Vasoconstriction resulting from dynamic membrane trafficking of TRPM4 in vascular smooth muscle cells

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Am J Physiol Cell Physiol 299: C682–C694, 2010. First published July 7, 2010; doi:10.1152/ajpcell.00101.2010.—The melastatin (M) transient receptor potential (TRP) channel TRPM4 mediates pressure and protein kinase C (PKC)-induced smooth muscle cell depolarization and vasoconstriction of cerebral arteries. We hypothesized that PKC causes vasoconstriction by stimulating translocation of TRPM4 to the plasma membrane. Live-cell confocal imaging and fluorescence recovery after photobleaching (FRAP) analysis was performed using a green fluorescent protein (GFP)-tagged TRPM4 (TRPM4-GFP) construct expressed in A7r5 cells. The surface channel was mobile, demonstrating a FRAP time constant of 168 ± 19 s. In addition, mobile intracellular trafficking vesicles were readily detected. Using a cell surface biotinylation assay, we showed that PKC activation with phorbol 12-myristate 13-acetate (PMA) increased (~3-fold) cell surface levels of TRPM4-GFP protein in <10 min. Similarly, total internal reflection fluorescence microscopy demonstrated that stimulation of PKC activity increased (~3-fold) the surface fluorescence of TRPM4-GFP in A7r5 cells and primary cerebral artery smooth muscle cells. PMA also caused an elevation of cell surface TRPM4 protein levels in intact arteries. PMA-induced translocation of TRPM4 to the plasma membrane was independent of PKCα and PKCβ activity but was inhibited by blockade of PKCδ with rottlerin. Pressure-myograph studies of intact, small interfering RNA (siRNA)-treated cerebral arteries demonstrate that PKC-induced constriction of cerebral arteries requires expression of both TRPM4 and PKCδ. In addition, pressure-induced arterial myocyte depolarization and vasoconstriction was attenuated in arteries treated with siRNA against PKCδ. We conclude that PKCδ activity causes smooth muscle depolarization and vasoconstriction by increasing the number of TRPM4 channels in the sarcolemma.

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STRONG EVIDENCE LINKS protein kinase C (PKC)-dependent regulation of vascular smooth muscle cell excitability and arterial tone with the melastatin (M) transient receptor potential (TRP) channel TRPM4 (22). Stimulation of PKC with the phorbol compound phorbol 12-myristate 13-acetate (PMA) increases the magnitude of whole cell TRPM4 currents in human embryonic kidney (HEK) cells overexpressing the channel (31) and in native cerebral artery smooth muscle cells (9). In addition, expression of TRPM4 is required for PMA-induced smooth muscle cell depolarization and vasoconstriction of cerebral arteries (9). However, it is not clear whether PKC increases TRPM4 activity by directly phosphorylating channel protein or indirectly by influencing downstream signaling pathways. Furthermore, multiple PKC isoforms are present in vascular smooth muscle cells (29, 39) and it is not known which is involved in TRPM4 regulation. These questions are of considerable interest because PKC (44) and TRPM4 (9, 10, 37) are critical mediators of arterial function.

Dynamic, directed movement of channel protein into and out of the plasma membrane is responsible for insulin-like growth factor-mediated activation of the canonical (C) TRP channel TRPC5 (4). Regulation of TRPM4 activity by trafficking-dependent mechanisms has not been extensively investigated. However, it was recently reported that defects in endocytosis of TRPM4 channel protein resulting from a point mutation contributes a familial form of conduction block in the heart (19). In addition, we found that the frequency of observation of TRPM4 channels in inside-out membrane patches pulled from native cerebral artery smooth muscle cells is increased approximately fourfold without alteration of basic biophysical properties following a brief pretreatment with PMA (10). Similar observations were reported for a TRPM4-like channel in human atrial cardiomyocytes (14). Enhanced frequency of channel observation in patch-clamp experiments following short-term exposure to PMA could be due to an increase in the density of TRPM4 protein at the cell surface. Thus, we hypothesized that rapid insertion of TRPM4 protein into the plasma membrane is a means of regulating channel activity, smooth muscle contractility, and vascular tone in response to PKC activation.

Cell surface biotinylation and total internal reflection fluorescence (TIRF) microscopy were used to examine the effects of PKC activity on TRPM4 accumulation at the plasma membrane of isolated smooth muscle cells. Findings of these studies show for the first time that TRPM4 is acutely inserted into the plasma membrane in response to PKCδ activity. Pressure-myograph studies demonstrate that PKCδ is critical for the maintenance of smooth muscle cell membrane potential and that PMA pressure-induced constriction of cerebral arteries requires the expression of both TRPM4 and PKCδ. We conclude that PMA-induced increases in PKCδ activity cause vasoconstriction by increasing the number of TRPM4 channels in the sarcolemma.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (250–350 g; Harlan, Indianapolis, IN) were used for these studies. Animals were deeply anesthetized with pentobarbital sodium (50 mg ip) and euthanized by exsanguination according to a protocol approved by the Institutional Ani-
cultural Care and Use Committee of Colorado State University. Brains were isolated in ice-cold MOPS-buffered saline [in mM: 3 MOPS (pH 7.4), 145 NaCl, 5 KCl, 1 MgSO4, 2.5 CaCl2, 1 KH2PO4, 0.02 EDTA, 2 pyruvate, 5 glucose, and 1% bovine serum albumin]. Cerebral and cerebellar arteries were dissected from the brain, cleaned of connective tissue, and stored in MOPS-buffered saline on ice before further manipulation.

