The Sonic Hedgehog Receptor Patched Associates with Caveolin-1 in Cholesterol-rich Microdomains of the Plasma Membrane

Heidi E. Karpen‡§, John T. Bukowski‡, Thomas Hughes¶, Jean-Philippe Gratton‖, William C. Sessa¶, and Mae R. Gailani**†

From the ‡Department of Pediatrics, United States Department of Agriculture/Agricultural Research Service Children’s Nutrition Research Center, Baylor College of Medicine and Texas Children’s Hospital, Houston, Texas 77030 and the Departments of ¶Ophthalmology and Visual Science and ‖Pediatrics and the **Department of Pharmacology, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut 06536

The Hedgehog signaling pathway is involved in early embryonic patterning as well as in cancer; however, little is known about the subcellular localization of the Hedgehog receptor complex of Patched and Smoothened. Since Hh has been found in lipid rafts in Drosophila, we hypothesized that Patched and Smoothened might also be found in these cholesterol-rich microdomains. In this study, we demonstrate that both Smoothened and Patched are in caveolin-1-enriched/raft microdomains. Immunoprecipitation studies show that Patched specifically interacts with caveolin-1, whereas Smoothened does not. Fractionation studies show that Patched and caveolin-1 can be co-isolated from buoyant density fractions that represent caveola/raft microdomains and that Patched and caveolin-1 co-localize by confocal microscopy. Glutathione S-transferase fusion protein experiments show that the interaction between Patched and caveolin-1 involves the caveolin-1 scaffolding domain and a Patched consensus binding site. Immunocytochemistry data and fractionation studies also show that Patched seems to be required for transport of Smoothened to the membrane. Depletion of plasmalemmal cholesterol influences the distribution of the Hh receptor complex in the caveolin-1-enriched/raft microdomains. These data suggest that caveolin-1 may be integral for sequestering the Hh receptor complex in these caveolin-enriched microdomains, which act as a scaffold for the interactions with the Hh protein.

The Hedgehog signaling pathway, first described in Drosophila and conserved in vertebrates, is fundamental in early embryonic patterning of many structures, including the neural tube, axial skeleton, limbs, and lungs. Sonic Hedgehog (Shh), the most studied of the three vertebrate homologs of Drosophila Hedgehog, is a secreted protein that acts on target cells to increase transcription of several genes, including members of the Wnt and transforming growth factor-β families, and its receptor Patched. Patched (Ptc) is predicted to encode a large transmembrane protein that acts as a negative regulator of the pathway. It associates with a second transmembrane protein, Smoothened (Smo), which is a positive regulator of the pathway. Prior genetic and biochemical studies indicate that the two proteins form an unusual complex at the membrane that is inactive in the absence of the Shh ligand. Once the Shh protein binds to Ptc, this relieves the inhibition of Smo (by unknown mechanisms) and allows transduction of the Hedgehog signal. Little is known about the structure or function of the Hedgehog receptor complex or the possible role of accessory proteins or lipids. The Ptc protein is predicted to have at least 12 transmembrane domains, and although it has little homology to other known receptors, it has been shown to directly bind Shh. The transmembrane domains of Ptc have a high degree of homology to the sterol-sensing domains of several proteins involved in cholesterol processing and trafficking, including NPC-1 (Niemann-Pick C protein-1), NPC-2, 3-hydroxy-3-methyl-glutaryl-CoA reductase, and SCAP (sterol regulatory element-binding protein cleavage-activating protein). Smo encodes a serpentine, seven-transmembrane protein with characteristics of a G-protein-coupled receptor, including a glycosylated extracellular N terminus. Smo, however, does not directly bind Shh. The interaction with Ptc seems to be mediated through the N-terminal domain and/or the first two transmembrane domains of Smo,1 with most of the signaling activity mediated through the third intracellular loop and the seventh transmembrane domain. The exact mechanism through which Smo transduces the Shh signal remains unclear, but it most probably involves a conformational change in the receptor complex, as the Ptc-Smo/sterol complex can be co-immunoprecipitated. The Shh protein undergoes autoproteolytic cleavage with concomitant attachment of a cholesterol moiety to the N-terminal component of the protein. This modified N-terminal product is responsible for most of the apparent biological activity of the Shh protein. The cholesterol modification is not absolutely required for binding to Ptc or for limited biological activity, as several model systems have shown a response utilizing a bacterially derived Shh-N fusion protein. It has been proposed,

Received for publication, November 30, 2000, and in revised form, February 28, 2001
Published, JBC Papers in Press, March 1, 2001, DOI 10.1074/jbc.M010832200

* This work was supported by the National Institutes of Health, the Swiss Federal Foundation, and the American Cancer Society and in part by the United States Department of Agriculture/Agricultural Research Service under Cooperative Agreement 58-6250-6001. The confocal microscope at Yale University School of Medicine was supported by the Fanny Rippel Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Deceased.

‡ To whom correspondence should be addressed: Children’s Nutrition Research Center, Baylor College of Medicine, 1100 Bates St., Suite 10070, Houston, TX 77030. Tel.: 713-798-7045; Fax: 713-798-7057; E-mail: hkarpen@bcm.tmc.edu.

§ The on-line version of this article (available at http://www.jbc.org) contains Supplemental Figs. 1–4.
however, that covalently linked cholesterol may modulate Shh activity, possibly through the sterol-sensing domains of Ptc, increasing the efficiency of signal transduction (6).

Caveolae are non-clathrin-coated invaginations of the plasma membrane that are important in endocytosis, cholesterol trafficking, and sequestering various lipid-modified signaling molecules in discrete microdomains. The caveolins, a family of three protein isoforms, are the major coat proteins of caveolae. Caveolin-1 directly binds to and transports cholesterol from the Golgi to the plasma membrane, and this association is required for caveolar formation (7–11). Caveolae are enriched in cholesterol and sphingolipids, are insoluble in non-ionic detergents such as Triton X-100, and can be isolated as low density buoyant membranes in the absence of detergents. Associated with these complexes are various lipid-modified signaling molecules, including Ha-Ras, endothelial nitric-oxide synthase, serine/threonine kinases, several G-protein α-subunits, and Src tyrosine kinases (reviewed in Ref. 12). It has been postulated that caveolae may be signaling centers for multiple pathways and may regulate cross-talk between different pathways. Caveolin, per se, may directly influence signaling by serving as a molecular scaffold for signaling complexes (13–15) or indirectly modulate signaling by influencing cholesterol trafficking.

