et al.*, 2005). Recent transcriptome analysis has shown that M1 mAChRs are expressed in epithelial cells to a degree similar to that appearing in the nervous system (see CHRM1 in GeneCards: www.genecards.org and Su *et al.*, 2004) with transcriptome analysis at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi>). In addition, M1 mAChR-deficient mice show a stronger response to amphetamines (van Koppen and Kaiser, 2003).

Prolonged exposure of mAChRs, as well as other GPCRs, to agonists typically results in the attenuation of their cellular responses and induces their sequestration in internal organelles. This sequestration may represent endocytosis of receptors via clathrin-coated vesicles (von Zastrow *et al.*, 1993; Tolbert and Lamah, 1996) or caveolae (Roettger *et al.*, 1995; Henley *et al.*, 1998; Dessy *et al.*, 2000; Lamb *et al.*, 2002; McFarland *et al.*, 2004), or it may be associated with conformational changes of the receptor in the PM that make it inaccessible to agonists (Roettger *et al.*, 1995). Endocytosis may play different roles for different GPCRs. For example, β2-adrenergic receptor is endocytosed and recycled back to the PM, where it is resensitized. The receptor is internalized

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Abbreviations used: AP, apical; BL, basolateral; CME, clathrin-mediated endocytosis; cav 1 and 2, caveolin 1 and 2; DRM, detergent-resistant membrane; MDCK, Madin-Darby canine kidney; PM, plasma membrane; GPCR, G-protein-coupled receptor; mAChR, muscarinic acetylcholine receptor.

by early-recycling-sorting endosomes, from which it is returned to the PM (Pitcher *et al.*, 1995). Internalization may also prolong desensitization of the M4 mAChR (Bogatkewitsch *et al.*, 1996; Lee *et al.*, 1998; Vogler *et al.*, 1998).

Many reports have shown the uncoupling of GPCR down-regulation and endocytosis. Specifically, deletion of a large portion of the third cytoplasmic loop of the M1 mAChRs strongly reduces agonist-induced M1 down-regulation without affecting receptor internalization in Y1 adrenal carcinoma cells. In JEG-3 cells, M1 mAChR undergoes agonist-induced down-regulation, but not internalization. Several substitution mutants of M1 mAChRs stably transfected in CHO cells are defective in down-regulation, but not agonist-induced receptor internalization (Shockley *et al.*, 1997). Thus, endocytosis appears to be segregated from the degradation process of the M1 receptor. The mechanism by which GPCRs, and specifically mAChRs, internalize has recently been the focus of a great deal of research, and the mechanism of internalization, CME or caveolae, appears to be dependent on the cell line under study (Silva *et al.*, 1986; Slowiejko *et al.*, 1996; Tolbert and Lameh, 1996; Feron *et al.*, 1997; Dessy *et al.*, 2000; Roseberry and Hosey, 2001).

Caveolae-dependent internalization of sphingolipids and sphingolipid-binding toxins (cholera toxin and shiga toxin), GPI-anchored proteins, the autocrine motility factor (AMF), endothelin, growth hormone, IL2 receptors, viruses [including simian virus (SV) 40], and bacteria has been well documented (Lamaze *et al.*, 2001; Le *et al.*, 2002; Pelkmans and Helenius, 2002; Conner and Schmid, 2003; Nabi and Le, 2003).

The caveolin proteins are the major constituents of cell-surface caveolae, a specialized form of detergent-resistant membranes (DRMs). Mammals express three isoforms, of which caveolin (cav) 1 and 3 are predominantly localized at the cell surface (Parton, 2003). Generally, cav 2 is colocalized with Golgi markers, unless targeted to the PM by oligomerization with cav 1 (Parolini *et al.*, 1999). Caveolins localized to the Golgi are mainly in the nonnumeric state and are detergent-soluble (Pol *et al.*, 2005). Caveolins, and specifically cav 1, have been extensively studied in polarized epithelial Madin-Darby canine kidney (MDCK) cells and localized to both apical and basolateral PM and Golgi (Kurzchalia *et al.*, 1992; Dupree *et al.*, 1993; Sargiacomo *et al.*, 1993; Parton, 1994; Parton *et al.*, 1994; Hailstones *et al.*, 1998; Vogel *et al.*, 1998). Expression of cav 1 causes the formation of caveolae and a lack of cav 1 or 3 results in loss of caveolae (Fra *et al.*, 1995; Vogel *et al.*, 1998). In polarized MDCK cells, cav 1 and 2 are found as hetero-oligomers on basolateral (BL) caveolae, whereas the apical (AP) membrane, where only cav 1 is present, lacks these caveolae (Scheiffele *et al.*, 1998; Vogel *et al.*, 1998; Lahtinen *et al.*, 2003). Cross-linking of apical raft proteins by primary and secondary antibodies resulted in cav 1 recruitment to the cluster followed by caveolae internalization (Verkade *et al.*, 2000). Expression of a cav 1 or 2 mutant prevents the formation of cav 1–2 hetero-oligomeric complexes and leads to intracellular retention of cav 2 and the disappearance of caveolae from the BL membrane (Dupree *et al.*, 1993; Scheiffele *et al.*, 1998; Vogel *et al.*, 1998). Expression of cav 2 results in its enrichment in BL internal vesicles. A study on epithelial FRT cells lacking both caveolins clearly shows that cav 2 is preferentially localized to the Golgi, whereas cav 1 is preferentially found at the PM (Mora *et al.*, 1999). Cav 1 has also been suggested to associate with bone morphogenetic protein (BMP) receptors on the PM. Binding receptor type-I (BRIa) associates with and shuttles between cav 1 α and β , depending on the presence of the BMP-2 ligand. In addition, the cav

1 β isoform inhibits BMP signaling, whereas the α isoform does not (Nohe *et al.*, 2005).

Clearly, the events that precede endocytosis and the mechanism by which GPCRs, and specifically mAChRs, are endocytosed are of prime interest, as this is the main mechanism for the attenuation of receptor signaling. Here we show that the M1 mAChR is localized to the lateral PM, proximal to the tight-junction complex, and internalized by the clathrin-dependent endocytosis system. We also provide preliminary evidence for cav 2 inhibition of receptor arrival at the PM and internalization, a phenomenon that may be abolished by cav 1.

MATERIALS AND METHODS

Materials

Chemicals and anti-Flag antibody were purchased from Sigma-Aldrich (Rehovot, Israel), unless otherwise specified. Growth media were purchased from Biological Industries (Beit Haemek, Israel). All fluorescent secondary antibodies and Alexa 594-transferrin were purchased from Molecular Probes (Eugene, OR). MG132 stock solution (20 mM in DMSO) was obtained from Calbiochem (San Diego, CA), X22 antibody against clathrin heavy chain was kindly provided by Frances Brodsky (UCSF, San Francisco, CA). Rat mAb against ZO-1 was obtained from Chemicon International (Temecula, CA) and GP135 was a kind gift from George Ojakian (SUNY, Brooklyn). Anti-caveolin 1 (clone pAb) and caveolin 2 (clone 65) were obtained from Transduction Laboratories (Lexington, KY), anti-cav 2, 12CA5 and 9E10 and mouse anti-ubiquitin (P4G7) antibodies were purchased from Covance (Berkeley CA), and anti-E-cadherin was obtained from Zymed (San Francisco, CA). M1 mAChR cDNA was obtained from UMR cDNA Resource Center (University of Missouri-Rolla, Rolla, MO), pDsRed2-Rab5a/7/9/11 (BD Biosciences Clontech, Palo Alto, CA) was kindly provided by Rick Pagano (Rochester, MN; Choudhury *et al.*, 2005). All images were compiled using the Adobe Photoshop (San Jose, CA), and/or Canvas software (ACD Systems International, Saanichton, British Columbia, Canada) and are representative of the original data.

Methods

DNA Constructs and Cloning. The M1 mAChR cDNA fused to triple hemagglutinin (HA) at the N-terminal was obtained from the UMR cDNA Resource Center (www.cdna.org). The cDNA was amplified using the following primers (5' primer: ACC ACG CAA GCT TAC CAT GTA CCC ATA CGA TGT TCC AGA TTA CG and 3' primer: ACC ACG CGG ATC CGC GCA TTG GCG CGA GGG AGT GCG GTG CAC G), followed by digestion with HindIII and BamHI, and cloned at the N-terminal of green fluorescent protein (GFP) in pCB7-GFP to obtain extracellular triple HA and C-terminal intracellular GFP. The construct was transfected into MDCK tet off cells (T23) and selected using hygromycin to produce stable MDCK T23 M1 mAChR.

Adenoviral Expression, Immunofluorescence, and Biochemical Assays. MDCK cells were grown as previously described (Altschuler *et al.*, 1999). Recombinant tetracycline-regulated adenovirus-expressing dominant dynamin I K44A, dominant-negative clathrin hub, and the wild-type form of cav 1 and 2 were produced and used as described previously (Altschuler *et al.*, 1998, 1999). The protein levels were regulated by the concentration of doxycycline (Dx), the amount of virus, and the length of time after infection and/or Dx removal, to obtain the minimal expression that results in an inhibitory or stimulatory effect on endocytosis and avoids toxic repercussions. Cells infected by recombinant adenovirus were incubated for 16–18 h to express recombinant proteins, except for the clathrin hub, which required 24–26-h incubation. We routinely used 60–90 pfu/cell to obtain the minimal functional effect. These conditions minimized the possibility of toxic effects and produced an adequate signal for our localization and functional studies. Controls in all of the experiments included cells that were 1) not infected, 2) infected but whose expression was fully repressed by 20 ng/ml Dx, or 3) infected with a control virus encoding β -galactosidase to identify possible viral effects. In all cases, Dx treatment resulted in the loss of any effect on endocytosis or redistribution of the M1 mAChRs. In biochemical or immunofluorescence experiments including carbachol, its concentration was 1 mM for the indicated periods. Images were taken using a Nikon TE-2000S (Melville, NY) inverted fluorescence microscope with a plan Apo 60 $\times$ objective lens (Nikon), equipped with a Z stepper and a Hamamatsu CCD ORCAII camera (Tucson, AZ). All images were deconvolved with SimplePCI software (Improvision, Coventry, United Kingdom). Endocytosis was performed according to loss-of-surface protocols. In brief, MDCK control cells and those expressing M1 mAChR were incubated with or without 1 mM carbachol for the indicated time periods, followed by rapid cooling of the cells. Subse-