Recombinant DNA. Full-length mouse TRPM4 cDNA in the vector pBluscript (a gift from Dr. Veit Flockerzi) was digested with Xba I and the resulting fragment was purified using a Qiagel Gel Extraction Kit (Qiagen) before ligation into the Nhe I site of pAcGFP1-N2 (Clontech) to fuse the green fluorescent protein (GFP) coding region to the COOH terminus of the channel. A stop codon resulting from the cloning procedure was removed using a Quikchange II XL Site-Directed Mutagenesis Kit (Strategene).

A7r5 cell culture. A7r5 cells (American Type Culture Collection) were cultured in Dulbecco’s 1X high glucose modified Eagle’s medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) and 0.5% penicillin-streptomycin (GIBCO). Cells were maintained in a 37°C incubator with 6% CO2, media were changed every 2 to 3 days, and cells were subcultured when confluent using 0.25% trypsin-EDTA (GIBCO).

Primary cerebral artery smooth muscle cell preparation and culture. To isolate smooth muscle cells, vessels were cut into 2-mm segments and placed in the following cell isolation solution (in mM): 60 NaCl, 80 Na-glutamate, 5 KCl, 2 MgCl2, 10 glucose, and 10 HEPES (pH 7.4). Arterial segments were initially incubated at room temperature in 1 ml/gm papain (Worthington), 1 mg/ml diithiothreitol, and 0.5 mM CaCl2 for 30 min, followed by 30 min of incubation at 37°C in 2 mg/ml type II collagenase (Worthington). The digested segments were then washed three times in cell isolation solution and triturated to release smooth muscle cells. Cells were cultured on 25-mm round glass coverslips in Smooth Muscle Cell Growth Medium (Genlantis) in an incubator maintained at 37°C with 6% CO2.

Transient DNA transfection. A7r5 cells and primary cerebral artery smooth muscle cells were transiently transfected with our TRPM4-GFP fusion protein with the aid of Effectene Transfection Reagent (Qiagen) according to the manufacturer’s instructions for adherent cells. Media were changed 24 h after transfection, and cells were imaged 3 days after transfection. A7r5 cells were selected for transfection when cultures were 70–80% confluent, and primary cerebral artery smooth muscle cells were transfected 1 day after proliferation was detected in culture (~1–2 wk).

Stable DNA transfection. A7r5 cells were transfected with TRPM4-GFP as described above using Effectene Transfection Reagent. Cultures showing expression of the tagged channel were maintained in Dulbecco’s 1X high glucose modified Eagle’s medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 0.5% penicillin-streptomycin (GIBCO), and 0.5 mM genetin (GIBCO). Cells were maintained as described above. Transfection efficiency was monitored using epifluorescence.

Live-cell confocal microscopy. A7r5 cells transiently transfected with TRPM4-GFP were imaged 3 days after transfection using an Olympus Fluoview 1000 confocal microscope equipped with an Ar (458/488/515) laser for detection of green fluorescent protein. A 405 nm diode laser-based SIM scanner coupled to the imaging system was used to photobleach a region of interest (ROI), and fluorescence recovery after photobleaching (FRAP) was monitored as a function of time during image acquisition as previously described (33). Cells were imaged at room temperature in a physiological imaging saline consisting of (in mM) 146 NaCl, 4.7 KCl, 2.5 CaCl2, 0.6 MgCl2, 1.6 NaHCO3, 0.15 NaH2PO4, 0.1 ascorbic acid, 8 glucose, and 20 HEPES (pH 7.4). Image analysis performed using FV1000 and Velocity 5.0.2 software.

Cell surface biotinylation. Membrane protein was quantitated using a cell surface biotinylation assay. A7r5 cells stably expressing TRPM4-GFP were cultured in the absence of serum 24 h before biotinylation to minimize PKC activity and simulate quiescence. Cells were pretreated with PMA (1 μM; 15 min; 37°C) or with the PKC-inhibiting compounds chelerythrine (1 μM), Gö6976 (100 nM), or rottlerin (30 μM) (30 min; 37°C) followed by treatment with PMA. Surface proteins were biotinylated, extracted, and purified using a Cell Surface Labeling Accessory Pack (Pierce) according to the manufacturer’s instructions. Isolated surface proteins were detected by Western blot analysis. Samples were boiled for 3 min and separated by SDS-PAGE on 7.5% Tris-HCl Ready-Gels (Bio-Rad), transferred onto polyvinylidene difluoride membranes, and probed with a goat polyclonal anti-TRPM4 antibody (1:250, Santa Cruz Biotechnology) or a rabbit polyclonal anti-GFP horseradish peroxidase (HRP)-conjugated antibody (1:250 Novus Biologicals). A donkey anti-goat IgG-HRP antibody was used as a secondary to the anti-TRPM4 antibody. Bound antibody was detected using ECL Chemiluminescent Substrate (Pierce) and an EpiChem II Darkroom system. Images were analyzed using LabWorks (4.0) and Scion Image software.

Biotinylation of surface protein in intact cerebral arteries was performed as previously described (1, 3). Briefly, cerebral arteries were cleaned of connective tissue. Arteries were incubated in a 1 mg/ml mixture each of EZ-Link Sulfo-NHS-LC-LC-Biotin and Maleimide-PEG2-Biotin reagents (Pierce) in phosphate-buffered saline (PBS, Invitrogen) at room temperature for 1 h. In the treatment group, PMA (100 nM) was added for the last 10 min of the incubation period. Control arteries received no PMA treatment. Arteries were then briefly washed with 100 mM glycine in PBS to remove any unbound biotin.