Since very little is known about the cellular localization of members of the Shh signaling pathway, we hypothesized that components of the pathway were in caveolae or lipid raft domains of cells. Recent data have shown that Ihh is trafficked to lipid rafts in Drosophila, most probably through its association with cholesterol (16). Given these recent data and our preliminary observations of the Ptc trafficking pattern, we hypothesized that the Shh receptor complex is also targeted to these cholesterol-rich microdomains on the plasma membrane through an association between the Shh receptor Ptc and perhaps caveolin-1. In this report, we show that Ptc and caveolin associate with each other in the caveolar/lipid raft fraction of the plasma membrane, and we show data strongly implicating cholesterol as a key player in the transport and, most likely, the function of the Hedgehog receptor.

**EXPERIMENTAL PROCEDURES**

**Constructs and Introduction of Mutations**—A full-length human Ptc-GFP2 construct (cDNA gift from Rune Toftgard) was made by creating an NheI/SalI cDNA fragment, ligating an 8-base pair linker to create an EcoRI site at the 5′-end, and then cloning this cDNA fragment into a mammalian GFP transfection vector (GFP vector C2, CLONTECH). This placed the GFP cDNA at the 5′-end of Ptc. The full-length human Smo-GFP construct was made by creating a HindIII/KpnI cDNA fragment (gift from Carol Wicking) and cloning it into a GFP transfection vector (GFP vector N3, CLONTECH), which placed the GFP cDNA at the 3′-end of Smo. The NheI/SalI Ptc cDNA fragment was also cloned into an expression vector (pcDNA3 mammalian expression vector; Prmega), and the HindIII/KpnI Smo cDNA fragment was cloned into a FLAG mammalian transfection vector (pFLAG-CMV5a, Sigma), which placed the FLAG tag at the 3′-end of the Smo cDNA. All constructs were sequenced to confirm that the GFP or FLAG tags were in frame. The Myc-tagged caveolin-1 construct was a gift from Dr. Michael Howard Hughes Institute) that introduced eight nucleotide changes into the wild-type sequence resulted in a cDNA that would encode the altered protein. Sequencing of the entire PtcBS-GFP construct (where PtcBS (is the caveolin-binding site on Ptc) confirmed the addition of the appropriate mutations. The altered construct was cloned into GFP vector C2 as described above (forward primer, 5′-GATCTCCTGGGAAAAGACCCGCACGTTTGCTCAACATGTATATAGTCACCC). The Smo cDNA fragment was prepared by G-A (for 1.5 h at 4 °C. Protein G-agarose (20 μl of a 50% suspension in PBS; Sigma) was added and rotated for 1 h at 4 °C. The beads were collected by centrifugation (brief pulse in a microcentrifuge) and then washed three times with lysis buffer. The beads were resuspended in 2× Laemmli loading buffer and boiled for 5 min, and the supernatant was loaded onto an SDS-polyacrylamide gel.

Samples were separated by SDS-polyacrylamide gel electrophoresis using a 6% gel for the Patched and Smothened proteins and a 15% gel for detection of the caveolin protein. The samples were transferred to nitrocellulose membrane (Biorad), incubated in blocking solution (PBS, 0.1% Tween, and 5% nonfat dry milk) for 1 h at room temperature, and then washed twice with PBS/Tween. The membrane was incubated with the primary antibody (anti-caveolin-1 antibody or anti-Ptc polyclonal antibody) in blocking solution, rotated for 1 h at room temperature, and then washed three to four times with PBS/Tween. The membrane was then incubated with the appropriate secondary antibody tagged with horseradish peroxidase, and the membranes were detected by chemiluminescence using the ECL detection system and reagents supplied by Amersham Pharmacia Biotech. The membrane was exposed to film for up to 20 min and then developed in an Eastman Kodak X-Omat M43A processor.

**Confocal Microscopy**—COS cells were grown to 60% confluence on 35-mm coverslip plates (Mattek Corp., Ashland, MA), transiently transfected using Effectene transfection reagent, and allowed to recover for 48 h before fixing and permeabilizing. Cells were examined live in a confocal microscope (Zeiss, 60× confocal objective; or Olympus, 60× confocal objective) and assessed for localization and trafficking of the Ptc protein. Multiple images were obtained from the same cell, and a three-dimensional reconstruction of the cell was performed (NIH Image software). Time series and photobleaching experiments were also performed.

**Immunocytochemistry**—COS cells were grown to 60–80% confluence in 6-well dishes containing untreated coverslips. They were transiently transfected and allowed to recover for 36–48 h. The cells were fixed and permeabilized with methanol at −20 °C for 2–4 min and then washed five times with PBS. Slides were blocked with goat serum; shaken for 1 h at room temperature; incubated with the appropriate primary antibody (anti-patched monoclonal antibody or anti-Ptc polyclonal antibody) in blocking solution, rotated for 1 h at room temperature, and then washed three to four times with PBS/Tween. The membrane was then incubated with the appropriate secondary antibody tagged with horseradish peroxidase, and the membranes were detected by chemiluminescence using the ECL detection system and reagents supplied by Amersham Pharmacia Biotech. The membrane was exposed to film for up to 20 min and then developed in an Eastman Kodak X-Omat M43A processor.

**Fractionation**—Fractionation experiments were performed using a non-detergent method for isolation of caveolin-1-enriched buoyant membranes (20) and modified as described (39). 100-mm plates of COS cells were transiently transfected with Ptc-GFP or PtcB31SMut and allowed to recover and express protein for 48 h. To avoid interference from up-regulation of endogenous Ptc, cells transfected with Smo-GFP alone were processed after 40 h. The plates were then

---

2 The abbreviations used are: GFP, green fluorescent protein; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; GST, glutathione S-transferase; MBCD, methyl-b-cyclodextrin.
washed three times with PBS on ice. Cells were lysed in a solution containing 500 mM Na₂CO₃ (pH 11.0) with protease inhibitors and incubated on ice for 10 min. The cells were scraped from the plate and homogenized in a Dounce homogenizer and then transferred to an Eppendorf tube and centrifuged at 1000 × g for 10 min at 4 °C. The supernatant was collected in a new Eppendorf tube and sonicated on ice. The supernatant was mixed with an equal volume of an 85% (w/v) sucrose/MB solution (MB = 25 mM A-morpholine-ethanesulfonic acid (pH 6.5) and 0.15 M NaCl), allowed to equilibrate for 2 h at 4 °C, and then placed at the bottom of a ultracentrifuge tube. An overlay of 6 ml of a 30% (w/v) sucrose/MB solution and then 3.5 ml of a 5% (w/v) sucrose/MB solution was added and centrifuged at 35,000 rpm for 18 h at 4 °C in a Sorvall UltraPro-8 using a TH641 rotor. The gradient was fractionated into 1-ml fractions taken from the top, mixed with an equal volume of 2× Laemmli loading buffer, and boiled for 5 min, and protein was separated by SDS-polyacrylamide gel electrophoresis. The gels were processed as described above, and the membranes were incubated with the anti-Ptc C terminus polyclonal antibody to detect Patched, with the anti-caveolin monoclonal antibody to detect caveolin-1, with the anti-FLAG M2 monoclonal antibody to detect Smo, with the anti-β-COP monoclonal antibody to detect the Golgi protein, and with the anti-BIP/GRP78 antibody to detect the ER.