quently, the receptor remaining on the PM was measured by the binding of iodinated anti-HA-12CA5 antibody to M1 mAChR on the BL PM. To calculate the amount of internalized M1 mAChR, the radioactivity bound to MDCK not expressing M1 mAChR was subtracted from the radioactivity bound to cells expressing M1 mAChR. Subsequently, the radioactivity associated with cells treated with carbachol was subtracted from the radioactivity of the untreated cells (Total Bound), yielding the amount of radioactivity representing internalized M1 mAChR. The radioactivity of the cells expressing M1 was in the range of 60,000–100,000 cpm per sample. The background was generally between 1 and 2% of the experimental total bound radioactivity and represented the nonspecific 12CA5 bound to cells not expressing the M1 mAChR. To obtain immunofluorescent transferrin or E-cadherin, the following experiment was conducted: briefly, MDCK cells were grown in Corning transwells (Corning Glassworks, Corning, NY) for 3 d, infected with the appropriate recombinant adenoviruses, and incubated for 18 h. The cells were then rinsed with ice-cold phosphate-buffered saline (PBS; containing 1 mM CaCl_2 and 1 mM MgCl_2), and then incubated with 40 μl of media containing Alexa 594-transferrin or anti-E-cadherin for 60 min at 4°C. Excess transferrin or E-cadherin antibody was removed by extensive washes. The bound antibody was fixed with paraformaldehyde (PFA) according to an immunofluorescence protocol (Altschuler *et al.*, 1999).

Equilibrium Flotations. Flotation of detergent-insoluble complexes was performed as described previously (Rouvinski *et al.*, 2003). In brief, MDCK cells, cultured for 3 d on 90-mm dishes (1×10^7 cells), were gently scraped, pelleted, and lysed with 550 μl of flotation lysis buffer (1% vol/vol Triton X-100 [TX-100] in TNE [150 mM NaCl, 25 mM Tris-HCl, 5 mM EDTA, pH 7.5]) on ice for 30 min. The lysates were spun at low speed (3000 rpm, 4°C, 5 min) in an Eppendorf centrifuge (Fremont, CA; 5417; $g_{\text{av}} = 960 \times g$) to generate a postnuclear supernatant (PNS). All subsequent steps were performed on ice. The lysates were adjusted to 35% (vol/vol) Nycodenz by adding an equal volume of ice-cold 70% Nycodenz dissolved in TNE. Each sample (900 ml) was loaded at the bottom of a TLS-55 tube (Beckman Instruments, Fullerton, CA). An 8–25% Nycodenz linear-step gradient in TNE was then laid above the lysate (200 μl each of 25, 22.5, 20, 18, 15, 12, and 8% Nycodenz). The tubes were spun at 55,000 rpm for 3 h, at 4°C in a TLS-55 rotor ($g_{\text{av}} = 200,000 \times g$). Twelve 180- μl fractions were collected from the top of the tube. The buoyant material migrated to low-density fractions 1–7.

Coimmunoprecipitation. Immunoprecipitation of the M1 mAChR was performed according to Lu *et al.* (2001). In brief, MDCK-M1 mAChR cells were infected with different caveolin adenoviruses followed by incubation for 18 h to allow expression. Subsequently, cells were scraped and lysed in 1 ml of RIPA buffer containing 50 mM Tris (pH 7.4), 135 mM NaCl, 1% TX-100, and 60 mM octylglucoside and supplemented with protease inhibitors (2 mM phenylmethylsulfonyl fluoride and 100 U/ml aprotinin) and 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and 20 mM NaF. Lysates were cleared by centrifugation at $12,000 \times g$ for 30 min at 4°C, and 20- μl aliquots were saved as total protein lysates for analyses. Supernatants were incubated with anti-HA antibody (12CA5, 1 μg) and protein A-Sepharose beads (20 μl packed beads) at 4°C for 90 min rocking. At the end of the incubation, beads were washed five times with RIPA lysis buffer. The resulting immunoprecipitated immunocomplexes were solubilized in 35 μl of high-pH sample buffer, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. The protein complex was detected by Western blot analysis and developed by ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

Transient Transfection of RFP-Rab cDNAs. Full-length wild-type Rab cDNAs (Rab5a, Rab11, Rab7, and Rab9) cloned into pDsRed2 were transiently transfected into MDCK-M1 mAChR cells using Metafecten Pro (Biontex, Martinsried/Planegg, Germany) according to the manufacturer's protocol at a ratio of 1 μg DNA to 3 μl metafecten for 4×10^5 MDCK cells. At this ratio we obtained 20–30% transfected cells and the endosome sizes were similar to those obtained with antibody to other endosomal markers such as EEA1.

RESULTS

To investigate the mechanism underlying M1 mAChR endocytosis and membrane traffic, we subcloned the gene encoding the receptor as a fusion with GFP to generate an in-frame fusion protein in which GFP is fused to the C terminus of M1 mAChR (see *Materials and Methods*). This construct places GFP after the C-terminal tail in the cytoplasmic environment. Thus, the GFP does not interfere with ligand binding or with the conformational changes that follow receptor activation. We then generated a stable cell line that modestly expresses the receptor.

A. Apical

B. Tight junction

C. Basolateral

Figure 1. The M1 muscarinic receptor localizes to the basolateral plasma membrane in MDCK cells. MDCK tet-off cells expressing GFP-tagged M1 mAChR were grown for 3 d in Corning transwells and processed for immunofluorescence. (A) Cells were stained for M1 mAChR (GFP, left column) and the apical marker gp135 (Alexa 594, right column). Sections were taken through the apical and basolateral domains as indicated. No M1 mAChR is seen within the apical domain. (B) Cells stained for M1 mAChR (GFP, left column) and the tight junction marker ZO1 (Alexa 594, right column). (C) Cells stained for M1 mAChR (GFP, left column) and transferrin bound to the basolateral membrane for 60 min at 4°C (Alexa 594, right column). Bar, 5 μm .

M1 Muscarinic Receptor Localizes to the Basolateral Plasma Membrane in MDCK Cells

To identify the subcellular epithelial structure to which M1 mAChR is localized, we performed immunofluorescence co-localization experiments with markers for the AP PM, tight junction and BL PM. Figure 1A shows an AP section decorated with the AP marker gp135 and the absence of GFP-M1 mAChR staining. A BL section, taken 4 μm below, shows the absence of gp135 and clear classic BL PM localization of the M1 mAChR. In Figure 1B, two sections taken at the level of the tight junction clearly show the ZO1 tight-junction marker. Both sections, taken 1 μm apart, reveal M1 mAChR. Note that the lower section is sharper, indicating that the receptor is localized in the BL region of the tight junction. In

Figure 2. The M1 muscarinic receptor is differentially distributed along the lateral membrane and is highly concentrated proximal to the tight junction. MDCK tet-off cells expressing GFP-tagged M1 mAChR were grown for 3 d in Corning transwells and processed for immunofluorescence. Cells were stained for M1 mAChR (GFP, left column) and with anti-E-cadherin (Alexa 594, right column). Sections were taken through the lateral domain every 2 μm . M1 mAChR is concentrated proximal to the tight junction and fades with distance. The numbers on the left represent distance in microns from the tight junction toward the basolateral PM. Bar, 5 μm .

Figure 1C, the M1 mAChR is completely colocalized with the transferrin bound to the BL-resident PM transferrin receptor. Moreover, the M1 mAChR is concentrated in punctate staining on the lateral membrane and is not evenly dispersed, indicative of the receptor's localization in a specific structure, such as clathrin-coated pits, rafts, or caveolae. To confirm the observed uneven distribution of M1 mAChR along the BL PM, we colocalized the receptor with E-cadherin, the latter known to be distributed evenly along the lateral membrane. Four lateral sections beginning 1 μm under the tight junction and spaced 2 μm apart are shown in Figure 2. The M1 mAChR is differentially distributed on the lateral membrane and mainly concentrated proximal to the tight junction. Its distribution declines as we proceed away from the tight junction, down the lateral membrane. We concluded that M1 mAChR is unevenly localized through the lateral PM, its highest concentration near the tight junction.

Rapid Endocytosis of the M1 Muscarinic Receptor

To gain an understanding of M1 mAChR endocytosis, we utilized both biochemical and microscopic approaches. In

the steady state, the M1 mAChR appeared to be on the lateral PM with only a modest amount of it internalized (Figure 1, B and C). Therefore, we treated the cells with the well-known muscarinic agonist carbachol at the saturating concentration of 1 mM and incubated them for different periods of time at 37°C. At the indicated time points, the cells were rapidly cooled and processed for microscopy or biochemical quantitation of endocytosed M1 mAChR. For the biochemical assay, we resorted to the loss-of-surface approach, in which the amount of receptor remaining on the surface at the indicated time points after agonist stimulation is quantitated. For quantitation, we iodinated a 12CA5 antibody. After treatment with or without carbachol, the cells were rapidly cooled and bound the iodinated 12CA5 antibody, on the BL PM, by means of the HA tag subcloned into the N-terminal extracellular domain of the receptor. In Figure 3A, very rapid internalization was seen in the first 2 min after agonist treatment. This rate accounted for ~15% of the total receptor bound per minute. Within 5 min of carbachol treatment, the internalization rate had declined dramatically, to ~3% per minute. We reasoned that the drop in internalization rate might be due to rapid recycling of the M1 mAChR returning to the BL PM.

To image carbachol-dependent endocytosis of M1 mAChR, cells treated with carbachol for the indicated periods of time were cooled and fixed with PFA, followed by immunocytochemistry. In Figure 3B, we show a lateral section proximal to the tight junction where both PM and endocytosed M1 mAChR are observed at maximum intensity. The steady-state distribution of M1 mAChR, shown in Figures 1, B and C, 2, and 3B (0 time), indicates that ~10–15% (as quantitated by ImageJ densitometry) of the total amount of receptor is in internal endosomal compartments, whereas most of it is concentrated on the lateral membrane. Constitutive agonist treatment (1 mM carbachol) resulted in rapid endocytosis: within 15 min, with about half of the receptor internalized. Within 30 min, most of it was internalized. Staining of the membrane by the receptor was minimal and unchanged from the 30-min time point through 45 and 60 min. In contrast to the microscopy-based assay, the biochemical assay showed a plateau at ~40–50% of the internalized receptors. This difference may be explained by the higher sensitivity of the iodinated antibody tracer or difficulty in detecting the recycling M1 mAChR. Nevertheless, the M1 mAChR internalized at a rate similar to that of the fast-internalizing receptors, such as transferrin and IgA receptors.