To determine total protein, biotinylated arteries were homogenized in a lysis buffer of the following composition: 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 0.1% SDS. Cellular debris was removed by centrifugation at 8,000 rpm for 10 min. Total protein was then determined to allow normalization for Avidin (Monomeric Avidin, Pierce) pull-down of biotinylated surface proteins. Following pull-down, the supernatant comprised the nonbiotinylated (cytosolic) protein fraction. Biotinylated surface proteins were eluted from the avidin beads by boiling in 1X Laemmli buffer containing 2-mercaptoethanol (2%). Surface and cytosolic proteins were analyzed using Western blotting and probed with rabbit polyclonal anti-TRPM4 antibody (1:500, Affinity BioReagents). Surface and cytosolic TRPM4 protein bands were analyzed using Quantity One software (Bio-Rad) and are expressed as percentage of total protein.

RNA interference and reverse permeabilization. Small interfering RNAs (siRNA) against TRPM4 and PKCδ were used to downregulate expression of these proteins. siRNA molecules purchased from Qiagen (10272280 [AllStars Negative Control], SI02868292 [Rn_Trpm4_1], SI02868313 [Rn_Trpm4_4], and SI03028502 [Rn_Prkcd_5_HPI]) were dissolved as instructed at a concentration of 20 μM in siRNA Suspension Buffer (Qiagen). Control siRNA or gene-specific siRNA molecules were introduced into intact cerebral arteries using a reversible permeabilization procedure (23). To permeabilize the arteries, segments were first incubated for 20 min at 4°C in the following solution (in mM): 120 KCl, 2 MgCl2, 10 EGTA, 5 Na2ATP, and 20 TES (pH 6.8). Arteries were then placed in a similar solution containing siRNA (40 nM) for 3 h at 4°C and then transferred to a third siRNA-containing solution with elevated MgCl2 (10 mM) for 30 min at 4°C. Permeabilization was reversed by placing arteries in a MOPS-buffered physiological siRNA-containing solution consisting of (in mM) 140 NaCl, 5 KCl, 10 MgCl2, 5 glucose, and 2 MOPS (pH 7.1, 22°C) for 30 min at room temperature. Ca2+ was gradually increased in the latter solution from nominally Ca2+-free to 0.01, 0.1, and 1.8 mM over a 45-min period. Following the reversible permeabilization procedures, arteries were organ cultured for 2 to 3 days in DMEM/F-12 culture media supplemented with 1-glutamine (2 mM) (GIBCO) and 0.5% penicillin-streptomycin (GIBCO). Arteries were then used for isolated vessel experiments or real-time RT-PCR.
Real-time RT-PCR. Arteries containing siRNA were enzymatically dissociated as described above, and RNA was immediately isolated and purified using an RNasy Protect Mini Kit (Qiagen). mRNA was synthesized into cDNA with the aid of an Omniscript Reverse Transcriptase Kit (Qiagen) using 100 ng RNA/reaction. Downregulation of TRPM4 or PKCδ was detected using a real-time SYBR Green detection assay (Bio-Rad), QuantiTect primers spanning an intron/exon boundary(ies) of TRPM4 or PKCδ (Qiagen), and an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Samples were normalized to β-actin, and cycling parameters were selected on the basis of the protocol for QuantiTect primer assays (Qiagen). β-Actin primers were designed on the basis of a published sequence (NM_031144) and were purchased from Integrated DNA Technologies. Sequences were as follows: 5'-TTGCTGACAGGATGCAAGAGGA-3' (forward) and 5'-ACTCTGCTTGGTGATCCACATCT-3' (reverse). Downregulation of target mRNA was calculated according to the Pfaffl method (35).

Whole cell patch-clamp studies. Patch-clamp studies were conducted using nontransfected A7r5 or A7r5 cells transiently transfected with TRPM4-GFP 3 days after transfection. TRPM4 currents were recorded using an Axopatch 200B amplifier equipped with a CV203BU headstage (Molecular Devices). Recording electrodes (resistance 3–5 MΩ) were pulled from borosilicate glass (1.5 mm OD, 1.17 mm ID; Sutter Instruments) and coated with wax to reduce capacitance. Currents were filtered at 1 kHz, digitized at 40 kHz, and stored for subsequent analysis. Clampex and Clampfit versions 9.0 (Molecular Devices) were used for data acquisition and analysis, respectively. All recordings were performed at room temperature. Cells were initially held at a membrane potential of 0 mV. The bathing solution contained (in mM) 146 NaCl, 5 CaCl2, 10 HEPES, and 10 glucose (pH 7.4). The pipette solution contained (in mM) 146 CsCl, 1 MgCl2, and 10 HEPES. The bathing and pipette solutions were supplemented with the potassium channel blocker tetraethylammonium (10 mM). Intracellular Ca2+ was 100 μmol/L. Currents were measured during voltage ramps between −100 and +100 mV and were normalized to membrane capacitance (Cm).

TIRF microscopy. A7r5 cells stably transfected with TRPM4-GFP, or A7r5 cells and primary cerebral artery smooth muscle cells transiently transfected with TRPM4-GFP were grown on 25-mm round coverslips and imaged at room temperature in a HEPES-buffered saline containing (in mM) 146 NaCl, 4.7 KCl, 2.5 CaCl2, 0.6 MgSO4, 1.6 NaHCO3, 0.15 Na2HPO4, 0.1 ascorbic acid, 8 glucose, and 20 HEPES (pH 7.4). Transiently transfected cells were imaged 3 days after transfection. TIRF images (200-ms exposure at 1 Hz) were acquired on a through-the-lens TIRF system built around an inverted microscope (Zeiss LSM). The spatially averaged fluorescence intensity (i.e., averaged intensity, focus, TIRF angle, and camera gain throughout each experiment) was determined using custom software written in the Salford Research Language. Inhibitory experiments were performed over the entire region of the cell imaged) was determined using Clampex and Clampfit software. Intracellular Ca2+ concentrations were calculated using an ImageJ version 1.42q (National Institutes of Health, Bethesda, MD).