**FIG. 1. Co-localization of Ptc and caveolin-1 by immunocytochemical staining.** In A, COS-1 cells were transfected with Ptc-GFP, fixed, and stained for native caveolin-1 (Cav) with the anti-caveolin-1 monoclonal antibody. Single channel confocal imaging shows a vesicular pattern, which seems to be typical of both Ptc and caveolin-1. Dual channel confocal imaging shows co-localization of Ptc and caveolin-1 both within the cell and at the plasma membrane (yellow). In B, COS cells transfected with Ptc-GFP and a Myc-tagged form of caveolin-1 (Cav-Myc) again showed co-localization of the two proteins by confocal microscopy.

**FIG. 2. Caveolin-1 co-immunoprecipitates with Ptc, but not with Smo.** In A, lysates of COS cells transiently transfected with Ptc-GFP and caveolin-Myc constructs were immunoprecipitated (IP) with the anti-caveolin-1 monoclonal antibody, and proteins were Western-blotted for Ptc (upper panel) or caveolin-1 (Cav; lower panel). Lane 1 shows co-immunoprecipitation of caveolin-1 and Ptc. Incubating lysates with a nonimmune mouse IgG (lane 2) did not result in the precipitation of either caveolin-1 or Ptc. Lane 3 shows Ptc in total cell lysates. In B, lysates of COS cells transiently transfected with Ptc-GFP and caveolin-Myc constructs were immunoprecipitated with the anti-Ptc C terminus antibody (Ptc CT Ab), and proteins were Western-blotted for caveolin-1 (upper panel) or Ptc (lower panel). Lane 1 again shows co-immunoprecipitation of Ptc and caveolin-1, whereas there was no precipitation of either Ptc or caveolin-1 using preimmune serum in lane 2. Lane 3 used the anti-caveolin-1 monoclonal antibody (MAB) to detect caveolin-1 in these cell lysates. In C, lysates of COS cells transiently transfected with the Smo-GFP and caveolin-Myc constructs were immunoprecipitated with the anti-Smo C terminus antibody, and proteins were Western-blotted for caveolin-1 (upper panel) or Smo (lower panel). No co-immunoprecipitation was detected between Smo and caveolin-1 in lane 1. The negative control in lane 2 used preimmune serum to precipitate nonspecific proteins. The positive control in lane 3 used the anti-caveolin-1 monoclonal antibody to detect caveolin-1 in these cell lysates. These data are representative of at least three experiments each.

**GST Fusion Proteins—**GST fusion constructs of full-length caveolin-1 and the caveolin-binding site (amino acids 81–101) were made by creating two BamHI/EcoRI cDNA fragments by polymerase chain reaction and cloning these fragments into a GST vector (pGEX-2TK, Amersham Pharmacia Biotech). A GST fusion protein of the putative PtcBS (amino acids 772–810) was made by creating EcoRI/BamHI cDNA fragments by polymerase chain reaction and cloning this fragment into the GST vector. The constructs were sequenced to confirm that the GST cDNA was in frame and then transformed into *Escherichia coli* DH5α cells. A 1:100 dilution of an overnight culture of each construct and the GST vector alone was grown in 100 ml of 2× yeast tryptone medium for 3 h at 37 °C and then induced with isopropyl-β-D-thiogalactopyranoside (0.5 mM) for 4 h at 37 °C. The bacteria were pelleted at 7700 × g for 10 min and washed with 3 ml of STE buffer (150 mM NaCl, 7.5 mM Tris (pH 8.0), and 3 mM EDTA). The bacteria were resuspended in 3 ml of STE buffer with lysozyme (100 μg/ml) and placed on ice for 15 min. Dithiothreitol to a final concentration of 5 mM, phenylmethylsulfonyl fluoride to a final concentration of 100 μM, N-lauroylsarcosine to a final concentration of 1.5%, and protease inhibitors were added, and the mixture was incubated on ice for 30 min. The lysate was homogenized with 15 strokes of a Dounce homogenizer and sonicated until clear. The lysate was centrifuged at 15,000 rpm in a Sorvall SS34 rotor for 15 min at 4 °C. The supernatant was removed, and Triton X-100 was added to a final concentration of 2%. Glutathione beads (100 μl of a 50% slurry in PBS) were added and incubated for 18 h at 4 °C. The beads were collected by centrifugation at 500 × g for 5 min and washed five times with STE buffer.

COS cells were transiently transfected with either the Patched or caveolin-1 construct and lysed 36 h later as described above. The GST beads (1–5 μg of protein) were incubated with cell lysate (300 μg of protein) for 2 h at 4 °C. The complexes were washed four times with wash buffer (50 mM Tris (pH 8.0), 400 mM NaCl, and 1 mM EDTA); the beads were resuspended in 2× Laemmli loading buffer, boiled for 5 min, and briefly centrifuged; and the supernatant was separated by SDS-
immunoprecipitation of Smo-FLAG results in co-immunoprecipitation of Ptc-GFP. Lane 1 shows the Western blot for Ptc after precipitation with the anti-Ptc antibody. Lane 3 shows the Western blot for Smo, shown in its two phosphorylation forms, after precipitation using the anti-Ptc C terminus antibody (Ptc CT). Lanes 2 and 4 are negative controls using preimmune serum. In C, COS-1 cells cotransfected with the Ptc-GFP (first panel) and Smo-FLAG (second panel) constructs display a vesicular pattern, with co-localization of Ptc and Smo seen both within the cell and at the membrane (third and fourth panels).

RESULTS

**Patched Associates with Caveolin-1**—Ptc and Smo proteins are difficult to study in untransfected mammalian cells due to their low base-line levels of expression. Others who have studied endogenous Ptc in *Drosophila* (17, 18) and epitope-tagged Ptc in mammalian cells (19) have shown that the majority of Ptc is found in intracellular vesicles, with a small proportion of the protein found at the membrane. To assess base-line localization and trafficking patterns for wild-type Ptc in our model system, we transiently transfected COS cells with a Ptc-GFP construct and examined living cells by confocal microscopy. As shown in Fig. 1A, wild-type Ptc-GFP was enriched in intracellular membranes, with the majority in the perinuclear region, reminiscent of the ER/Golgi (see also Supplemental Figs. 3 (lower panel) and 4 (upper panel)). Using time-lapsed microscopy, these small packets of protein trafficked from the Golgi to and along the cell membrane (Ptc.mov). The vesicular pattern displayed by Ptc-GFP was consistent across cell lines (Chinese hamster ovary, Madin-Darby canine kidney, HepG2, and the medulloblastoma cell line Daoy) and was consistent in cells transfected with untagged Ptc detected by immunocytochemistry using the anti-Ptc C terminus antibody (Supplemental Fig. 1A). A three-dimensional reconstruction of images obtained from a Z-series suggested that a portion of the Ptc protein localized at or just under the plasma membrane. The pattern was reminiscent of proteins associated with caveolae and their major structural protein, caveolin-1.