M1 Muscarinic Receptor Rapidly Recycles to the Lateral Membrane

To shed more light on the route of M1 mAChR after endocytosis and to better understand the amount of receptor recycling to the lateral membrane that could not be observed in the presence of agonist, the following experiment was performed. M1 mAChR-expressing cells were treated for 10 min with agonist, and then the agonist was extensively washed out, and the cells were chased for different periods of time. As shown in Figure 4, within 10 min the M1 mAChR internalized into endosomal compartments. After removal of the agonist, the M1 mAChR receptor rapidly recycled to the lateral membrane. Within the first 10 min, most of the receptor had returned to the lateral PM. The last remains of internalized receptor were observed in endosomal structures 20 min after the removal of agonist; at 30, 40, and 50 min of agonist removal, all of the receptor was observed on the lateral membrane. Thus, the M1 mAChR receptor rap-

Figure 3. The M1 muscarinic receptor rapidly endocytoses from the lateral membrane. MDCK tet-off cells expressing HA-tagged M1 mAChR-GFP were grown for 3 d in Corning transwells. The cells were then treated with 1 mM carbachol for the indicated times and either (A) processed biochemically for the amount of internalized receptor or (B) processed for immunofluorescence. The amount of internalized receptor was calculated by quantifying the receptor remaining on the PM as the binding of iodinated anti-HA antibody (12CA5) to untreated control cells and treated cells. The percent M1 mAChR endocytosis was calculated by the loss-of-surface method (see *Materials and Methods*). Experiments were performed in triplicate.

Figure 4. The M1 muscarinic receptor rapidly recycles back to the lateral membrane. MDCK tet-off cells expressing M1 mAChR-GFP were grown for 3 d in Corning transwells. The cells remained untreated (Control) or were treated with 1 mM carbachol (Carb) for 10 min followed by extensive washes and chasing without carbachol for the indicated times. The cells were then fixed and processed for immunofluorescence and stained for M1 mAChR (GFP). Sections were taken through the lateral domain near the tight junction. Representative images are shown. In the absence of carbachol, M1 mAChR rapidly recycled back to the lateral PM. Bar, 2 μ m.

cate (a representative experiment is shown) or (B) processed for immunofluorescence. Cells were stained for M1 mAChR (GFP). Sections were taken through the lateral domain near the tight junction. Representative images are shown. M1 mAChR was rapidly endocytosed and remained intracellular in the presence of carbachol. Bar, 2 μ m.

Figure 5. The M1 muscarinic receptor endocytoses through a dynamin-clathrin-dependent pathway. MDCK tet-off cells expressing HA-tagged M1 mAChR-GFP were grown for 3 d in Corning transwells. Control cells (uninfected) and cells expressing the dominant-negative dynamin I K44A mutant (50 pfu/cell) for 18 h and the dominant-negative clathrin hub mutant (150 pfu/cell) for 24 h were either (A) used to monitor the amount of M1 mAChR accumulating

idly endocytoses from and rapidly recycles back to the lateral PM.

Mechanism Governing M1 Muscarinic Receptor Endocytosis

To investigate the mechanism by which the M1 mAChR is internalized, we adopted a dominant-negative approach, i.e., expression of mutant forms of polypeptides that are known to participate in and inhibit specific endocytosis pathways. In Figure 5A, it can be seen that expression of dynamin I K44A, a dominant-negative mutant known to inhibit both clathrin- and caveolin-based endocytosis pathways, increased the amount of receptor located at the BL PM to fivefold that of the control cells. This was indicated by an increase in the binding of iodinated anti-HA antibody. Similar expression of the clathrin hub mutant also resulted in a two- to threefold increase in M1 mAChR on the lateral PM. When endocytosis was inhibited by expressing the dominant-negative polypeptides, receptors continued to be delivered to the PM, either from the biosynthetic pathway or by bypassing the inhibitory effect and recycling back to the PM. Under these conditions, receptors accumulated on the PM without being internalized. The fivefold dynamin-dependent and two- to threefold clathrin-dependent M1 mAChR accumulation on the BL PM indicates that endocytosis was severely inhibited by the expression of these constructs. Western blot and incubation of the cells with proteasome inhibitor show that the inhibition of endocytosis inhibits lysosomal degradation of the receptor (Shmuel and Altschuler, unpublished results).

To confirm these observations and to better observe the internalization of the M1 mAChR, the cells were treated with agonist, followed by incubation at 37°C for the indicated time periods. At each time point, the cells were cooled, fixed, and processed for immunofluorescence. Close scrutiny at 0 time (Figure 5B; before the addition of agonist) in cells expressing dynamin or clathrin dominant-negative mutants shows the disappearance of internalized M1 mAChR endosomal staining. This observation indicates that the internal staining in the control cells was the result of basal internalization of the receptor rather than of receptor transport via the biosynthetic pathway on its way to the BL membrane. This means that the dominant-negative con-

on the PM through binding of ^{125}I -12CA5 antibody at 4°C to the lateral PM (*t* test between control and dynamin I K44A and with clathrin hub performed and *p* value indicated in graph, ***p* < 0.01 and **p* < 0.05) or (B) incubated with 1 mM carbachol for 10 or 60 min, or left untreated (control) as indicated, and processed for immunofluorescence. The cells were stained for M1 mAChR (GFP). Sections were taken through the lateral domain in proximity to the tight junction. In A, both dynamin and clathrin mutants are seen to cause a dramatic increase in M1 mAChR on the lateral PM. Images in B show complete inhibition of endocytosis by the dynamin I dominant-negative construct and partial inhibition of endocytosis by the clathrin hub mutant. Arrowheads indicate punctate accumulation of M1 mAChR on the lateral PM. Representative images are shown. Bar, 2 μm . (C) Cell expressing the dominant-negative dynamin I K44A as described were not treated or were treated with 1 mM carbachol (Carb) for 5 or 15 min and processed for immunofluorescence. Cells were stained for M1 mAChR (GFP, left column) or with anti-clathrin heavy chain X22 antibody (Alexa 594, right column). Sections were taken through the lateral domain near the tight junction. The dominant-negative dynamin I completely inhibited M1 mAChR endocytosis; clathrin was redistributed and revealed very small punctate staining within minutes of carbachol treatment. Representative images are shown. Bar, 2 μm .

structs inhibited the basal internalization of M1 mAChR. After 10 min of agonist treatment, the dominant-negative dynamin I-expressing cells showed almost no internalization of M1 mAChR; after 60 min, minimal receptor internalization was observed, with a large amount of receptor decorating the lateral PM. These results indicate that the dominant-negative dynamin construct severely inhibited receptor endocytosis. Cells expressing the dominant-negative clathrin construct showed reduced internalization at both 10 and 60 min. They also showed increased punctate staining at the lateral PM (Figure 5B, arrowheads), suggesting accumulation in the clathrin-coated pits.

To more closely observe the distribution of the M1 mAChR in relation to clathrin, dominant-negative dynamin I K44A was coexpressed; this slowed the rate of internalization, enabling us to capture the colocalization of the receptor with clathrin. As seen in Figure 5C, in the absence of agonist, M1 was present mainly on the lateral PM, and clathrin was observed as punctate staining in the cytoplasm. Despite the expression of the dominant-negative dynamin construct, clathrin did not accumulate on the PM. Expression of the dominant-negative dynamin showed that agonist treatment for a period of 5 or 15 min causes redistribution of clathrin to the PM with a significant increase in colocalization with the M1 mAChR at the 15-min time point. These results pinpoint the involvement of the CME pathway in M1 mAChR receptor internalization.

Involvement of Caveolins in M1 Muscarinic Receptor Internalization

Mounting evidence for the involvement of caveolae in GPCR endocytosis (Wyse *et al.*, 2003; Chini and Parenti, 2004; McFarland *et al.*, 2004), together with our observation that the clathrin dominant-negative construct has a weaker inhibitory effect than the dynamin dominant-negative construct, raises the possibility that caveolae-dependent endocytosis may be involved in the M1 mAChR internalization process. This is in light of the effect of dynamin in caveolae-dependent endocytosis (Nabi and Le, 2003; Shajahan *et al.*, 2004) and the increasing evidence of caveolae involvement in GPCR internalization. To test this hypothesis, we expressed cav 1, cav 2, or cav 1 and 2 together in MDCK cells expressing M1 mAChR for 18 h and assayed M1 mAChR receptor endocytosis. Owing to the elevated sensitivity of MDCK cells to the expression of polypeptides such as caveolins, we used a recombinant adenoviral infection system (Mora *et al.*, 1999). Infection of MDCK cells with recombinant adenoviruses for cav 1 and 2 for 16–20 h had no effect on the polarity of the cells, as indicated by the expected localization of several polar markers such as E-cadherin, P58, ZO1, and gp135 (unpublished data). As shown in Figure 6A, expression of cav 1 had no effect on M1 mAChR endocytosis. On the other hand, expression of cav 2 inhibited M1 mAChR endocytosis by as much as 70%. This inhibitory effect was relieved by coexpression with cav 1, suggesting that M1 mAChR associates with cav 2 on the PM, preventing receptor internalization. When both caveolins were expressed, cav 2 preferentially bound cav 1 and released M1 mAChR, enabling its internalization.