Isolated vessel experiments. Arterial segments treated with siRNA were cleaned and transferred to a vessel chamber (Living Systems Instrumentation). The proximal end of the vessel was cannulated with a glass micropipette and secured, the lumen was rinsed, and the distal end of the vessel was cannulated and secured. Vessels were pressurized to 20 mmHg with physiological saline solution (PSS) (in mM): 119 NaCl, 4.7 KCl, 1.8 CaCl2, 1.2 MgSO4, 24 NaHCO3, 0.2 KH2PO4, 1.6 glucose, 1.1 EDTA, and superfused with warm (33°C) PSS aerated with a normoxic gas mixture (21% O2, 6% CO2, 73% N2). Following a 15-min equilibration period, intraluminal pressure was slowly increased to 100 mmHg, vessels were stretched to remove bends, and pressure was reduced to 20 mmHg for an additional 15-min equilibration period. Inner diameter was continuously monitored using video microscopy and edge-detection software (Ionoptix). To determine constrictor responses, vessels were pressurized to 60 mmHg and PMA (0.1 μM) was added to the superfusate. PMA-induced constrictor responses were normalized to baseline diameter. To assess myogenic tone, vessels treated with control PKCδ-specific siRNA were subjected to a series of pressure steps between 20 and 100 mmHg and spontaneous myogenic tone was allowed to develop at each step until a stable diameter was achieved (∼3 min). After completion of the pressure-response curve, intraluminal pressure was maintained at 20 mmHg and vessels were superfused with Ca2+-free PSS (in mM): 119 NaCl, 4.7 KCl, 1.2 MgSO4, 24 NaHCO3, 0.2 KH2PO4, 10.6 glucose, 3 EDTA, and 0.15 diltiazem. The pressure-response curve was repeated under Ca2+-free conditions to obtain passive responses. Myogenic tone was calculated as the percent difference in diameter observed for Ca2+-containing vs. Ca2+-free PSS at each pressure. Values of n refer to the number of arteries used for a particular experimental group.

Smooth muscle cell membrane potential. For measurement of smooth muscle cell membrane potential, cerebral arteries treated with either control or PKCδ-specific siRNA were cannulated, pressurized, and superfused with warm (37°C) PSS. Vascular smooth muscle cells...
were impaled through the adventia with glass intracellular microelectrodes (tip resistance 100–200 MΩ). A WPI Intra 767 amplifier was used for recording membrane potential. Analog output from the amplifier recorded using IonOptix Software (sample frequency 60 Hz). Criteria for acceptance of membrane potential recordings were as follows: 1) an abrupt negative deflection of potential as the microelectrode was advanced into a cell; 2) stable membrane potential for at least 30 s; and 3) an abrupt change in potential to ~0 mV after the electrode was retracted from the cell. Vascular smooth muscle cell membrane potential was recorded at intraluminal pressures of 20 and 80 mmHg.

Calculations and statistical analysis. All data are means ± SE. Values of n refer to number of arteries for isolated vessel experiments, number of cells for live-cell imaging experiments, or number of experiments for cell surface biotinylation or real-time RT-PCR. Statistical methods conform to the recommendations of the editors of Circulation Research (43). A level of \( P \leq 0.05 \) was accepted as statistically significant for all experiments.

RESULTS

TRPM4 channels are mobile. To investigate the effects of PKC activity on the subcellular localization of TRPM4 channels, GFP was fused to the carboxyl terminus of TRPM4 (TRPM4-GFP) and expressed in the smooth muscle-derived A7r5 cell line. As illustrated in Fig. 1, A and B, TRPM4-GFP was present on the cell surface and within the cytoplasm, where it appeared to be located within small vesicles. A7r5 cells expressing TRPM4-GFP were patch clamped in the conventional whole cell mode. Cation currents recorded from these cells exhibited outward rectification (Fig. 1C), rapid...
rundown, and dependence on intracellular Ca\(^{2+}\) for activation. These electrophysiological characteristics are similar to those of native TRPM4 channels under whole cell patch-clamp conditions (22, 30), suggesting that addition of the GFP tag does not interfere with channel activity.

TRPM4-GFP protein appears to be present in two distinct compartments, being expressed not only on the cell surface but also within intracellular vesicles reminiscent of a reserve storage pool. Live-cell FRAP analysis was employed to investigate the mobility of TRPM4-GFP within these two locations. Illustrated in Fig. 1, D-G, is a FRAP experiment targeting the diffuse fluorescence and punctate intracellular vesicles. Fluorescence returned to a bleached ROI (Fig. 1E, dashed circle) over \(\sim 5\) min (Fig. 1, F and G, and Supplemental Movie S1; supplemental material for this article is available online at the Journal website) with a mean time constant (\(\tau\)) of 168 \pm 19 s \((n = 5)\) (Fig. 1H), indicating that TRPM4-GFP is mobile. In addition, the intracellular vesicles were observed moving through the bleached ROI possibly delivering channel protein to the cell surface during the recovery phase (arrow, Fig. 1F). The diffusion coefficient of TRPM4-GFP is 0.02 \pm 0.01 \(\mu m^2/s\), comparable to that of voltage-dependent K\(^+\) Kc2.1 channels (0.03 \pm 0.02 \(\mu m^2/s\)) (41). Mobile vesicles outside the FRAP ROI are readily observed in Supplemental Movie S1.

