Basal protein kinase Cδ activity is required for membrane localization and activity of TRPM4 channels in cerebral artery smooth muscle cells

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The melastatin (M) transient receptor potential channel (TRP) channel TRPM4 is a critical regulator of vascular smooth muscle cell membrane potential and contractility. We recently reported that PKCδ activity influences smooth muscle cell excitability by promoting translocation of TRPM4 channel protein to the plasma membrane. Here we further investigate the relationship between membrane localization of TRPM4 protein and channel activity in native cerebral arterial myocytes. We find that TRPM4 immunolabeling is primarily located at or near the plasma membrane of freshly isolated cerebral artery smooth muscle cells. However, siRNA mediated downregulation of PKCδ or brief (15 min) inhibition of PKCδ activity with rottlerin causes TRPM4 protein to move away from the plasma membrane and into the cytosol. In addition, we find that PKCδ inhibition diminishes TRPM4-dependent currents in smooth muscle cells patch clamped in the amphotericin B perforated patch configuration. We conclude that TRPM4 channels are mobile in native cerebral myocytes and that basal PKCδ activity supports excitability of these cells by maintaining localization of TRPM4 protein at the plasma membrane.

Introduction

The melastatin (M) transient receptor potential (TRP) channel TRPM4 is present and functional in vascular smooth muscle cells where it is responsible for pressure-induced cerebral artery myocyte membrane potential depolarization and vasoconstriction.1,2 Furthermore, expression of the channel is necessary for autoregulation of cerebral blood flow.3 Because TRPM4 plays a critical role in vascular physiology, a major focus of our lab is to elucidate how the channel is regulated in native smooth muscle cells. TRPM4 is selective for monovalent cations and requires high levels of intracellular Ca2+ for activation.4,5 In addition, TRPM4 channels are sensitive to protein kinase C (PKC) activity1,6,7 and mediate vascular smooth muscle cell depolarization and vasoconstriction in response to phorbol 12-myristate 13-acetate (PMA).8 We recently reported that PMA-induced elevation of PKCδ activity increases the amount of TRPM4 protein present at the cell surface, a response that is associated with increased membrane excitability and vasoconstriction.9 These findings suggest that PKCδ activity supports TRPM4-dependent membrane depolarization by promoting trafficking of channel protein to the plasma membrane.9 Here we present additional data demonstrating a link between PKCδ-dependent membrane localization of TRPM4 channel protein and cation current activity in native cerebral artery smooth muscle cells.

Results

Inhibition of PKCδ expression or activity disrupts membrane localization of TRPM4 in native cerebral artery smooth muscle cells. To determine the effects of PKCδ expression on the subcellular localization of TRPM4, isolated cerebral arteries were treated with siRNA selective for PKCδ and cultured for 48 hours to allow downregulation. Our prior
The study demonstrates that this treatment effectively reduces expression of PKCδ mRNA and protein. Following this treatment, the arteries were enzymatically dispersed and smooth muscle cells were immobilized on glass slides, fixed and immunolabeled for TRPM4. To determine the subcellular distribution of TRPM4 protein in this preparation, membrane fluorescence (FM) and total fluorescence (FT) were determined using Image J from regions of interest encompassing either the plasma membrane or total cytosol of the cell, respectively. Using this technique, an FM/FT value greater than 1.0 indicates that the fluorescence signal is concentrated at or near the plasma membrane, whereas a value of less than 1.0 signifies that fluorescence is uniformly distributed throughout the cell.

We found that in cells from arteries treated with control (non-silencing) siRNA, TRPM4 protein fluorescence was primarily localized to the plasma membrane (FM/FT = 1.4 ± 0.03, n = 30 cells; Fig. 1A), whereas channel protein was uniformly distributed throughout the cytosol in cells obtained from PKCδ siRNA-treated groups (FM/FT = 0.9 ± 0.02; n = 30; Fig. 1B). These findings indicate that PKCδ expression is necessary for maintaining TRPM4 channels at the plasma membrane in cerebral artery smooth muscle cells.

Discussion

Recent reports demonstrate that TRPM4 is an important regulator of cerebral artery function. Antisense and siRNA-mediated downregulation of the channel in intact cerebral arteries attenuates pressure and PMA-induced membrane potential depolarization and vasoconstriction. These findings are supported by a recent study showing that in isolated cerebral arteries at physiological intraluminal pressure, selective pharmacological inhibition of TRPM4 hyperpolarizes the smooth muscle cell membrane potential to nearly to the K+ equilibrium potential and essentially abolishes myogenic tone. In addition, antisense-mediated downregulation of TRPM4 expression in vivo impairs autoregulation of cerebral blood flow, highlighting the physiological significance.
of the channel. Given the central role of TRPM4 in regulation of smooth muscle excitability and contractility, understanding the channel’s regulation under native conditions is critically important.