Next, we performed studies to determine if Ptc and caveolin-1 co-localize. Immunofluorescent microscopy of cells transfected with the Ptc-GFP cDNA (Fig. 1A, left panel) and immunolabeled for endogenous caveolin-1 (middle panel) demonstrated co-localization of the two proteins (right panel). In addition, cotransfection of a Myc-tagged version of caveolin-1 (caveolin-Myc) and Ptc-GFP constructs showed that the proteins co-localized both within the cell and at the plasma membrane (Fig. 1B). To examine if these proteins can interact biochemically, we performed co-immunoprecipitation in COS cells transiently transfected with Ptc-GFP and caveolin-Myc. As shown in Fig. 2 (A and B), immunoprecipitation of caveolin-1 (left panel) resulted in the co-association of Ptc, and immunoprecipitation of caveolin-1 (middle panel), resulted in the association of caveolin-1. To investigate the potential interaction between Smo and caveolin-1, co-immunoprecipitation experiments were performed on lysates of COS-1 cells cotransfected with Smo-GFP and caveolin-Myc. Western blotting of the immunoprecipitates (Fig. 2C) showed that there was no association between these two proteins, and additional immunocytochemistry studies failed to show co-localization between the two proteins (Supplemental Fig. 2).

**Smoothened Interacts with Patched, but Does Not Interact with Caveolin-1**—It is known that several G-protein-coupled receptors are sequestered in a latent phase in caveolar or raft microdomains of the plasma membrane (9, 14, 15). Because Smo has significant homology to G-protein-coupled receptors, we sought to determine if the presence of Smo is necessary for the interaction between Ptc and caveolin-1. Transfection of Smo-GFP into COS cells (Fig. 3A, right panel) resulted in a more uniform intracellular distribution compared with Ptc (left and middle panels). There was no detectable Smo protein at the membrane, suggesting that most of the expressed protein was in the ER and Golgi (Supplemental Figs. 3B and 4, upper panels). However, cotransfection of FLAG-tagged Smo (Smo-FLAG) (Fig. 3C, second panel) with Ptc-GFP (first panel) dem-
studies). Similar to prior lower panel Western blotting with the anti-Ptc C terminus antibody (upper panel) and upper panel sent sequential (top to bottom) fractions that were processed for Western fractions. In density fractions, as well as layers that represent the Golgi and ER density fractions 3 and 4. Ptc was also found to localize to these buoyant fractions. In cells that were cotransfected, however, whereas caveolin continued to be isolated in the buoyant fractions (fraction 4). In cells that were cotransfected, however, Smo trafficked as a complex to the membrane, we hypothesized that Smo alone would not be found in the caveolin-enriched buoyant fractions, whereas Ptc and Smo would co-fractionate when both were transfected into cells. COS-1 cells were transfected with either Smo-FLAG alone (40 h) or Ptc-GFP and Smo-FLAG (48 h). The Smo-FLAG-alone cells were processed a few hours earlier to avoid possible interference from up-regulated endogenous Ptc, as Smo is a positive regulator of this pathway. Cells were processed again by a non-detergent method and centrifuged through a continuous sucrose gradient. Western blotting of sequential fractions from cells transfected with Smo alone (Fig. 4B) showed that the majority of the Smo protein was isolated in the heavier fractions (fractions 9–11), whereas caveolin-1 continued to be isolated in the buoyant fractions (fraction 4). In cells that were cotransfected, however, Smo co-fractionated with both Ptc and caveolin in the buoyant fractions (Fig. 4C), with a significant amount of protein still found in the ER/Golgi fractions as well. This again supports the idea that Ptc and Smo traffic as a complex and can be found together in the caveolin-enriched raft domains.

**Interaction between Patched and Caveolin-1 Involves the Putative Caveolin-1 Scaffolding Domain and a Consensus Binding Motif in Patched**—Caveolin-1 interacts with various proteins through an intracellular region termed the caveolin-1 scaffolding domain (amino acids 82–101) (reviewed in Ref. 12). To assess whether the interaction between Ptc and caveolin-1 is mediated through this scaffolding domain, GST fusion proteins of PtcBS (amino acids 788–798) or with GST alone. PtcBS was sufficient to specifically bind full-length caveolin-1 in these cell lysates, whereas there was no binding with GST alone. Endothelial cell lysate was used as a positive (Pos) control for the presence of caveolin-1.

Western blot analysis of sequential fractions from cells transfected with Ptc-GFP alone for 48 h shows that Ptc co-distributes and recruits Smo into caveolin-enriched buoyant membranes (see Fig. 4A). Consistent with the confocal imaging studies, the majority of Ptc co-fractionated with Golgi and ER markers (lanes 8–11); however, a significant amount of Ptc was also found in fractions 3 and 4, confirming that Ptc localizes to the same microdomains as caveolin-1. Similar results were obtained when these experiments were repeated on cells transfected with both Ptc and caveolin-Myc constructs.

Because our confocal microscopy data suggested that Ptc and Smo trafficked as a complex to the membrane, we hypothesized that Smo alone would not be found in the caveolin-enriched buoyant fractions, whereas Ptc and Smo would co-fractionate when both were transfected into cells. COS-1 cells were transfected with either Smo-FLAG alone (40 h) or Ptc-GFP and Smo-FLAG (48 h). The Smo-FLAG-alone cells were processed a few hours earlier to avoid possible interference from up-regulated endogenous Ptc, as Smo is a positive regulator of this pathway. Cells were processed again by a non-detergent method and centrifuged through a continuous sucrose gradient. Western blotting of sequential fractions from cells transfected with Smo alone (Fig. 4B) showed that the majority of the Smo protein was isolated in the heavier fractions (fractions 9–11), whereas caveolin-1 continued to be isolated in the buoyant fractions (fraction 4). In cells that were cotransfected, however, Smo co-fractionated with both Ptc and caveolin in the buoyant fractions (Fig. 4C), with a significant amount of protein still found in the ER/Golgi fractions as well. This again supports the idea that Ptc and Smo traffic as a complex and can be found together in the caveolin-enriched raft domains.