PKC activation rapidly increases TRPM4 protein levels at the cell surface. Previous work suggests that PKC activation increases TRPM4 current density (9, 31) and depolarizes smooth muscle (9). While the FRAP data indicate that TRPM4 channel localization within the cell is dynamic, these experiments do not address the issue of membrane insertion. We used a cell surface biotinylation assay to test the hypothesis that PKC activity induces translocation of cytoplasmic TRPM4 channels to the sarcolemma. A7r5 cells stably transfected with TRPM4-GFP were briefly (15 min) treated with PMA (1 \(\mu M\)) to stimulate PKC activity. Surface proteins were biotinylated immediately following PMA treatment, isolated on Neutravidin columns, and subjected to Western blot analysis using anti-GFP (Fig. 2A) or anti-TRPM4 antibodies. Protein bands with identical electrophoretic mobility (~160 kDa) were detected with anti-TRPM4 and anti-GFP antibodies (Supplemental Fig. S1), confirming that the identity of this protein is TRPM4-GFP. TRPM4-GFP band density was approximately threefold greater in cells treated with PMA compared with unstimulated control cells, demonstrating that PKC activity induces rapid accumulation of TRPM4-GFP protein at the cell surface (Fig. 2, A and B). PMA-induced increases in TRPM4-GFP at the plasma membrane were inhibited by pretreating cells with the pan-specific PKC inhibitor chelerythrine (1 \(\mu M\); 30 min) (Fig. 2, A and B). These findings demonstrate that stimulation of PKC activity causes an increase in plasma membrane levels of TRPM4-GFP protein in <15 min. The finding that PKC inhibition decreased the surface expression relative to control suggests that, under basal conditions, TRPM4 cycles between cytoplasmic and surface compartments. The kinetics of PMA-induced increases in surface protein suggest that increased levels of TRPM4 at the membrane result from a dynamic transport process rather than elevated gene expression.

TIRF microscopy was used to quantitate sarcolemmal expression of TRPM4 following PKC stimulation in living cells. Consistent with the findings of biotinylation experiments, PMA (1 \(\mu M\)) administration caused a rapid (maximal after \(\sim 10\) min) increase (~3-fold) in surface fluorescence of TRPM4-GFP-expressing A7r5 cells (Fig. 2, C and D). This increase consistently began within 4 min of PMA application. Fluorescence did not change in unstimulated control cells during a 10-min recording period (Fig. 2, C and D). Increases in cell-surface fluorescence in response to PMA were not observed in nontransfected A7r5 cells, nor in TRPM4-GFP-expressing cells pretreated with chelerythrine (1 \(\mu M\), 30 min) (Fig. 2E). In addition, the inactive analog 4a-PMA (1 \(\mu M\)) did not alter cell surface TRPM4-GFP fluorescence (Fig. 2E).

Importantly, no increase in surface fluorescence was detected following PMA administration in cells transfected with a GFP-tagged voltage-dependent K\(^+\) channel (Kc2.1-GFP) or a TRPC3-yellow fluorescent protein fusion protein (Fig. 2E), demonstrating that PKC activity does not increase cell surface levels of all membrane proteins.

To investigate TRPM4 membrane trafficking in a more physiological setting, primary cerebral artery myocytes were cultured and transfected with TRPM4-GFP (Fig. 3, A and B). Cells were immunostained for smooth muscle \(\alpha\)-actin to confirm identity (Fig. 3C). PMA (1 \(\mu M\)) administration caused a rapid increase in surface fluorescence in these cells (Fig. 3, D–G, and Supplemental Movie S2). The time course of PMA-induced TRPM4 plasma membrane translocation in primary cerebral artery smooth muscle cells (Fig. 3F) was similar to that observed in A7r5 cells (Fig. 2D). Our findings demonstrate that TRPM4 protein is transported to the sarcolemma in response to PKC activity in primary vascular smooth muscle cells as well as A7r5 cells. These are the first observations linking membrane trafficking of TRPM4 channels in vascular smooth muscle cells with a depolarizing vasoconstrictor stimulus.

PKC stimulates acute trafficking of TRPM4 to the cell surface in intact cerebral arteries. Using a recently described method (1, 3), cell surface biotinylation experiments were performed to examine TRPM4 channel localization in resistance-size (~200 \(\mu m\) diameter) cerebral arteries. These experiments show that ~63\% of TRPM4 protein is located in the arterial smooth muscle plasma membrane (Fig. 4B). Interestingly, plasma membrane TRPM4 appears to be of a slightly higher molecular weight than cytosolic TRPM4 (Fig. 4A). Preincubation of the TRPM4 primary antibody with an antigenic peptide abolished detection of both the membrane and cytosolic bands (Supplemental Fig. S2), suggesting that the protein bands represent different forms of TRPM4, perhaps resulting from differences in posttranslational modification. Consistent with data obtained in cultured smooth muscle cells, PMA (0.1 \(\mu M\)) induced a rapid (10 min) increase in plasma membrane TRPM4 to ~73\% of total TRPM4 protein and a proportional decrease in cytosolic TRPM4 in intact cerebral arteries (Fig. 4B). Prior publications using this technique demonstrate that the intracellular protein heat shock protein 90 is not detected in surface fractions from biotin-treated arteries (1, 3), indicating that intracellular proteins are not labeled. Thus, these findings demonstrate that endogenously expressed TRPM4 channels traffic to the membrane in response to increased PKC activity in arterial tissue.
PKCδ mediates dynamic trafficking of TRPM4 protein to the cell surface. Vascular smooth muscle cells express numerous PKC isoforms (29, 39) including the conventional (Ca^{2+}-dependent) isoforms PKCα, PKCβ, and PKCγ, the atypical (Ca^{2+}-independent) isoforms PKCδ and PKCε, and the novel isoform PKCζ. Selective pharmacological inhibitors were used to investigate which PKC isoform(s) mediate PMA-induced translocation of TRPM4 protein to the cell surface. PKCα and PKCβ are selectively inhibited by Gö6976 (IC_{50} = 3 nM) (25). Pretreatment of A7r5 cells expressing TRPM4-GFP with Gö6976 (100 nM, 30 min) did not diminish PMA-induced increases of TRPM4 protein in cell surface biotinylation assays.
Fig. 3. TRPM4 translocates to the cell surface in response to PKC activity in primary rat cerebral artery smooth muscle cells. A: DIC. B: GFP fluorescence. C: α-actin immunostaining of a primary rat cerebral artery myocyte transiently transfected with TRPM4-GFP. Bar = 10 μm. D and E: representative TIRF images of a primary cerebral artery smooth muscle cell expressing TRPM4-GFP before (D) and 10 min after (E) administration of PMA (1 μM). Bar = 5 μm. F: levels of fluorescence vs. time for TIRF recordings of primary cerebral artery myocytes transiently transfected with TRPM4-GFP. Controls are unstimulated cells vs. cells treated with PMA (1 μM). G: summary data depicting change in fluorescence (F/F₀) of primary cerebral artery smooth muscle cell transfected with TRPM4-GFP in response to PMA (1 μM); n = 4 cells (control), n = 3 cells (PMA). *P ≤ 0.05 vs. control.