Next, we examined the effect of caveolins on the delivery of the M1 mAChR to the lateral PM. M1 mAChR arrives at the lateral PM via the biosynthetic pathway or from recycling endosomes—common endosomes as part of the recycling pathway. In this experiment, radioactive anti-HA antibody was applied to the BL PM, which binds an HA tag placed on the extracellular domain of the receptor. The amount of radioactivity indicates the amount of receptor on

Figure 6. Endocytosis of the M1 muscarinic receptor is affected by expression of caveolins 1 and 2. MDCK tet-off cells expressing HA-tagged M1 mAChR-GFP were grown for 3 d in Corning transwells. Control cells (uninfected) and cells expressing caveolin 1 or caveolin 2 or coexpressing both caveolins (50 pfu/cell of each construct) for 18 h were then (A) incubated with 1 mM carbachol for 10 min and processed biochemically for the amount of receptor remaining on the PM through the binding of iodinated anti-HA antibody (12CA5) to untreated, control cells and treated cells or (B) used to monitor the amount of M1 mAChR accumulating on the PM through binding of ^{125}I -12CA5 antibody at 4°C to the lateral PM. (A) Percent M1 mAChR endocytosis was calculated by the loss of surface method. Experiments were performed in triplicate, and a representative experiment is shown; (B) *t* test was performed, and *p* value is indicated in graph (***p* < 0.01 and **p* < 0.05). Caveolin 2 expression resulted in a marked reduction in endocytosis of the M1 mAChR. Expression of both caveolin 1 and 2 abolished the caveolin 2-dependent inhibition of M1 mAChR endocytosis. Experiments were performed in triplicate, and a representative experiment is shown.

the BL PM. Figure 6B shows that expression of cav 1 slightly increased the amount of M1 mAChR on the BL PM. Conversely, expression of cav 2 reduced the amount of M1 mAChR on the surface by as much as 45% compared with the control and by 55% compared with cav 1-expressing cells. Coexpression of both cav 1 and 2 abolished the effects of the caveolins, bringing the amount of surface-localized M1 mAChR to close to control levels. This indicates that the effect achieved mainly by cav 2 is antagonized by cav 1.

Both inhibitory effects, on endocytosis and on the presence of M1 mAChR on the BL PM, were manifested upon expression of cav 2 and relieved by expression of cav 1. This raises the possibility of an indirect or direct association between cav 2 and M1 mAChR, which is terminated by the preferential association of cav 1 with cav 2.

The extreme effect on M1 mAChR imposed by the expression of cav 2 and relieved by that of cav 1 suggests that the receptor may be shuttling in and out of DRMs on the PM during the recycling or while in the biosynthetic pathway. To address this possibility, we studied the flotation properties of the M1 mAChR in TX-100 extracts from MDCK cells expressing cav 1, cav 2, or both caveolins. As can be seen in Supplementary Figure 1A, cav 1 was localized in DRM, whereas cav 2 was not. Subsequently (Supplementary Figure 1B), the M1 mAChR remained excluded from the DRMs. These results led us to assume that the effect of the caveolins may be mediated in the presence of activated receptor by its agonist. As can be seen in Supplementary Figure 1C, neither agonist treatment nor expression of cav 2 followed by agonist treatment caused the receptor to migrate into the DRMs. Nevertheless, expression of cav 2 along with agonist treatment caused a minor but noticeable shift toward the lighter fractions (fractions 7–10), which are not classical DRMs. These experiments thus rule out the likelihood that the effect of cav 2 on M1 mAChR occurs mainly within the DRM. We hypothesize that cav 2 associates with M1 mAChR along the biosynthetic pathway and on the lateral membrane outside of the DRM. The cav 2-M1 mAChR association competes with cav 1's association with cav 2. When the latter occurs, cav 2 becomes enriched in the DRM, and M1 mAChR is released and free to be endocytosed by the clathrin-mediated machinery.

Newly Synthesized Caveolins 1 and 2 Affect Localization of the M1 Muscarinic Receptor

To further study the effect of caveolins on M1 mAChR trafficking, we first examined the localization of the former in MDCK cells that do not express M1 mAChR. Figure 7A shows that each caveolin, when expressed alone for 16 h, was predominantly localized to the intracellular compartments. When both cav 1 and cav 2 were coexpressed, in addition to their intracellular endosomal localization, they were significantly localized to the lateral PM (Figure 7A, arrowheads). These results are in agreement with those of previous studies using the adenoviral delivery of caveolins (Parolini *et al.*, 1999).

To study the effect of caveolin expression on the distribution of M1 mAChR, we infected MDCK cells stably expressing M1 mAChR with adenovirus carrying caveolins under a tet-regulated promoter. As shown in Figure 7B, expression of cav 1 or 2 did not alter the BL localization of the M1 mAChR. On closer inspection, cells expressing cav 2 showed, in addition to the PM localization, intracellular localization of the M1 mAChR. Intracellular M1 mAChR colocalized extensively with cav 2. When both caveolins were expressed, M1 mAChR was localized to intracellular compartments, in addition to its localization in the lateral PM (Figure 7B, arrowheads). These results indicate that cav 2, being in the intracellular pathway of the M1 mAChR endocytic pathway, has the potential to affect M1 mAChR trafficking.

To further study M1 mAChR trafficking of agonist-activated receptor, cells expressing M1 mAChR and newly synthesized caveolins were incubated with agonist (1 mM carbachol) for 15 min or for 15 min with a 45-min chase period without agonist. This enabled us to assay the effect of the

caveolins on the internalization as well as on the recycling of the receptor back to the lateral PM. A 15-min agonist treatment resulted in complete internalization of the receptor, with none remaining on the lateral PM (Figure 7C Control). Figures 3B, 4, and 5B show that when M1 mAChR was treated with agonist (when caveolins are not coexpressed), a fraction of the receptor decorated the lateral PM. When cav 1 was expressed, no receptor decorated the PM, indicating that in the absence of cav 1, the M1 mAChR rapidly internalizes and a fraction of it (within 15 min of agonist treatment) is recycled to the lateral PM. When caveolins were present, they attenuated receptor transport within the intracellular compartment (Figure 7C). A 45-min chase enabled complete recycling of the receptor to the lateral PM (Figure 4). In Figure 7D, a fraction of the receptor is seen recycling back to the lateral PM (arrows), whereas the main fraction accumulates in a tight perinuclear compartment, colocalizing with intracellular caveolins. Higher expression of the caveolins resulted in complete cessation of receptor recycling to the PM, whereas a lower level of caveolin expression revealed the appearance of some recycled receptor on the lateral PM. This finding indicates that at a low level of caveolin expression, some receptors elude the interaction with caveolin and recycle back to the lateral PM. On the other hand, at a higher level of caveolin expression, all of the M1 mAChR is associated with caveolins and cannot recycle back to the lateral membrane. These findings support our concept that caveolins attenuate M1 mAChR recycling back to the lateral PM by direct or indirect association.

Intracellular Transport of the M1 Muscarinic Receptor

Our observation that cav 1 and 2 attenuate M1 mAChR either on the BL PM or at intracellular locations motivated us to establish the intracellular pathway of the M1 mAChR and to assay the effect of caveolins on its intracellular transport. Therefore, we focused on one biosynthetic pathway and three endocytic pathways: the epithelial-specific biosynthetic pathway to the BL PM through Rab11-positive endosomes, the recycling pathway starting with arrival at BL early endosomes (Rab5), the degradative-lysosomal pathway through late endosomes (Rab7), and the Rab9-dependent pathway that acts at the late endosomes to rescue membrane proteins back to the TGN (*trans*-Golgi network). Specifically, we colocalized the M1 mAChR with different endogenous Rab proteins that are well-established markers for the different endosomal compartments in both nonpolarized and polarized epithelial cells (Mostov *et al.*, 2000; Zerial and McBride, 2001; Pfeffer, 2005; Rodriguez-Boulan *et al.*, 2005). All the antibodies to the different Rab proteins revealed very faint signal in immunofluorescent experiments, and we could not establish colocalization with the different Rab proteins being studied. We therefore transiently expressed Rab proteins fused to red fluorescent protein (RFP). In general, we attempted to express low levels of the Rab proteins in order to minimize their effect on the secretory system where they may cause enlargement of the relevant endosome (Stenmark *et al.*, 1994). Expression of all Rabs (Rab5, 7, 9, and 11) trapped the M1 mAChR in the corresponding endosomal compartment, even in cells that had not been treated with carbachol. This indicated that a fraction of the M1 mAChR, which is independent of agonist treatment is in transit through the endocytic system, in both the recycling pathway and the degradative pathway.

In Figure 8A we show that in the absence of agonist, a small fraction of the M1 mAChR colocalizes with Rab5 and, interestingly, all Rab5 early endosomes contain M1 mAChR. After a 10-min treatment with the agonist carbachol, all the

Figure 7. Transient expression of caveolins in MDCK cells. MDCK tet-off cells expressing the M1 mAChR-GFP (B–D) or not expressing the receptor (A) were grown for 3 d in Corning transwells and then infected with adenovirus for expression of caveolin 1, caveolin 2, or both caveolins (50 pfu/cell) for 18 h. The cells were then processed for immunofluorescence. (A) Tracing of caveolin (cav) localization in the absence of M1 mAChR or (B–D) tracing of caveolin (Alexa 594, right panel) localization in the presence of M1 mAChR (GFP, left panel). (B) Cells in the absence of the agonist carbachol. Cells expressing caveolin 2 or coexpressing caveolin 1 and 2 show intracellular and PM accumulation of the receptor. Cells expressing Caveolin 1 alone did not alter receptor lateral localization. (C) Cells were treated with 1 mM carbachol for 15 min, fixed, and processed for immunofluorescence. (D) Cells were treated with 1 mM carbachol for 15 min followed by a chase of 45 min without carbachol, fixed, and processed for immunofluorescence. Cells expressing cav 2 (marked by an asterisk) showed a low level of caveolin 2 and as a result, the receptor accumulated on the PM (arrowheads). Sections taken through the lateral domain near the tight junction. Images show that caveolins are mainly localized in intracellular compartments with little caveolin on the PM. Coexpression of caveolins 1 and 2 resulted in increased PM localization of the receptor. Because of expression of the caveolins, the M1 mAChR was retained in intracellular compartments. When both caveolins were expressed, low caveolin expression revealed the receptor on the PM; when a high level of caveolins was expressed, all the receptors appeared intracellularly. Arrowheads indicate punctate accumulation of M1 mAChR on the lateral PM, arrows point to sites of colocalization of the receptor and caveolins. Representative images are shown. Bar, 2 μ M.