**Interaction between Patched and Caveolin-1 Involves the Putative Caveolin-1 Scaffolding Domain and a Consensus Binding Motif in Patched**—Caveolin-1 interacts with various proteins through an intracellular region termed the caveolin-1 scaffolding domain (amino acids 82–101) (reviewed in Ref. 12). To assess whether the interaction between Ptc and caveolin-1 is mediated through this scaffolding domain, GST fusion proteins of the full-length caveolin-1 and the region of caveolin encoding the scaffolding domain (amino acids 82–101) as well as GST alone were incubated with lysates of COS-1 cells ex-
Many proteins that can potentially bind to caveolin-1 contain a specific caveolin-binding sequence motif that may facilitate interaction with the caveolin-1 scaffolding domain, but not with GST alone. Approximately 10–30% of input Ptc interacted with the GST-caveolin fusion proteins.

We mutated the sequence by replacing the underlined aromatic amino acids with the amino acid alanine. Previous studies have shown that these substitutions inhibit the functional interaction between endothelial nitric-oxide synthase and caveolin-1 (12). The altered Ptc construct, PtcBSMut, was cloned into the GFP vector and transiently transfected into COS cells. Cells were examined live under the confocal microscope and compared with wild-type images. Unlike wild-type Ptc, which accumulated in the perinuclear region and moved to and from the membrane, the PtcBSMut protein accumulated in the same region, but failed to traffic throughout the cell (Fig. 6A, left panel). Immunocytochemistry studies of PtcBSMut and caveolin-My also failed to show significant co-localization of the two proteins at the membrane, but did show some overlap within the perinuclear region (Fig. 6A, right panel). Co-immunoprecipitation studies on lysates derived from cells cotransfected with PtcBSMut and caveolin-1 showed that, despite altering this putative binding site, PtcBSMut and caveolin-1 continued to associate, but to a lesser degree (Fig. 6B). Fractionation of these cells revealed some PtcBSMut protein in the lipid raft component (Fig. 6C, middle panel), but this seemed decreased in comparison with wild-type Ptc-GFP (upper panel). This suggests that this binding site is important for the association between Ptc and caveolin-1, but that additional sites of interaction or accessory proteins may be necessary for the interaction of the proteins in vivo.

Role of Cholesterol in Trafficking of the Hedgehog Receptor to Lipid Rafts on the Plasma Membrane—Cholesterol is a key component of lipid rafts in mammalian cells, and similar sterols seem to function as a necessary component in Drosophila raft formation (16). Prior studies in mammalian cells using methyl-β-cyclodextrin (MBCD) have shown that cholesterol depletion abrogates the formation of caveolae, which is reversed upon cholesterol replacement (38). Due to the similarities between Ptc and several other proteins involved in cholesterol biosynthesis and transport (1, 2) and the role of caveolin-1 in cholesterol trafficking (8–11), we hypothesized that cholesterol might be involved in the transport of the Ptc-Smo-caveolin-1 complex to caveolae and/or insertion into the plasma membrane. We transfected COS cells with Ptc-GFP or PtcBSMut and Smo-FLAG and then treated them with serum depletion and/or MBCD. Confocal microscopy of cells transfected with wild-type Ptc-GFP and Smo-FLAG showed the receptor complexes localized to discrete vesicles, which remained intracellular and co-localized with Golgi markers (Fig. 7A, left and middle panels). During live confocal imaging, there was little movement of these vesicles to the plasma membrane in the serum-depleted cell group and no movement of these vesicles in the MBCD-treated group. This abnormal trafficking pattern was reversed after serum repletion (Fig. 7A, right panel). In contrast, cells transfected with PtcBSMut and Smo-FLAG showed the formation of complexes in intracellular vesicles that were unable to traffic to the membrane (Fig. 7B, left and middle panels) even after cholesterol replacement (right panel). This indicates that cholesterol is most likely involved in the transport of the Ptc-Smo complex to the membrane. The failure of PtcBSMut to traffic correctly after replacement of cholesterol may be due to the fact that the mutated caveolin-1-binding site is also located in the sterol-sensing domain of Ptc.

Detergent-free methods of caveolar isolation have shown that ~90% of caveolin-1 in the cell is associated with lipid rafts (24, 25) and that cholesterol is necessary for the insertion of caveolin-1 into these rafts. We expected treatment with MBCD to cause a significant decrease in the amount of Ptc and caveolin-1 recovered from the lipid raft fraction of those cells. We performed detergent-free isolation and sucrose gradient frac-

Pressing the Ptc-GFP construct. As shown in Fig. 5A, Ptc in cell lysates interacted with full-length caveolin-1 and the caveolin-1 scaffolding domain, but not with GST alone. Approximately 10–30% of input Ptc interacted with the GST-caveolin fusion proteins.
...middle panels repletion. Similar to base-line studies using PtcBSMut constructs, the protein was not seen to traffic out of the Golgi to the plasma membrane (right panel). After 4 h of MBCD treatment, there was no evidence of Ptc protein trafficking from the Golgi to the plasma membrane (middle panel). The effects on trafficking seen with serum depletion or MBCD treatment were completely reversed after serum repletion. Cells were examined by confocal microscopy under each of these conditions. After 24 h of serum depletion, very little Ptc protein was seen trafficking to the plasma membrane (left panel). After 4 h of MBCD treatment, there was no evidence of Ptc protein trafficking from the Golgi to the plasma membrane (right panel). This pattern was not reversed by serum repletion (right panel).}

**FIG. 7.** Serum depletion/methyl-β-cyclodextrin treatment of cell transfected with Ptc-GFP or PtcBSMut-GFP: role of cholesterol in trafficking. In A, COS-1 cells expressing Ptc-GFP were subjected to serum depletion (SD) or MBCD (CD) treatment, followed by serum repletion. Cells were examined by confocal microscopy under each of these conditions. After 24 h of serum depletion, very little Ptc protein was seen trafficking to the plasma membrane (left panel). After 4 h of MBCD treatment, there was no evidence of Ptc protein trafficking from the Golgi to the plasma membrane (middle panel). The effects on trafficking seen with serum depletion or MBCD treatment were completely reversed after serum repletion (right panel). In B, COS-1 cells expressing PtcBSMut were subjected to serum depletion or MBCD treatment, followed by serum repletion. Similar to base-line studies using PtcBSMut constructs, the protein was not seen to traffic out of the Golgi to the plasma membrane (left and middle panels). This pattern was not reversed by serum repletion (right panel).

**DISCUSSION**

In this report, we provide evidence supporting the concept that components of the Hh signaling pathway reside in caveolin-enriched microdomains. This assertion is supported by data demonstrating co-localization of Ptc with the caveolar coat protein caveolin-1 and a smaller proportion of Ptc-GFP were recovered in the raft fractions. In cells treated with MBCD, both caveolin-1 and Ptc were shifted from the raft fractions to the heavier membrane fractions. This further suggests that cholesterol is important for the correct trafficking of both Ptc and caveolin-1 to caveolar/lipid rafts.