(Fig. 5, A and B) or increases in cell surface fluorescence in TIRF experiments (Fig. 5, C and D). Interestingly, PMA-induced increases in fluorescence were greater for cells treated with Gö6976 (Fig. 5D), suggesting that PKCα or PKCβ might oppose PMA-mediated translocation of TRPM4 to the plasma membrane.

The compound rottlerin is reported to inhibit PKCδ more effectively than other PKC isoforms (IC₅₀ for PKCδ = 6 μM vs. 30 μM for PKCα) (13). In TIRF experiments, pretreatment with rottlerin (30 μM, 30 min) abolished PMA-induced increases in cell surface TRPM4-GFP fluorescence (Fig. 5, C and E), suggesting that the activity of PKCδ stimulates transport of TRPM4 protein to the plasma membrane.

PKCδ influences smooth muscle cell membrane potential. An RNA interference (RNAi)-based approach to silence expression of these proteins in intact cerebral arteries was used to investigate the functional consequences of PKCδ-dependent membrane translocation of TRPM4. Expression of TRPM4 or
PKCδ in cultured arteries exposed to gene-specific siRNA was reduced by ~70% compared with levels in vessels treated with negative control siRNA (Fig. 6A). A recent report from our laboratory demonstrates that siRNA treatment causes a corresponding decrease in TRPM4 proteins levels (11). In addition, PKCδ immunofluorescence was decreased by ~55% in smooth muscle cells isolated from arteries treated with PKCδ siRNA, demonstrating that this treatment decreases protein levels (Fig. 6B).

Our prior work demonstrates that TRPM4 is required for pressure-induced smooth muscle cell depolarization and myogenic constriction of cerebral arteries (10). PKCδ activity increases the number of TRPM4 channels in the plasma membrane, suggesting that this isoform also influences smooth muscle resting membrane potential and pressure-induced arterial tone. This hypothesis was tested by determining the effects of PKCδ siRNA treatment on smooth muscle cell membrane potential in intact cerebral arteries. Microelectrode recordings from pressurized vessels demonstrate that the membrane potential of myocytes in arteries treated with PKCδ siRNA is significantly hyperpolarized compared with controls at intraluminal pressures of 20 and 80 mmHg (Fig. 6, C and D). The mean resting membrane potentials for control siRNA-treated arteries is $-52.8 \pm 4.1$ mV at 20 mmHg vs. $-39.7 \pm 1.9$ mV at 80 mmHg, and for PKCδ siRNA-treated arteries, $-69.7 \pm 4.0$ at 20 mmHg vs. $-51.9 \pm 2.9$ at 80 mmHg ($n = 5$ arteries for all groups). These findings demonstrate that PKCδ plays an important role in maintaining depolarized membrane potentials in cerebral myocytes in pressurized arteries.

**TRPM4 and PKCδ influence vasomotor responses.** RNAi-treated cerebral vessels were also used to examine the importance of TRPM4 and PKCδ in vasomotor responsiveness. Arteries treated with TRPM4 siRNA exhibited reduced PMA (0.1 μM)-induced vasoconstriction compared with control siRNA-treated vessels (Fig. 7, A, B, and E). Pretreatment with the putative TRPM4 inhibitor 9-phenenthrol (12) (20 and 60 μM) also resulted in diminished PMA-induced vasoconstriction compared with controls (Fig. 7, C and E). Furthermore, RNAi-mediated downregulation of PKCδ also attenuated PMA-induced constriction of cerebral arteries compared with controls (Fig. 7, D and E). The mean luminal diameters of arteries used in these experiments before and after administration of PMA are shown in Supplemental Table S1. These findings demonstrate that expression and activity of TRPM4 and PKCδ are required for vasoconstriction of cerebral arteries following administration of PMA, supporting the concept that PKCδ-mediated transport of TRPM4 protein to the cell surface regulates this response.