M1 mAChR are localized to the Rab5 early endosomal compartments; after an additional 20 min of incubation in the presence of the agonist, a significant fraction of the receptor has left the Rab5 early endosomes and appears in other endosomal compartments. In contrast to the complete internalization of the M1 mAChR with 30 min of agonist, in the presence of elevated amounts of Rab5, a small amount of the receptor is recycled back to the PM and retained there. This may be explained by the known inhibitory effect of Rab5 in its GDP state on endocytosis (Stenmark *et al.*, 1994; Barbieri *et al.*, 2000; Galperin and Sorkin, 2003). Expression of the caveolins did not alter the route or the extent of the M1 mAChR colocalization with Rab5. In addition, cells expressing cav 2 continued to retain the receptor on either the BL PM or in intracellular compartments.

In epithelia, membrane proteins in their biosynthetic pathway and targeted to the basolateral PM may leave the TGN, fuse with the common/recycling endosomes and be targeted to the BL PM or, like E-cadherin, be transported to Rab11-positive endosomes and then delivered to the BL PM (Lock and Stow, 2005). To address this issue, we colocalized M1 mAChR with Rab11-RFP. In Figure 8B, we show that a fraction of the M1 mAChR colocalizes with Rab11. Expression of cav 1, 2, or both caveolins together with M1 mAChR did not alter the receptor's route or extent of endocytosis. The Rab11 localization may indicate recycling through a Rab11-positive endosome, or that M1 mAChR, in its biosynthetic pathway, arrives at a Rab11-positive endosome after its exit from the TGN.

In epithelial cells, neurons, and nonpolarized cells, Rab7 and Rab9 mark the late endosome compartment. Rab7 spe-

Figure 8. Transient expression of Rab proteins in MDCK-M1 muscarinic receptor cells. MDCK tet-off cells expressing M1 mAChR-GFP were grown for 1 d in Corning transwells and then transiently transfected with the indicated Rab construct and further grown for 2 more days. Cells were then infected with adenovirus for expression of caveolin 1, caveolin 2, or both (50 pfu/cell) for 18 h. Cells were either not treated or treated with carbachol agonist for 10 or 30 min as indicated. The cells were then processed for immunofluorescence. (A–D) Tracing of M1 mAChR (green) and (A) Rab5a (red), (B) Rab11 (red), (C) Rab7 (red), and (D) Rab9 (red). Stimulated and nonstimulated M1 mAChR utilizes both a short recycling pathway (BL early endosome to common endosome) and a long recycling pathway (BL early endosome to late endosomes to TGN and through Rab11-positive endosomes back to the BL PM) and is also delivered to the lysosomes through late endosomes. Representative images are shown. Bar, 2 μ m.

cifically marks the late endosome domain that concentrates proteins destined for lysosomal degradation and is involved in late endosome biogenesis (Feng *et al.*, 1995; Kamei *et al.*, 1999; Bucci *et al.*, 2000). In Figure 8C, M1 mAChR and Rab7 reveal significant colocalization. A closer look at the cells treated with the agonist reveals that many of the intracellular endosomal structures are not labeled with Rab7, indicating that a significant fraction of the M1 mAChR does not utilize the lysosomal degradative pathway. Expression of Rab7 does not alter the effect of cav 2 and its rescue by cav 1. Membrane proteins arriving at the late endosome may escape degradation by sorting to the Rab9-positive domain of the late endosome and being transported to the TGN, from which they can once more be transported to the BL PM. In Figure 8D, cells that have been treated with agonist or left untreated reveal high colocalization between the M1 mAChR and Rab9. Expression of caveolins did not affect the extent of colocalization, as evidenced by analyses of multiple experiments.

These results signify that the M1 mAChR utilizes two recycling pathways: a short pathway through early endosomes and common endosomes and a long pathway through early endosomes, late endosomes, the TGN, and returning to the BL PM (see Figure 11). The latter pathway enables lysosomal targeting as well as sorting away from the lysosomes of receptors that were misrouted earlier in the pathway.

The M1 Muscarinic Receptor Interacts with Caveolins

The inhibitory effect of cav 2 expression on M1 mAChR endocytosis and its rescue by expression of cav 1 (Figure 6), followed by the prominent colocalization of caveolins with M1 mAChR (Figure 7), led us to examine the interaction between caveolins and the receptor. For this purpose, we adopted an established method for the coimmunoprecipitation of GPCRs and cav 1 (Lu *et al.*, 2001). MDCK-M1 mAChR expressing cav 1, 2, or both were either not treated or treated with the agonist carbachol for 60 min and processed for coimmunoprecipitation. Western blot analysis of total ly-

Figure 9. The M1 muscarinic receptor interacts with both caveolin 1 and 2. MDCK tet-off cells expressing HA-tagged M1 mAChR-GFP were grown for 3 d and subsequently either not infected (Control) or infected with caveolin 1, caveolin 2, or both (50 pfu/cell of each construct) and incubated for 18 h for expression. The cells were then incubated in media containing 1 mM carbachol for 60 min. M1 mAChR was immunoprecipitated using anti-HA against the extracellular HA of the M1 mAChR. (A) Total lysate (2%) was separated by SDS-PAGE and reacted with anti-HA. (B) Immunoprecipitate was reacted with anti-HA and reveals the precipitated M1 mAChR. (C) Immunoprecipitate was reacted with caveolin 1-specific antibody. (D) Immunoprecipitate was reacted with caveolin 2-specific antibody. Molecular weights are shown on the left, and the identified protein complexes are indicated on the right. Both caveolins, but to a larger extent caveolin 2, stimulate oligomerization of M1 mAChR, maintaining a constant amount of monomeric receptor. The M1 mAChR interacts with monomeric and oligomeric caveolin 1 and with monomeric caveolin 2, and to a lesser extent with caveolin 1 homo- and hetero-dimers. Caveolin 2 prevents caveolin 1 oligomerization.

sates revealed that the expression of caveolins does not alter the amount of monomeric M1 mAChR but increases the amount of oligomeric M1 mAChR, with the highest amount of the latter in cells expressing cav 2. These oligomers are stable to SDS and heat in high-pH sample buffer, similar to the oligomers of cav 1. Treatment of the cells for 60 min with the agonist carbachol resulted in increased oligomerization

in control cells but had no effect on the oligomerization of caveolin-expressing cells (Figure 9, A and B). To assay the interaction of caveolins with M1 mAChR, we immunoprecipitated the latter with anti-HA, which reacts with the HA tag located in the extracellular domain of the receptor. The immunoprecipitate was separated by SDS-PAGE and reacted with cav 1- and cav 2-specific antibodies. The results, shown in Figure 9, C and D, indicate that independent of agonist treatment, cav 1 in both its monomeric and oligomeric forms interacts with M1 mAChR. Importantly, and independently of M1 mAChR, the cav 1 oligomer is disrupted in the presence of cav 2: the latter completely abolishes the cav 1 oligomer, leaving most of the caveolins as monomers with some homo-dimers (cav 1::cav 1) and some hetero-dimers (cav 1::cav 2; Figure 9, C and D). Both caveolins instigate the oligomerization of M1 mAChR, whereas the expression of cav 2, which does not oligomerize (like cav 1), stimulates a large amount of M1 mAChR oligomerization. The expression of cav 1 together with cav 2 reduces the amount of M1 mAChR oligomer (compared with oligomer stimulated by cav 2 alone). Most of the M1 mAChR interaction takes place with monomeric cav 2, with some interaction with dimeric cav 2 and heteromeric cav 1::cav 2. We hypothesize that the initial interaction of the M1 mAChR stimulates the oligomerization process to molecular weights similar to those of the caveolin oligomers.

To find out where the caveolin-M1 mAChR interaction takes place—on the PM or in an intracellular compartment, we took advantage of the HA tag positioned in the extracellular domain of the M1 mAChR. We incubated the anti-HA antibody on the BL PM of the cells at 4°C for 60 min, thereby binding the PM fraction of M1 mAChR with the anti-HA antibody. After an extensive wash, we lysed the cells and precipitated the PM fraction of M1 mAChR. The supernatant containing the remainder of the M1 mAChR, consisting mainly of M1 mAChR localized to intracellular compartments, was precipitated by another round of incubation of the unbound cell extract and anti-HA and protein A-Sepharose beads. As shown in Figure 10B (total, right panel), both endogenous and recombinant cav 1 form oligomers, and expression of cav 2 significantly reduces the number of cav 1 oligomers. The interaction with M1 mAChR occurs in intracellular compartments and not on the PM. On the other hand, cav 2 (endogenous and recombinant) interacts with M1 mAChR at both the PM and in intracellular compartments. Interestingly, both cav 1 and 2 are tyrosine phosphorylated when not bound to M1 mAChR. When associated, however, both caveolins are dephosphorylated. These results, together with our observation that cav 2 (but not cav 1) inhibits M1 mAChR endocytosis and that cav 1 abolishes cav 2's inhibition, suggest a scenario in which cav 2 associates with M1 mAChR on the PM and thus inhibits its ligand-dependent recruitment to the clathrin-coated pit. Cav 1 may associate with cav 2 and thus disrupt cav 2's association with M1 mAChR.

DISCUSSION

In this investigation, we show the involvement of both the clathrin endocytic machinery and caveolins in regulating endocytosis and intracellular transport of the M1 muscarinic receptor.