The Hh receptor complex is an interesting and unusual heteromer in that Ptc functions as the receptor domain for the Hh ligand, and Smo functions as the signaling domain for the complex. Little is known about the interactions between these two subunits, which compose the Hh receptor complex. Analysis of tumors suggests that Ptc functions not only at critical points in development, but also as a tumor suppressor where loss of both copies is required for tumor formation (29–31). Activating mutations in Smo have also been described that implicate it as a potential oncogene (32, 33). Current knowledge has suggested that Ptc and Smo exist as a complex on the membrane, where Ptc inhibits the tonic activity of Smo in the absence of the Hh signal. Upon binding of Hh to its receptor Ptc, this inhibition of Smo is released, through unknown mechanisms, and Smo is then free to transduce the Hh signal. It is thought that, in the absence of Ptc, Smo alone is localized to the plasma membrane, where its signal transduction is not under Hh control. Our initial data from transfections of Ptc and Smo indicate that, in the absence of Ptc, Smo does not traffic to the membrane at all, but remains intracellular (Fig. 3). Confocal microscopy of living cells transfected with Ptc alone shows that it is able to traffic efficiently to the membrane from the Golgi without Smo. The results of our fractionation studies (Fig. 4) show that Ptc alone co-fractionates with caveolin-1 in the lipid raft compartment, whereas Smo alone does not. When Smo is cotransfected with Ptc, however, Smo is found to co-fractionate with Ptc and caveolin in the buoyant membrane fractions. Experiments with various Ptc mutants also show that despite very abnormal trafficking patterns exhibited by some of the Ptc mutants, Smo consistently co-localizes with Ptc. Collectively, these data suggest that Ptc and Smo form a complex very early on, most probably in the Golgi, and are trafficked intact to lipid-rich microdomains on the plasma membrane (Fig. 9).

The raft hypothesis proposed by Simons and Ikonen (26) postulates that these lateral assemblies, composed of glycosphingolipids and cholesterol, function to aggregate certain proteins while excluding others. These rafts form a liquid order phase whose formation is driven by the interactions of the specific lipids involved. Caveolae, on the other hand, are specialized raft structures that are flask-shaped invaginations of the plasma membrane and are coated by caveolins. Indeed, caveolin-1 and -2 are necessary for the assembly of caveolae (36). In this study, we have used a well described detergent-free method for isolating caveolin-1-enriched membranes and lipid rafts. The strength of this method is that it can discern between buoyant membranes (caveolae/rafts) and heavy membranes...
the major structural protein in caveolae and is found in abundance in adipocytes, endothelial cells, and fibroblasts (10). The caveolin gene family has been conserved from Caenorhabditis elegans to humans, underscoring its evolutionary importance. Caveolin proteins form homo- and hetero-oligomers that directly bind and require cholesterol for insertion into the membrane. Caveolin-1 forms a hairpin-like structure, with the central portion of the protein inserted into the membrane and the N and C termini located in the cytoplasm (10). Work on heterotrimeric G-proteins as well as Ha-Ras, endothelial nitric-oxide synthase, and others has shown that caveolin-1 interacts with these proteins through an area termed the caveolin-1 scaffolding domain (amino acids 82–101) (12–14). Our GST fusion protein studies have shown similar interactions between Ptc and caveolin-1, in which the full-length caveolin-1 protein and the caveolin-1 scaffolding domain were able to bind Ptc from transfected cell lysates, whereas GST alone did not. Analysis of proteins that interact with caveolin through its scaffolding domain have shown that these proteins contain a common sequence motif (φXφXXXfφ, φXXXXfφ, or φXfφXXXfφXf, where φ is Trp, Tyr, or Phe) (23). Sequence analysis of Ptc revealed that it contains such a motif (φXXfXXXfφ) in the region of its seventh transmembrane domain, within the region of its sterol-sensing domain. As shown in Fig. 6, mutation of this binding motif by replacement of the aromatic amino acids with alanine produced a Ptc protein that was unable to traffic out of the Golgi complex to the membrane. However, mutation of these residues did not completely abrogate the association between Ptc and caveolin-1, indicating that other domains in Ptc, other proteins, and/or lipids may be involved in this interaction. It is known that caveolin-1 directly binds cholesterol and requires cholesterol for insertion into the plasma membrane and that its expression is regulated at the transcriptional level by cholesterol (8, 11, 34, 35). Our initial studies using serum depletion and MBCD treatment suggest that it is quite likely that cholesterol is integral for the correct trafficking of the Hh receptor complex to the plasma membrane as well. This hypothesis is also supported by the similarities between Ptc and NPC-1 and the role of NPC-1 in cholesterol trafficking (1, 2). The similarities between Ptc and NPC-1 raise interesting ideas about how Ptc traffics to the membrane and the role of its sterol-sensing domains in its interactions with Hh. Others have postulated that the Ptc sterol-sensing domain is probably important for binding of the Hh ligand, but it may also be critical for trafficking and localization of the receptor complex to caveolar domains of the plasma membrane.

Hh itself is an unusual morphogen in that it possesses an autocatalytic cleavage domain that cleaves the protein and covalently links cholesterol to the N terminus, forming the active portion. Recent data have shown that Hh is also modified by the addition of palmitate or other acyl residues to a Cys residue on the N terminus that seem to anchor Hh in the outer leaflet of the membrane bilayer, making it function like a glycosylphosphatidylinositol-anchored protein (16). This raises the conundrum of how a protein anchored in the lipid bilayer can function as a secreted morphogen. Sequestration of Ptc in these lipid-rich microdomains may be a method for the cell to separate this signaling pathway from others and to promote the interaction between Ptc and Shh.

Work on signaling molecules such as Ha-Ras, Src tyrosine kinases, endothelial nitric-oxide synthase, and G-protein-coupled receptors has shown that caveolin-1 plays an important role in holding these signaling molecules in a latent phase (13–15). Other studies have shown that this latent phase is also required for interaction of some of these molecules with caveolin-1 and that this interaction can be abolished by conversion of caveolin-1 to lipids.
the signaling molecule to the activated form (20, 37). Collectively, results from several labs suggest that Ptc sequesters Smo in an inactive state in the absence of the Hh ligand. An alternative interpretation, based on our studies, is that caveolin-1 and cholesterol in signal transduction, stimulated by the Hh receptor complex and the biogenesis of these components, are clearly trafficked through an exocytic pathway to the plasma membrane. Additional studies examining the roles of caveolin-1 and cholesterol in signal transduction, stimulated by the Hh receptor complex and the biogenesis of these components, are clearly warranted.

Acknowledgments—We thank Dr. Michael Lisanti for the full-length human Ptc cDNA, and Carol Wicking for the full-length Smo cDNA. We also thank Dr. Gzegorsz Sowa for initial help with fractionation studies.