The effects of siRNA-mediated PKCδ downregulation on pressure-induced constrictor responses were also investigated. We found that arteries treated with control siRNA develop myogenic tone (Fig. 7F, top), whereas arteries treated with PKCδ siRNA tended to passively dilate in response to stepwise increases in intraluminal pressure (Fig. 7F, bottom). Myogenic tone was significantly less for arteries treated with PKCδ siRNA compared with controls, indicating that PKCδ expression is required for myogenic constriction of cerebral arteries (Fig. 7G). These findings demonstrate that, like TRPM4 (10), PKCδ plays an important role in the regulation of smooth muscle cell membrane potential and myogenic tone.

The findings of this study are consistent with the hypothetical signaling pathway shown in Fig. 8. We propose that PMA and perhaps intraluminal pressure stimulate PKCδ activity, which serves to promote translocation of TRPM4 to the plasma membrane, where the channels are available to be activated by inositol 1,4,5-trisphosphate (IP$_3$) receptor (IP$_3$R)-mediated Ca$^{2+}$ release (11). The resulting increased Na$^+$ current density depolarizes the sarcolemma, activating voltage-dependent Ca$^{2+}$ channels (VDCC) and causing vasoconstriction (Fig. 8).

**DISCUSSION**

The current study examined the novel hypothesis that trafficking of TRPM4 channel protein to the plasma membrane contributes to arterial constriction in response to PKC activity. The major findings reported here are as follows: 1) TRPM4 channels tagged with GFP appear to be localized in structures that appear to be mobile transport vesicles; 2) PKC activity causes a rapid increase in cell surface levels of TRPM4 protein in A7r5 cells, primary cerebral artery smooth muscle cells, and intact cerebral arteries; 3) trafficking of TRPM4 to the membrane is independent of PKCδ and PKCβ activity but is inhibited by a PKCδ blocker; 4) downregulation of PKCδ results in smooth muscle cell hyperpolarization; 5) PMA-induced vasoconstriction of intact cerebral arteries requires expression and activity of TRPM4 and PKCδ; and 6) PKCδ is involved in myogenic vasoconstriction. We propose that PKCδ activity promotes translocation of TRPM4 to the plasma membrane, increasing the number of channels available for activation by intracellular Ca$^{2+}$ release events (Fig. 8). These data are the
first observations linking trafficking of TRPM4 protein to the plasma membrane with vasoconstrictor stimuli and demonstrate a novel mechanism for controlling smooth muscle membrane potential and arterial tone.

Many PKC-dependent mechanisms influence smooth muscle cell contractility. PKC activity contributes to vaso-motor tone (15, 20, 34) by enhancing the sensitivity of the arterial smooth muscle contractile apparatus to intracellular Ca\(^{2+}\) and by depolarizing the sarcolemma to activate Ca\(^{2+}\) influx via VDCCs (5, 17, 24, 28). In addition, PKC increases the activity of VDCCs (2). Under physiological conditions, PKC activity is increased by agonist binding to G\(_{q}\) protein-coupled cell surface receptors. Stimulation of these receptors increases the activity of phospholipase C, an enzyme that cleaves the membrane phospholipid phosphatidylinositol 4,5-bisphosphate into the second messenger molecules diacylglycerol (DAG) and IP\(_3\). DAG is an endogenous activator of several PKC isoforms, including PKC\(\alpha\) and PKC\(\delta\). Our prior work (9) and the current study show that TRPM4 contributes to smooth muscle cell depolarization and vasoconstriction in response to the PKC activator PMA. The earlier study did not investigate which of the multiple PKC isoforms that are present in vascular smooth muscle cells (29, 39) is responsible for activation of TRPM4. The current findings show that silencing of PKC\(\delta\) expression diminished PMA-induced constriction of cerebral arteries by \(\sim 50\%\), suggesting significant involvement of this isoform. Furthermore, we find that the membrane potential of smooth muscle cells in cerebral arteries treated with siRNA against PKC\(\delta\) is hyperpolarized by an average of 17 mV when intraluminal pressure is 20 mmHg and by 12.2 mV when intraluminal pressure is 80 mmHg. These data suggest that basal PKC\(\delta\) activity is an important mediator of smooth muscle membrane potential. We also find that, in agreement with a recent report using pharmacological inhibition of PKC\(\delta\) activity (18), expression of the
PKCδ isoform is required for myogenic vasoconstriction. Because PKCδ, but not PKCα nor PKCβ, activity is required for translocation of TRPM4 to the plasma membrane, we conclude that PKCδ activity causes TRPM4 protein to accumulate at the cell surface, which serves to depolarize the membrane and elicit vasoconstriction.

Steady-state levels of cell surface proteins are dictated by the relative kinetics of delivery and removal mechanisms. Prior studies show that increased protein delivery is responsible for elevated surface expression and activation of a number of TRP channels. For example, activation of ionic currents by dynamic transport of TRP channel protein to the plasma membrane was first described by Bezzerides and colleagues (4). This study showed that epidermal growth factor caused rapid increases in TRPC5 protein levels at the surface of HEK cells by a vesicular trafficking mechanism. Increased levels of TRPC5 protein at the membrane resulted in the onset of a nonselective cation current, demonstrating a novel mechanism for regulating the activity of these channels (4). A further report from the Clapham laboratory used simultaneous TIRF microscopy and patch-clamp electrophysiology to show that TRPM7, but not TRPC5 or TRPV5, channels accrue at the plasma membrane of A7r5 cells and simultaneously activate a cation current in response to laminar shear stress (32). Our findings demonstrate that TRPM4 levels at the cell surface are increased by PKC activity. PKC activity reportedly regulates dynamic surface trafficking of other channels (16, 21, 46), including N-methyl-D-aspartate receptors in rat hippocampal neurons (21), the voltage-dependent Ca^{2+} channel Ca_{v2} in Aplysia peptideric neurons (46), and ATP-sensitive K⁺ channels in neurons and cardiac myocytes (16). These prior studies also show that soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent exocytosis (21) and actin polymerization (46) are involved in PKC-mediated translocation of these channels to the plasma membrane. Proteins required for SNARE-dependent exocytosis are present in smooth muscle cells (38), and further studies are...
needed to evaluate how these pathways influence TRPM4 trafficking in arterial myocytes. It is also feasible that increased steady-state levels of TRPM4 protein at the plasma membrane result from PKC-dependent decreases in channel removal. This possibility is supported by a recent study demonstrating that defects in removal of TRPM4 channels from the plasma membrane in Purkinje fibers is associated with progressive familial heart block type I.