Endocytosis of the M1 Muscarinic Receptor

We show that M1 mAChR is localized to a restricted subdomain of the lateral PM near the tight junction (Figures 1 and 2). On agonist treatment, clathrin is redistributed to the

Figure 10. On the basolateral plasma membrane, the M1 muscarinic receptor interacts only with caveolin 2. MDCK tet-off cells expressing HA-tagged M1 mAChR-GFP were grown for 3 d on 24-mm transwells and subsequently either not infected (Control) or infected with caveolin 1, caveolin 2, or both (50 pfu/cell of each construct), then incubated for 18 h for expression. The PM fraction of M1 mAChR was then immunoprecipitated by incubating the anti-HA antibody at the BL PM for 60 min at 4°C. Excess antibody was washed, cells were lysed, protein A beads were added, and the receptor was precipitated. Lysate containing intracellular unbound receptor was precipitated with another round of antibody and protein A beads. Immunoprecipitate (left panel) and total lysates (right panel) reacted with either (A) anti-myc identifying recombinant caveolins, (B) specific anti-caveolin 1 identifying endogenous and recombinant caveolin 1, (C) specific anti-caveolin 2 identifying endogenous and recombinant caveolin 2, or (D) anti-phosphotyrosine (4G10) identifying tyrosine-phosphorylated immunoprecipitated proteins. Identified protein complexes are indicated on the right. On the PM, only caveolin 2 interacts with the M1 mAChR, in intracellular compartments both caveolins interact with the receptor, and caveolin 1 interacts in its monomeric and oligomeric forms. Caveolins interacting with M1 mAChR are not tyrosine-phosphorylated. (The four bands that appear on the left in D are the heavy chain.)

lateral PM (Figure 5C), and M1 mAChR endocytosis occurs from this region of the lateral PM (Figure 3B). The M1 mAChR internalizes through the clathrin-mediated pathway into the endosomal structures (Figure 5). The internalization rate of the M1 mAChR is similar to that of rapidly internalizing receptors such as transferrin and IgA receptor. Removal of agonist from the media results in rapid recycling of the receptor to the lateral domain, from which it was internalized (Figure 4). Further studies showed that expression of cav 2, which localizes mainly to the intracellular compartment and was previously shown to be in the TGN with a

minute amount on the lateral membrane, results in the marked reduction of M1 mAChR endocytosis and retention of newly synthesized, as well as internalized receptor in intracellular membranes (Figures 6 and 8 and Mora *et al.*, 1999). Expression of cav 1 relieves cav 2's inhibitory effect, most likely due to dimerization of cav 1 with cav 2 (Figure 10, B and C, total lysates), the former competing with M1 mAChR and enabling the receptor's internalization. Cav 2's association with the M1 mAChR is restricted to non-DRMs, as shown by the absence of both cav 2 and M1 mAChR in the Triton-insoluble fractions of the Nycodenz gradients (Supplemental Figure 1, B and C) and as supported by the observation that within the Golgi, caveolins are in a monomeric state and detergent-soluble, enabling them to associate with other polypeptides (Pol *et al.*, 2005).

Differential Localization of M1 Muscarinic Receptor

Most BL-targeted membrane proteins are distributed evenly along the lateral membrane, as shown for E-cadherin (Figure 2). In contrast, the M1 mAChR was highly concentrated proximal to the tight junction and was almost totally absent distal to it (Figure 2). Several studies have shown that delivery of membrane proteins to the BL PM occurs through a targeting apparatus termed exocyst (Novick *et al.*, 1980). The exocyst is a 750-kDa, eight-subunit proteinaceous apparatus that regulates the polarized delivery of membrane proteins to the PM. In polarized MDCK epithelial cells, the exocyst (also termed Sec6/8) complex is localized to the lateral membrane near the tight-junction complex (Grindstaff *et al.*, 1998; Lipschutz *et al.*, 2000), and this region has been shown to be an area of active exocytosis from the common recycling endosome and TGN (Kreitzer *et al.*, 2003). The mammalian exocyst complex, like its yeast counterpart, is important for specifying exocytic sites leading to localized membrane growth (Matern *et al.*, 2001; Yeaman *et al.*, 2004). We speculate that the M1 mAChR's restricted localization to this site enables its rapid endo- and exocytosis, similar to the pre-synaptic neurotransmitter release apparatus, but differs in that the receptor in its resting state is at the PM (Choudhury *et al.*, 2006).

Caveolin 2 Inhibits Caveolin 1 Oligomers

Cav 1 has been shown to oligomerize into large SDS-insoluble structures. These structures range between 200 and 600 kDa in size, appear in vivo in cell lines that express endogenous cav 1, and are induced by the expression of recombinant cav 1. Cav 1 oligomerization takes place shortly after synthesis and most likely within the TGN, because exit of caveolin is unusually slow relative to other secretory proteins (Ren *et al.*, 2004). The stability of the SDS-resistant oligomers is dependent on the palmitoylation of three cysteine residues within the C-terminal cytoplasmic tail of cav 1 (Monier *et al.*, 1995, 1996). A large alanine mutation scan performed on cav 1 has shown that of 29 mutated residues, 14 result in aberrant oligomerization and transport out of the TGN (Ren *et al.*, 2004), indicating that much of the structure of cav 1 is dedicated to the oligomerization that also dictates the exit from the TGN. Cav 2 does not oligomerize independently of cav 1 and does not form SDS-resistant structures. Moreover, mutated cav 1 (cav 1 DGV) retains cav 2 at the Golgi and prevents its oligomerization. Although cav 1 has been shown to draw cav 2 into oligomers that later form increased amounts of caveolae at the BL PM (Lahtinen *et al.*, 2003), in our system, cav 2 did not oligomerize, even in the presence of cav 1 expression (Figures 10C and 11C). Interestingly, we demonstrated that cav 2 completely eliminates the oligomers formed by cav 1. The monitoring of oligomers

Figure 11. Working model for the caveolin-dependent trafficking of M1 muscarinic receptor. M1 mAChR in its biosynthetic pathway arrives at the TGN where it is believed to oligomerize in a caveolin-dependent manner. After its exit from the TGN, the receptor arrives at the Rab11-positive common endosome and is delivered to the PM in proximity to the tight junction. A fraction of the M1 mAChR that is concentrated in this area is bound to caveolin 2, most likely in an oligomeric form. In the presence of caveolin 1, however, caveolin 1 associates with caveolin 2, which in turn releases M1 mAChR, enabling the latter's endocytosis through the clathrin-mediated endocytosis machinery. The receptor is delivered to the BL early endosome (Rab5) where it can either recycle back through the "short pathway" and be delivered to the common endosome and then back to the BL PM or take the "long pathway", to be delivered to the late endosome (Rab7 and 9), from which it will utilize the Rab9-dependent pathway and be delivered to the TGN. From the TGN, the receptor will follow a route similar to that of the proteins in the biosynthetic pathway. Alternatively, at the late endosome, the receptor destined for degradation will follow the Rab7 pathway and be delivered to the lysosome.

is performed either by following SDS resistance in high-pH sample buffer by boiling or in neutral pH buffer without boiling (Monier *et al.*, 1995) or by sucrose gradients (Lahtinen *et al.*, 2003). Assuming that the difference between our results, obtained using high-pH sample buffer followed by boiling and those shown by Lahtinen and colleagues are due to the oligomerization-assay methods applied, we conclude that the oligomers formed by both cav 1 and 2 may indicate different biophysical characteristic than those formed by cav 1 alone. This difference in the presence of different lipid contents in the membrane may have effects on the structure and mobility of caveolae.

Clathrin- and Caveolin-mediated Internalization

The biochemical and immunofluorescence data of our study revealed that expression of dominant-negative clathrin heavy chain and dynamin results in inhibition of M1 mAChR endocytosis (Figure 5). Moreover, addition of agonist (carbachol) resulted in redistribution of clathrin in cells expressing the M1 mAChR (Figure 5C) but had no effect on clathrin in cells that did not express the receptor (unpublished data), promoted the accumulation of M1 mAChR in typical clathrin punctate staining along the lateral PM, and increased the oligomeric form of M1 mAChR (Figure 9, A and B) and its monoubiquitination (Shmuel and Altschuler, unpublished data). Consequently, we conclude that the M1 mAChR internalizes through a clathrin-mediated pathway independent of agonist treatment. This conclusion is in agreement with previous ones obtained using nonpolarized HEK 293 cells, in which the M1 mAChR revealed dynamin I-dependent internalization (Lee *et al.*, 1998; Vogler *et al.*, 1998) and colocalization with the clathrin and AP2 machinery on the PM (Tolbert and Lameh, 1996). Because of increasing evidence for caveolae-dependent internalization of GPCRs, including adenosine A1, β_1 adrenergic, bradykinin B2, and endothelin type A receptors (Chini and Parenti, 2004), as well as caveolin's interaction with membrane and cytosolic signaling molecules such as Na^+/K^+ -ATPase (Wang *et al.*, 2004), we tested the possible role of caveolins in

M1 mAChR endocytosis. Surprisingly, expression of cav 2 dramatically inhibited endocytosis of the receptor and coexpression of cav 1 and 2 reversed this inhibitory effect (Figure 6A). We showed that both caveolins have the capacity to associate with the receptor (Figures 10 and 11), but on the lateral PM only cav 2 associates with M1 mAChR. Expression of cav 2 stimulated M1 mAChR oligomerization to a higher extent than that of cav 1, but cav 2 itself did not oligomerize (Figure 9, A and B). This cav 2 association and receptor oligomerization prevented the receptor's agonist-induced internalization. If cav 2 acts to directly inhibit the endocytic machinery, the amount of receptor localized to the membrane should increase. However, just the opposite occurred: cells expressing cav 2 presented far less receptor on the membrane (Figure 6B), suggesting that cav 2 also inhibits receptor delivery to the PM within the biosynthetic and recycling pathways (Choudhury *et al.*, 2006). In Figure 7, A and B, it can be seen that cav 2 is largely localized to intracellular compartments and is markedly colocalized with the M1 mAChR. As a result, cav 2 expression inhibited endocytosis (Figure 7C), as well as recycling (compare Figure 7C with Figure 4) and delivery of the receptor through the biosynthetic pathway (Figure 7B). This phenomenon was reversed when cav 1 was coexpressed with cav 2. The intracellular retention by cav 2 and its release is in agreement with previous work that showed cav 2 within the TGN and its transport to the lateral PM in polarized MDCK cells (Mora *et al.*, 1999). A Nycodenz gradient of Triton-insoluble fractions revealed that the cav 2 association with M1 mAChR occurs outside the DRM and caveolae (Supplemental Figure 1, B and C). These observations suggest a novel role for cav 2 as a chaperone for membrane proteins, which is regulated by cav 1 outside of the DRMs. The association of cav 1 with cav 2 may compete for the association of cav 2 with the membrane protein or cause its dissociation from the membrane protein. This would enable the receptor to continue in its membrane transport (Figure 7). The recently described nonacidic cav 1-rich intracellular compartments, termed caveosomes, may be the site in which caveolins