REFERENCES

1. Carstea, E. D., Morris, J. A., Coleman, K. G., Loftus, S. K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M. A., Pavan, W. J., Krizman, D. B. Nagle, J., Polymoroperoupolous, M. H., Sturley, S. L., Joannou, Y. A., Higgins, M. E., Comly, M., Conney, A., Brown, A., Kaneski, C. R., Blanchette-Mackie, E. J., Deyeer, N. K., Neufeld, E. B., Chang, T.-Y., Liecm, L., Strauss, J. R., III, Ohno, K., Zeigler, M., Cermi, R., Sokol, J., Markle, O'Neill, R. R., van der Horste, P., and Toftgard, R. (1996) J. Biol. Chem. 271, 120, 118–119

2. Loftus, S. K., Morris, J. A., Carstea, E. D., Gu, J., Cummings, C., Brown, A., Ellison, J., Ohno, K., Rosenfeld, M. A., Tagle, D. A., Pentchev, P. G., and Pavan, W. J. (1997) Science 277, 232–235

3. Murone, M., Rosenthal, A., and de Sauvage, F. J. (1999) Curr. Biol. 9, 76–84

4. Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Penchina, D., Goddard, A., Phillips, H., Noll, M., Hooper, J. E., de Sauvage, F., and Rosenthal, A. (1996) Nature 384, 129–134

5. Porter, J. A., Ekker, S. C, Park, W. J., von Kessler, D., Dean, M., and Toftgard, R. (1996) Nature 387, 424–431

6. Pepinsky, R. B., Zeng, C., Wen, D., Rayhorn, P., Baker, D. P., Williams, K. P., Bixler, S. A., Ambrose, C. M., Garber, E. A., Miatkowski, K., Taylor, F. R., Wang, K. A., and Goldes, A. (1996) J. Biol. Chem. 271, 14037–14045

7. Uittenbogaard, A., Ying, Y., and Smart, E. J. (1998) J. Biol. Chem. 273, 6525–6532

8. Murata, M., Pentchev, P. G., and Tagle, D. A. (1997) Science 278, 76–84

9. Kurchalik, T. V., and Parton, R. G. (1999) Curr. Opin. Cell Biol. 11, 424–431

10. Parton, R. G. (1996) Curr. Opin. Cell Biol. 8, 542–548

11. Fielding, C. J., and Fielding, P. E. (1997) J. Lipid Res. 38, 1503–1521

12. Okamoto, T., Schlegel, A., Scherer, P. E., and Lisanti, M. P. (1998) J. Biol. Chem. 273, 5419–5422

13. Garcia-Cardenas, G., Martasek, P., Siler Master, B. S., Skidd, P. M., Lisanti, M. P., and Stahle-Backdahl, M. (1997) J. Biol. Chem. 272, 25437–25440

14. Roy, S., Luetterforst, R., Harding, A., Appolloni, A., Etheridge, M., Sturley, S. L., Joannou, Y. A., and Toftgard, R. (1999) J. Cell Biol. 140, 105–115

15. Sternberg, P. W., and Schmid, S. L. (1999) Nat. Cell Biol. 1, 535–537

16. Rietveld, A., Neutz, S., Simons, K., and Eaton, S. (1999) J. Biol. Chem. 274, 12049–12054

17. Capdevila, J., Purente, F., Sampedro, J., Alonso, J. L., and Guerrero, I. (1994) Development 120, 987–998

18. Taylor, A. M., Nakano, Y., Mohler, J., and Ingham, P. W. (1993) Mech. Dev. 42, 89–96

19. Incardona, J. P., and Eaton, S. (2000) Curr. Opin. Cell Biol. 12, 193–203

20. Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M., and Lisanti, M. P. (1997) J. Biol. Chem. 272, 6525–6533

21. Smart, E. J., Ying, Y., Mineo, C., and Anderson, R. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10104–10108

22. Deleted in proof

23. Couet, J., Li, S., Okamoto, T., Ikekuz, T., and Lisanti, M. P. (1997) J. Biol. Chem. 272, 6525–6533

24. Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R., and Anderson, R. G. (1992) Cell 68, 673–682

25. Smart, E. J., Ying, Y. S., Conrad, P. A., and Anderson, R. G. (1994) J. Cell Biol. 127, 1185–1197

26. Simons, K., and Ikonen, E. (1997) Nature 387, 569–572

27. Deleted in proof

28. Deleted in proof

29. Gailani, M. R., Stable-Backdahl, M., Leffell, D. J., Glynn, M., Zababoupolous, P. G., Pressman, C., Unden, A. B., Dean, M., Brash, D. E., Bale, A. E., and Toftgard, R. (1996) Nat. Genet. 14, 78–81

30. Hahn, H., Wicking, C., Zababoupolous, P. G., Gailani, M. R., Shanley, S., Chidamharam, A., Vorechovsky, I., Holmberg, E., Unden, A. B., Gillies, S., Negus, K., Smyth, I., Pressman, C., Leffell, D. J., Gerrard, B., Goldstein, A. M., Wainwright, B., Toftgard, R., Chenevix-Trench, G., Dean, M., and Bale, A. E. (1996) Cell 86, 841–851

31. Hahn, H., Wojiowski, L., Miller, G., and Zimmer, A. (1999) J. Mol. Med. 77, 459–468

32. Lam, C. W., Xue, J., Ts, K. F., Ng, H. K., Lee, K. C., Yuen, N. W., Lim, P. L., Chan, L. Y., Tong, S. F., and McCormick, F. (1999) Oncogene 18, 833–836

33. Xie, J., Murone, M., Loh, S. M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J. M., Lam, C. W., Hynes, M., Goddard, A., Rosenthal, A., Epstein, E. H., and de Sauvage, F. J. (1998) Nature 391, 90–92

34. Hailstones, D., Sleer, L. S., Parton, R. G., and Stanley, K. K. (1998) J. Lipid Res. 39, 369–379

35. Fielding, C. J., Bist, A., and Fielding, P. E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3753–3758

36. Li, S., Galatii, F., Volonte, D., Sargiacomo, M., Engeland, J. A., Das, K., Scherer, P. E., and Lisanti, M. P. (1998) FEBS Lett. 434, 127–134

37. Li, S., Couet, J., and Lisanti, M. P. (1996) J. Biol. Chem. 271, 21982–21989

38. Puruchi, T., and Anderson, R. G. (1998) J. Biol. Chem. 273, 21099–21104

39. Sowa, G., Liu, J., Papapetropoulos, A., Rex-Haffner, M., Hughes, T. E., and Sessa, W. C. (1999) J. Biol. Chem. 274, 22524–22531
The Sonic Hedgehog Receptor Patched Associates with Caveolin-1 in Cholesterol-rich Microdomains of the Plasma Membrane
Heidi E. Karpen, John T. Bukowski, Thomas Hughes, Jean-Philippe Gratton, William C. Sessa and Mae R. Gailani

J. Biol. Chem. 2001, 276:19503-19511.
doi: 10.1074/jbc.M010832200 originally published online March 1, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010832200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2001/05/30/276.22.19503.DC1

Supplemental material:
http://www.jbc.org/content/suppl/2001/03/05/M010832200.DC1

This article cites 36 references, 18 of which can be accessed free at
http://www.jbc.org/content/276/22/19503.full.html#ref-list-1
Additions and Corrections

Vol. 276 (2001) 19503–19511
The sonic hedgehog receptor patched associates with caveolin-1 in cholesterol-rich microdomains of the plasma membrane.