Fig. 7. TRPM4 and PKCδ influence vasomotor responses. A–D: vasoconstriction in response to PMA (0.1 μM) for cerebral arteries treated with control siRNA (A), TRPM4 siRNA (B), the TRPM4 blocker 9-phenenthrol (60 μM) (9-phen; C), or PKCδ siRNA (D). E: summary data of the effects of TRPM4 siRNA, 9-phenenthrol, and PKCδ siRNA on PMA-induced vasoconstriction; n = 5 for each group. *P ≤ 0.05 vs. control. F: representative recording of changes in diameter in response to step-wise increases in luminal pressure for cerebral arteries treated with control (top) or PKCδ siRNA (bottom). Vessels were initially pressurized to 10 mmHg and then subjected to a series of 20-mmHg step increases in pressure to 110 mmHg. G: myogenic tone (normalized to diameter under Ca²⁺-free conditions) in response to increasing luminal pressure for cerebral arteries treated with control siRNA or PKCδ siRNA; n = 10 for control, n = 9 for PKCδ siRNA-treated vessels. *P ≤ 0.05 vs. control siRNA-treated arteries.
(PFHBI) (19). Patients with an autosomal-dominant form of PFHBI express TRPM4 with a missense mutation at amino acid 19 (19). This mutation diminishes deSUMOylation, resulting in impaired endocytosis and elevated levels of channel protein in the plasma membrane (19). In light of the current findings, it is possible that impaired endocytosis of TRPM4 in arterial myocytes could contribute to familial forms of hypertension. Dynamic regulation of TRPM4 protein levels in the plasma membrane may prove to be a critical factor in the regulation of smooth muscle cell membrane potential, because arterial myocytes have a very high input resistance (5–15 GΩ) (6, 7, 36, 40) and small changes in current density resulting from modest changes in cell surface protein levels can have large effects on resting membrane potential. Although further investigation is required to determine the relative importance of the delivery versus retrieval mechanism, our data clearly demonstrate that cell surface levels of TRPM4 are increased in response to PKCδ activity and this mechanism influences smooth muscle membrane potential and vascular tone.

TRPM4 channels are not constitutively active, and delivery of channel protein to the plasma membrane alone is unlikely to initiate cation currents. Elevated levels of intracellular Ca\(^{2+}\) (>1 μM) are required to activate TRPM4 (10, 22, 30). Recent findings from our laboratory demonstrate that Ca\(^{2+}\) released from IP\(_3\)Rs on the sarcoplasmic reticulum (SR) activate sustained TRPM4 currents in cerebral artery smooth muscle cells under physiological recoding conditions (11). Ca\(^{2+}\)-dependent regulation of TRPM4 is complex and may involve interaction with Ca\(^{2+}\)/calmodulin complexes or direct binding of Ca\(^{2+}\) to the channel (31). The Nilius laboratory demonstrated that PKC activation resulted in increased whole cell TRPM4 current density when intracellular [Ca\(^{2+}\)] was clamped at levels from 1 to 1,000 μM, suggesting that PKC enhances the sensitivity of TRPM4 to Ca\(^{2+}\)-dependent activation (31). Our current findings suggest that PKCδ activation inserts additional channels in the membrane that are then available for stimulation by Ca\(^{2+}\) released from IP\(_3\)Rs on the SR. This conclusion is consistent with our earlier observation that treatment with PMA increases the frequency of observation of TRPM4 channels in inside-out membrane patches pulled from cerebral myocytes without changing the current-voltage relationship, single-channel conductance, or open probability (NP\(_{o}\)) of the channel when intracellular Ca\(^{2+}\) was 100 μM (10). Experiments reported in prior studies did not investigate channel trafficking and are unable to distinguish between an authentic increase in channel Ca\(^{2+}\) sensitivity and an elevation in membrane channel protein. These two mechanisms are not mutually exclusive, and our new data suggest a possible alternative interpretation for earlier observations.

The mammalian TRP superfamily consists of 28 members (27 in humans where TRPC2 is a nonfunctional pseudogene) (42). TRP genes are expressed as six-transmembrane domain subunits, and functional channels are formed from the assembly of four subunits (42). In native cells, certain TRP channel subunits can combine to form heteromultimeric channels with properties that are distinct from homomeric channels assembled from the same subunits (8). This has been particularly well described for TRPC channel subunits (26). Although heteromultimeric channels composed of TRPM4 subunits have not been reported, our findings do not rule out the possibility that heteromultimeric channels involving TRPM4 exist in smooth muscle cells and are trafficked to the membrane in response to PKCδ activity.

In conclusion, the findings reported here suggest that dynamic trafficking of TRPM4 channel protein to the cell surface contributes to smooth muscle membrane depolarization in response to PKCδ activity. The signaling pathways responsible for channel translocation present a novel opportunity for investigation of pathophysiological conditions involving excessive membrane excitability, such as systemic hypertension and cerebral vasospasm.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
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