retain the intracellular M1 mAChR. The observations that SV40 traffics through this compartment on its way to the endoplasmic reticulum and that it is retained there for several hours support the notion that caveosomes regulate the kinetics of membrane trafficking by acting as an intermediate station that retains membrane proteins rather than redirecting them to other subcellular compartments (Pelkmans *et al.*, 2001; Tagawa *et al.*, 2005). Caveosome biogenesis and its behavior in photobleaching experiments as a static structure places it outside the conventional trafficking pathway shown here for M1 mAChR. The lack of M1 mAChR intracellular endosomal structure, which appears in the cells expressing caveolins (Figure 7) but seems to be reduced in cells expressing both caveolins and the different Rabs (Figure 8), may result from the effects of Rab protein expression. We hypothesize that the Rabs redirect the receptor from the caveosome to the conventional membrane-transport pathway. A more attractive site for accumulating M1 mAChR as a monomer and as an oligomer is the TGN, in which cav 1 oligomerizes, and therefore the intracellular site in which M1 mAChR oligomerizes as well. The TGN is also a site common to both the biosynthetic and long recycling pathways of the receptor (Figure 11). The caveolin-trapping mechanism that operates both on the PM independent of DRMs, and in intracellular compartments on the M1 mAChR was observed to stimulate the receptor's mono- and polyubiquitination, maintaining a fixed amount of monomeric receptor nonubiquitinated (Shmuel and Altschuler, unpublished data). This may serve as a novel mechanism to maintain a fixed pool of receptors available for agonist activation. The oligomerized and ubiquitinated pool may later be either degraded or released into the available pool on the lateral PM. This mechanism may be utilized to extend signaling at the PM or inhibit recycling and thus inhibit potential resensitization. Further research is warranted to characterize the intracellular compartment, the mechanism by which cav 2 regulates M1 mAChR transport, and ubiquitination in relation to caveolins. Our observations, together with accumulated findings related to trafficking in epithelia obtained from experiments in MDCK cells (Rodriguez-Boulant *et al.*, 2005) and from recent experiments with caveolins (Pelkmans *et al.*, 2001; Pelkmans and Helenius, 2002; Tagawa *et al.*, 2005), support a working model in which the M1 mAChR utilizes several routes that maintain a constant amount of available monomeric receptor. This is achieved by caveolin-dependent oligomerization, ubiquitination, and intracellular transport. In summary, M1 mAChR remains near the tight junction and may associate with cav 2. When agonist is applied, the receptor, if not associated with cav 2, internalizes through CME. The release of M1 mAChR from cav 2 on the PM or within intracellular locations may be promoted by the appearance and association of cav 1 with cav 2. The internalized M1 mAChR arrives at the BL early endosome, may be sorted to either a short recycling pathway through the common endosomes, or follow a long recycling pathway through late endosomes, the TGN, common endosomes and back to the BL PM. At the late endosomes, the receptor may either sort to the TGN or to lysosomal degradation. This event is most likely dependent on the ubiquitination state of the receptor (Figure 11).

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REFERENCES

- Altschuler, Y., Barbas, S. M., Terlecky, L. J., Tang, K., Hardy, S., Mostov, K. E., and Schmid, S. L. (1998). Redundant and distinct functions for dynamin-1 and dynamin-2 isoforms. *J. Cell Biol.* 143, 1871–1881.
- Altschuler, Y., Liu, S., Katz, L., Tang, K., Hardy, S., Brodsky, F., Apodaca, G., and Mostov, K. (1999). ADP-ribosylation factor 6 and endocytosis at the apical surface of Madin-Darby canine kidney cells. *J. Cell Biol.* 147, 7–12.
- Barbieri, M. A., Roberts, R. L., Gumusboga, A., Highfield, H., Alvarez-Dominguez, C., Wells, A., and Stahl, P. D. (2000). Epidermal growth factor and membrane trafficking. EGF receptor activation of endocytosis requires Rab5a. *J. Cell Biol.* 151, 539–550.
- Bogatkewitsch, G. S., Lenz, W., Jakobs, K. H., and Van Koppen, C. J. (1996). Receptor internalization delays m4 muscarinic acetylcholine receptor resensitization at the plasma membrane. *Mol. Pharmacol.* 50, 424–429.
- Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J., and van Deurs, B. (2000). Rab 7, a key to lysosome biogenesis. *Mol. Biol. Cell* 11, 467–480.
- Chini, B., and Parenti, M. (2004). G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there? *J. Mol. Endocrinol.* 32, 325–338.
- Choudhury, A., Marks, D. L., and Pagano, R. E. (2005). Use of Rab GTPases to study lipid trafficking in normal and sphingolipid storage disease fibroblasts. *Methods Enzymol.* 403, 166–182.
- Choudhury, A., Marks, D. L., Proctor, K. M., Gould, G. W., and Pagano, R. E. (2006). Regulation of caveolar endocytosis by syntaxin 6-dependent delivery of membrane components to the cell surface. *Nat. Cell Biol.* 8, 317–328.
- Conner, S. D., and Schmid, S. L. (2003). Regulated portals of entry into the cell. *Nature* 422, 37–44.
- Dessy, C., Kelly, R. A., Balligand, J. L., and Feron, O. (2000). Dynamin mediates caveolar sequestration of muscarinic cholinergic receptors and alteration in NO signaling. *EMBO J.* 19, 4272–4280.
- Dupree, P., Parton, R. G., Raposo, G., Kurzchalia, T. V., and Simons, K. (1993). Caveolae and sorting in the trans-Golgi network of epithelial cells. *EMBO J.* 12, 1597–1605.
- Enriquez de Salamanca, A., Siemasko, K. F., Diebold, Y., Calonge, M., Gao, J., Juarez-Campo, M., and Stern, M. E. (2005). Expression of muscarinic and adrenergic receptors in normal human conjunctival epithelium. *Invest. Ophthalmol. Vis. Sci.* 46, 504–513.
- Feng, Y., Press, B., and Wandering-Ness, A. (1995). Rab7, an important regulator of late endocytic membrane traffic. *J. Cell Biol.* 131, 1435–1452.
- Feron, O., Smith, T. W., Michel, T., and Kelly, R. A. (1997). Dynamic targeting of the agonist-stimulated m2 muscarinic acetylcholine receptor to caveolae in cardiac myocytes. *J. Biol. Chem.* 272, 17744–17748.
- Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1995). De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin. *Proc. Natl. Acad. Sci. USA* 92, 8655–8659.
- Galperin, E., and Sorkin, A. (2003). Visualization of Rab5 activity in living cells by FRET microscopy and influence of plasma-membrane-targeted Rab5 on clathrin-dependent endocytosis. *J. Cell Sci.* 116, 4799–4810.
- Grindstaff, K. K., Yeaman, C., Anandasabapathy, N., Hsu, S. C., Rodriguez-Boulant, E., Scheller, R. H., and Nelson, W. J. (1998). Sec6/8 complex is recruited to cell-cell contacts and specifies transport vesicle delivery to the basal-lateral membrane in epithelial cells. *Cell* 93, 731–740.
- Hailstones, D., Sleer, L. S., Parton, R. G., and Stanley, K. K. (1998). Regulation of caveolin and caveolae by cholesterol in MDCK cells. *J. Lipid Res.* 39, 369–379.
- Henley, J. R., Krueger, E. W., Oswald, B. J., and McNiven, M. A. (1998). Dynamin-mediated internalization of caveolae. *J. Cell Biol.* 141, 85–99.
- Kamei, T., Matozaki, T., Sakisaka, T., Kodama, A., Yokoyama, S., Peng, Y. F., Nakano, K., Takaiishi, K., and Takai, Y. (1999). Coendocytosis of cadherin and c-Met coupled to disruption of cell-cell adhesion in MDCK cells—regulation by Rho, Rac and Rab small G proteins. *Oncogene* 18, 6776–6784.
- Kreitzer, G., Schmoranz, J., Low, S. H., Li, X., Gan, Y., Weimbs, T., Simon, S. M., and Rodriguez-Boulant, E. (2003). Three-dimensional analysis of post-Golgi carrier exocytosis in epithelial cells. *Nat. Cell Biol.* 5, 126–136.
- Kurzchalia, T. V., Dupree, P., Parton, R. G., Kellner, R., Virta, H., Lehnert, M., and Simons, K. (1992). VIP21, a 21-kD membrane protein is an integral component of trans-Golgi-network-derived transport vesicles. *J. Cell Biol.* 118, 1003–1014.
- Lahtinen, U., Honsho, M., Parton, R. G., Simons, K., and Verkade, P. (2003). Involvement of caveolin-2 in caveolar biogenesis in MDCK cells. *FEBS Lett.* 538, 85–88.