Heidi E. Karpen, John T. Bukowski, Thomas Hughes, Jean-Philippe Gratton, William C. Sessa, and Mae R. Gailani

Dr. Gailani's name was misspelled. The correct name is shown above.

Vol. 276 (2001) 22544–22552
S100A13 participates in the release of fibroblast growth factor 1 in response to heat shock in vitro.

Matteo Landriscina, Raffaela Soldi, Cinzia Bagalà, Isabella Micucci, Stephen Bellum, Francesca Tarantini, Igor Prudovsky, and Thomas Maciag

One affiliation was omitted from the list of author affiliations. The additional affiliation, for all authors, is as follows: Center for the Biophysical Sciences, University of Maine, Orono, Maine 04469.

Vol. 276 (2001) 3999–4011
Molecular and functional characterization of a family of rat brain T-type calcium channels.

John E. McRory, Celia M. Santi, Kevin S. C. Hamming, Janette Mezeyova, Kathy G. Sutton, David L. Baillie, Anthony Stea, and Terrance P. Snutch

Pages 4006–4008, Figs. 6, 7, and 8: There are several errors in these figures.

Figs. 6 and 8: The Greek letter τ was omitted on several of the axes.

Fig. 7: The axis labels were omitted. Fig. 7A, the x axis should read Vc (mV) and the y axis normalized conductance. Fig. 7B, the x axis should read prepulse potential (mV), and the y axis should read normalized current. Fig. 7, C–E, the x axis should read Vc/prepulse potential (mV), and the y axis should read normalized conductance/current.

Fig. 8: The plot legend should indicate that $\alpha_{1H}$ is labeled as a square and $\alpha_{11}$ as a triangle. The correct figures and legends are the following.
FIG. 6. Voltage dependence of kinetics parameters of activation and inactivation. A, current traces illustrating a comparison of the temporal course of \( \alpha_{I1} \), \( \alpha_{H1} \), and \( \alpha_{H1} \). Traces were taken at the peak of the IV, normalized and superimposed for comparison. B–D, plot of mean values for activation constant (\( \tau_{act} \)) against command voltage (\( V_c \)) for \( \alpha_{I1} \) (\( n = 4 \)), \( \alpha_{H1} \) (\( n = 13 \)), and \( \alpha_{H1} \) (\( n = 9 \)). Smooth lines correspond to an exponential fit of the data with an e-fold change per 9.34, 15.8, and 13.27 mV for \( \alpha_{I1} \), \( \alpha_{H1} \), and \( \alpha_{H1} \), respectively. E–G, plot of mean values for inactivation time constant (\( \tau_{inact} \) obtained by fitting a single exponential to the decay phase of calcium current) against command voltage for \( \alpha_{I1} \) (\( n = 5 \)), \( \alpha_{H1} \) (\( n = 4 \)), and \( \alpha_{H1} \) (\( n = 9 \)). Smooth lines indicate single exponential voltage-dependence of \( \tau_{inact} \) with e-fold change per 5.16, 7.85, and 7.46 mV for \( \alpha_{I1} \), \( \alpha_{H1} \), and \( \alpha_{H1} \), respectively. Error bars represent S.E. H, plot of mean values of \( \tau_{act} \) and \( \tau_{inact} \) at −25 mV.
FIG. 7. Voltage-dependent activation and steady-state inactivation of $\alpha_{1I}$, $\alpha_{1G}$, and $\alpha_{1H}$ calcium currents. 

A, activation curves. The current amplitude was converted to conductance by assuming a calcium reversal potential extrapolated from the linear, positive slope region of the $I-V$ curve. The conductance at each potential was normalized to the maximum conductance and was averaged for each step potential. The symbols represent pooled data from $\alpha_{1I}$ (filled triangles, $n = 5$), $\alpha_{1G}$ (filled circles, $n = 4$), and $\alpha_{1H}$ (filled squares, $n = 9$). Solid lines represent the fitting with Boltzmann equations with half-activation voltages ($V_{0.5a}$) of $-60.7$, $-51.73$, and $-43.15$ mV and slope factors ($k_a$) of 8.39, 6.53, and 5.34 for $\alpha_{1I}$, $\alpha_{1G}$, and $\alpha_{1H}$, respectively.

B, steady-state inactivation curves. The membrane potential was stepped to $-30$ mV from holding potentials ranging from $-120$ to $-50$ mV. The normalized peak amplitude of the currents elicited by the test pulse to $-30$ mV was plotted as a function of the holding potential. These data were fitted with a Boltzmann equation (smooth curves). Half-inactivation voltage ($V_{0.5i}$) and slope factor ($k_i$) were $-93.2$ mV and 4.7 ($n = 6$), $-85.4$ mV and 5.4 ($n = 5$), and $-73.9$ mV and 2.76 ($n = 4$) for $\alpha_{1I}$ (open triangles), $\alpha_{1G}$ (open circles), and $\alpha_{1H}$ (open squares), respectively.

C–E, activation and inactivation curves were plotted in the same graphic and expanded to show window currents for each channel: $\alpha_{1I}$ (C), $\alpha_{1G}$ (D), and $\alpha_{1H}$ (E).
FIG. 8. Voltage dependence of deactivation kinetics. A, plot of mean deactivation time constants ($\tau_{\text{deact}}$) against repolarization potentials. Data represent mean and S.E. for the following number of cells: $\alpha_{1I}$ ($n = 6$), $\alpha_{1G}$ ($n = 3$), and $\alpha_{1H}$ ($n = 4$). Deactivation time constants were determined by fitting tail currents (B–D) with a single exponential. B–D, representative calcium current tail traces of $\alpha_{1I}$ (B), $\alpha_{1G}$ (C), and $\alpha_{1H}$ (D). Currents were evoked using the following voltage protocols: a 9-ms step to $-40$ mV for $\alpha_{1G}$, a 20-ms step to $-50$ mV for $\alpha_{1I}$, and a 6-ms step to $-30$ mV for $\alpha_{1H}$ followed by repolarization to potentials from $-120$ to $-40$ mV.