- Lamaze, C., Dujeancourt, A., Baba, T., Lo, C. G., Benmerah, A., and Dautry-Varsat, A. (2001). Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Mol. Cell* 7, 661–671.
- Lamb, M. E., Zhang, C., Shea, T., Kyle, D. J., and Leeb-Lundberg, L. M. (2002). Human B1 and B2 bradykinin receptors and their agonists target caveolae-related lipid rafts to different degrees in HEK293 cells. *Biochemistry* 41, 14340–14347.
- Le, P. U., Guay, G., Altschuler, Y., and Nabi, I. R. (2002). Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum. *J. Biol. Chem.* 277, 3371–3379.
- Lee, K. B., Pals-Rylaarsdam, R., Benovic, J. L., and Hosey, M. M. (1998). Arrestin-independent internalization of the m1, m3, and m4 subtypes of muscarinic cholinergic receptors. *J. Biol. Chem.* 273, 12967–12972.
- Lipschutz, J. H., Guo, W., O'Brien, L. E., Nguyen, Y. H., Novick, P., and Mostov, K. E. (2000). Exocyst is involved in cystogenesis and tubulogenesis and acts by modulating synthesis and delivery of basolateral plasma membrane and secretory proteins. *Mol. Biol. Cell* 11, 4259–4275.
- Lock, J. G., and Stow, J. L. (2005). Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin. *Mol. Biol. Cell* 16, 1744–1755.
- Lu, M. L., Schneider, M. C., Zheng, Y., Zhang, X., and Richie, J. P. (2001). Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation. *J. Biol. Chem.* 276, 13442–13451.
- Matern, H. T., Yeaman, C., Nelson, W. J., and Scheller, R. H. (2001). The Sec6/8 complex in mammalian cells: characterization of mammalian Sec3, subunit interactions, and expression of subunits in polarized cells. *Proc. Natl. Acad. Sci. USA* 98, 9648–9653.
- Matsui, M., Yamada, S., Oki, T., Manabe, T., Taketo, M. M., and Ehler, F. J. (2004). Functional analysis of muscarinic acetylcholine receptors using knock-out mice. *Life Sci.* 75, 2971–2981.
- McFarland, M. J., Porter, A. C., Rakhshan, F. R., Rawat, D. S., Gibbs, R. A., and Barker, E. L. (2004). A role for caveolae/lipid rafts in the uptake and recycling of the endogenous cannabinoid anandamide. *J. Biol. Chem.* 279, 41991–41997.
- Monier, S., Dietzen, D. J., Hastings, W. R., Lublin, D. M., and Kurzchalia, T. V. (1996). Oligomerization of VIP21-caveolin in vitro is stabilized by long chain fatty acylation or cholesterol. *FEBS Lett.* 388, 143–149.
- Monier, S., Parton, R. G., Vogel, F., Behlke, J., Henske, A., and Kurzchalia, T. V. (1995). VIP21-caveolin, a membrane protein constituent of the caveolar coat, oligomerizes in vivo and in vitro. *Mol. Biol. Cell* 6, 911–927.
- Mora, R., Bonilha, V. L., Marmorstein, A., Scherer, P. E., Brown, D., Lisanti, M. P., and Rodriguez-Boulant, E. (1999). Caveolin-2 localizes to the golgi complex but redistributes to plasma membrane, caveolae, and rafts when co-expressed with caveolin-1. *J. Biol. Chem.* 274, 25708–25717.
- Mostov, K. E., Verges, M., and Altschuler, Y. (2000). Membrane traffic in polarized epithelial cells. *Curr. Opin. Cell Biol.* 12, 483–490.
- Nabi, I. R., and Le, P. U. (2003). Caveolae/raft-dependent endocytosis. *J. Cell Biol.* 161, 673–677.
- Nohe, A., Keating, E., Underhill, T. M., Knaus, P., and Petersen, N. O. (2005). Dynamics and interaction of caveolin-1 isoforms with BMP-receptors. *J. Cell Sci.* 118, 643–650.
- Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* 21, 205–215.
- Parolini, I. *et al.* (1999). Expression of caveolin-1 is required for the transport of caveolin-2 to the plasma membrane. Retention of caveolin-2 at the level of the golgi complex. *J. Biol. Chem.* 274, 25718–25725.
- Parton, R. G. (1994). Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. *J. Histochem. Cytochem.* 42, 155–166.
- Parton, R. G. (2003). Caveolae—from ultrastructure to molecular mechanisms. *Nat. Rev. Mol. Cell Biol.* 4, 162–167.
- Parton, R. G., Joggerst, B., and Simons, K. (1994). Regulated internalization of caveolae. *J. Cell Biol.* 127, 1199–1215.
- Pelkmans, L., and Helenius, A. (2002). Endocytosis via caveolae. *Traffic* 3, 311–320.
- Pelkmans, L., Kartenbeck, J., and Helenius, A. (2001). Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat. Cell Biol.* 3, 473–483.
- Pfeffer, S. (2005). A model for Rab GTPase localization. *Biochem. Soc. Trans.* 33, 627–630.
- Phatarpekar, P. V., Durdan, S. F., Copeland, C. M., Crittenden, E. L., Neece, J. D., and Garcia, D. M. (2005). Molecular and pharmacological characterization of muscarinic receptors in retinal pigment epithelium: role in light-adaptive pigment movements. *J. Neurochem.* 95, 1504–1520.
- Pitcher, J. A., Touhara, K., Payne, E. S., and Lefkowitz, R. J. (1995). Pleckstrin homology domain-mediated membrane association and activation of the beta-adrenergic receptor kinase requires coordinate interaction with G beta gamma subunits and lipid. *J. Biol. Chem.* 270, 11707–11710.
- Pol, A., Martin, S., Fernandez, M. A., Ingelmo-Torres, M., Ferguson, C., Enrich, C., and Parton, R. G. (2005). Cholesterol and fatty acids regulate dynamic caveolin trafficking through the Golgi complex and between the cell surface and lipid bodies. *Mol. Biol. Cell* 16, 2091–2105.
- Ren, X., Ostermeyer, A. G., Ramcharan, L. T., Zeng, Y., Lublin, D. M., and Brown, D. A. (2004). Conformational defects slow Golgi exit, block oligomerization, and reduce raft affinity of caveolin-1 mutant proteins. *Mol. Biol. Cell* 15, 4556–4567.
- Rodriguez-Boulant, E., Kreitzer, G., and Musch, A. (2005). Organization of vesicular trafficking in epithelia. *Nat. Rev. Mol. Cell Biol.* 6, 233–247.
- Roettger, B. F., Rentsch, R. U., Pinon, D., Holicky, E., Hadac, E., Larkin, J. M., and Miller, L. J. (1995). Dual pathways of internalization of the cholecystokinin receptor. *J. Cell Biol.* 128, 1029–1041.
- Roseberry, A. G., and Hosey, M. M. (2001). Internalization of the M2 muscarinic acetylcholine receptor proceeds through an atypical pathway in HEK293 cells that is independent of clathrin and caveolae. *J. Cell Sci.* 114, 739–746.
- Rouvinski, A., Gahali-Sass, I., Stav, I., Metzger, E., Atlan, H., and Taraboulos, A. (2003). Both raft- and non-raft proteins associate with CHAPS-insoluble complexes: some APP in large complexes. *Biochem. Biophys. Res. Commun.* 308, 750–758.
- Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M. P. (1993). Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J. Cell Biol.* 122, 789–807.
- Scheiffele, P., Verkade, P., Fra, A. M., Virta, H., Simons, K., and Ikonen, E. (1998). Caveolin-1 and -2 in the exocytic pathway of MDCK cells. *J. Cell Biol.* 140, 795–806.
- Shajahan, A. N., Timblin, B. K., Sandoval, R., Tiruppathi, C., Malik, A. B., and Minshall, R. D. (2004). Role of Src-induced dynamin-2 phosphorylation in caveolae-mediated endocytosis in endothelial cells. *J. Biol. Chem.* 279, 20392–20400.
- Shen, Y., Xu, L., and Foster, D. A. (2001). Role for phospholipase D in receptor-mediated endocytosis. *Mol. Cell Biol.* 21, 595–602.
- Shockley, M. S., Burford, N. T., Sadee, W., and Lameh, J. (1997). Residues specifically involved in down-regulation but not internalization of the m1 muscarinic acetylcholine receptor. *J. Neurochem.* 68, 601–609.
- Silva, W. I., Andres, A., Schook, W., and Puszkun, S. (1986). Evidence for the presence of muscarinic acetylcholine receptors in bovine brain coated vesicles. *J. Biol. Chem.* 261, 14788–14796.
- Slowiejko, D. M., McEwen, E. L., Ernst, S. A., and Fisher, S. K. (1996). Muscarinic receptor sequestration in SH-SY5Y neuroblastoma cells is inhibited when clathrin distribution is perturbed. *J. Neurochem.* 66, 186–196.
- Stenmark, H., Parton, R. G., Steele-Mortimer, O., Lutcke, A., Gruenberg, J., and Zerial, M. (1994). Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. *EMBO J.* 13, 1287–1296.
- Su, A. I. *et al.* (2004). A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc. Natl. Acad. Sci. USA* 101, 6062–6067.
- Tagawa, A., Mezzacasa, A., Hayer, A., Longatti, A., Pelkmans, L., and Helenius, A. (2005). Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters. *J. Cell Biol.* 170, 769–779.
- Tolbert, L. M., and Lameh, J. (1996). Human muscarinic cholinergic receptor Hm1 internalizes via clathrin-coated vesicles. *J. Biol. Chem.* 271, 17335–17342.
- van Koppen, C. J., and Kaiser, B. (2003). Regulation of muscarinic acetylcholine receptor signaling. *Pharmacol. Ther.* 98, 197–220.
- Verkade, P., Harder, T., Lafont, F., and Simons, K. (2000). Induction of caveolae in the apical plasma membrane of Madin-Darby canine kidney cells. *J. Cell Biol.* 148, 727–739.
- Vogel, U., Sandvig, K., and van Deurs, B. (1998). Expression of caveolin-1 and polarized formation of invaginated caveolae in Caco-2 and MDCK II cells. *J. Cell Sci.* 111(Pt 6), 825–832.
- Vogler, O., Bogatkevitch, G. S., Wriske, C., Krummenier, P., Jakobs, K. H., and van Koppen, C. J. (1998). Receptor subtype-specific regulation of muscarinic acetylcholine receptor sequestration by dynamin. Distinct sequestration of m2 receptors. *J. Biol. Chem.* 273, 12155–12160.
- von Zastrow, M. (2003). Mechanisms regulating membrane trafficking of G protein-coupled receptors in the endocytic pathway. *Life Sci.* 74, 217–224.

- von Zastrow, M., Link, R., Daunt, D., Barsh, G., and Kobilka, B. (1993). Subtype-specific differences in the intracellular sorting of G protein-coupled receptors. *J. Biol. Chem.* 268, 763–766.
- Wang, H., Haas, M., Liang, M., Cai, T., Tian, J., Li, S., and Xie, Z. (2004). Ouabain assembles signaling cascades through the caveolar Na⁺/K⁺-ATPase. *J. Biol. Chem.* 279, 17250–17259.
- Wyse, B. D., Prior, I. A., Qian, H., Morrow, I. C., Nixon, S., Muncke, C., Kurzchalia, T. V., Thomas, W. G., Parton, R. G., and Hancock, J. F. (2003). Caveolin interacts with the angiotensin II type 1 receptor during exocytic transport but not at the plasma membrane. *J. Biol. Chem.* 278, 23738–23746.
- Yeaman, C., Grindstaff, K. K., and Nelson, W. J. (2004). Mechanism of recruiting Sec6/8 (exocyst) complex to the apical junctional complex during polarization of epithelial cells. *J. Cell Sci.* 117, 559–570.
- Zerial, M., and McBride, H. (2001). Rab proteins as membrane organizers. *Nat. Rev. Mol. Cell Biol.* 2, 107–117.