TRPM4 channels have a complex relationship with Ca\textsuperscript{2+}. High levels of intracellular Ca\textsuperscript{2+} are required for activation,\textsuperscript{4,5} although prolonged exposure to high Ca\textsuperscript{2+} concentrations results in rapid inactivation in conventional whole cell and inside-out patch clamp configurations.\textsuperscript{6,8} Channel activity can be rescued under these conditions if phospholipase C (PLC) activity is blocked or if the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)\textsubscript{2}) is included in the patch pipette.\textsuperscript{11,12} These findings suggest that PtdIns(4,5)\textsubscript{2} depletion from the plasma membrane resulting from Ca\textsuperscript{2+}-dependent PLC activity is responsible for rapid TRPM4 inactivation. Recently, using the amphotericin B perforated patch clamp configuration, our laboratory showed that TRPM4 currents could be recorded from native arterial smooth muscle cells for as long viable seals could be maintained (up to ~30 min).\textsuperscript{10} The perforated patch configuration allows voltage clamp to be maintained with minimal disturbance to intracellular Ca\textsuperscript{2+} signaling dynamics and is more representative of native conditions compared with conventional and inside-out patch clamp. We reported that in perforated-patched cerebral arterial myocytes, TRPM4 activity is dependent on release of Ca\textsuperscript{2+} from inositol trisphosphate receptors located on the sarcoplasmic reticulum, producing currents that we refer to as TICCs.\textsuperscript{8} These findings suggest that transient elevations in localized [Ca\textsuperscript{2+}] in smooth muscle cells activate sustained TRPM4 currents under physiological conditions, whereas persistently elevated intracellular [Ca\textsuperscript{2+}] associated with conventional whole cell and inside-out configurations results in rapid inactivation. We conclude that the rapid channel inactivation reported in the earlier studies is an artifact of the patch clamp methods used to record channel activity and not an inherent property of the channel itself.

Reports indicate that PKC activity elevates TRPM4 current density by increasing the apparent sensitivity of the channel to intracellular Ca\textsuperscript{2+}. Our recent study demonstrates that stimulation of PKC\textgreek{d} activity with PMA increased TRPM4 protein levels at the plasma membrane, suggesting that increases in the Ca\textsuperscript{2+} sensitivity of the channel results from increased amounts of TRPM4 protein at the cell surface.\textsuperscript{9} In other words, when PKC\textgreek{d} activity is elevated, more channels are available at the plasma membrane for Ca\textsuperscript{2+}-dependent activation. These findings are consistent with prior reports showing that PMA administration increases the frequency of observation of TRPM4 currents from inside-out membrane patches pulled from human atrial cardiomyocytes and native cerebral artery smooth muscle cells.\textsuperscript{8} Remarkably, in the current study, we find that only brief (15 min) inhibition of PKC\textgreek{d} activity dramatically alters the location of TRPM4 in native cerebral arterial myocytes. These findings suggest that TRPM4 channel protein is very mobile in these cells, and that the channel rapidly cycles into and out of the plasma membrane. Our findings are consistent with the possibilities that PKC\textgreek{d} activity is required for membrane insertion, or that PKC\textgreek{d} activity impairs removal of channel protein from the plasma membrane. These two proposed mechanisms are not mutually exclusive and further investigation is needed to define the exact molecular mechanisms involved.

The current findings also show that TRPM4 channels are located primarily on the plasma membrane of smooth muscle cells in unpressurized arteries cultured in the absence of serum for 48 hours, suggesting that basal activity of the kinase is sufficient to maintain the bulk of TRPM4 protein at the plasma membrane. These findings are consistent with our prior results showing that the pan-specific PKC\textgreek{d} inhibitor chelerythrine diminished baseline cell surface levels of a TRPM4-GFP construct in serum-starved A7r5 cells.\textsuperscript{9} Furthermore, using the amphotericin B perforated patch clamp method we show here that the PKC\textgreek{d} inhibitor rottlerin also decreases TRPM4-dependent TICC activity in native cerebral artery myocytes. Thus, PKC\textgreek{d} inhibition disrupts the subcellular distribution TRPM4 and decreases activity of the channel, indicating that membrane localization is
necessary for normal channel activity. This finding is consistent with our prior reports showing that membrane depolarization and vasoconstriction in response to PMA-induced PKC activation requires TRPM4 expression and that downregulation of PKCδ hyperpolarizes the smooth muscle cell plasma membrane and blunts PMA and pressure-induced vasoconstriction. Our findings indicate that PKCδ supports membrane excitability and contractility of vascular smooth muscle cells by maintaining TRPM4 channel protein at the plasma membrane.

TRPM4 gain of function mutations, resulting in increased cell-surface density of TRPM4 protein in Purkinje fibers, contribute to some forms of familial cardiac conduction block. Our findings are consistent with the possibility that similar mechanisms involving either TRPM4 or PKCδ could contribute to cardiovascular diseases involving elevated smooth muscle cell excitability such as hypertension, stroke or cerebral vasospasm.

Material and Methods

Animals. Male Sprague-Dawley rats (250–350 g; Harlan) 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 Animal Care and Use Committees (IACUC) of Colorado State University. Brains were isolated in the following solution (in mM): 110 KCl, 2 MgCl₂, 10 glucose and 10 HEPES (pH 7.2). Arterial segments were initially incubated for 20 minutes 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 hours at 4°C and then transferred to a third siRNA-containing solution with elevated MgCl₂ (10 mM) for 30 minutes 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 minutes 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 minute period. Following the reversible permeabilization procedures, arteries were organ cultured for two to three days in D-MEM/F-12 culture media supplemented with L-glutamine (2 mM) (Gibco) and 0.5% penicillin-streptomycin (Gibco). Arteries were used for smooth muscle cell isolation.

Immunocytochemistry. Cells were enzymatically dissociated as described above, and allowed to adhere to glass slides for 20 minutes at 4°C. Cells were fixed with 4% formaldehyde for 10 minutes, permeabilized with cold methanol (-80°C), blocked with 2% bovine serum albumin and incubated with primary antibody (rabbit anti-TRPM4; Abcam ab63080; 1:100 dilution) overnight at 4°C. Cells were subsequently washed and incubated with appropriate fluorescent secondary antibody (anti-rabbit conjugated to Texas Red; Santa Cruz sc-2780; 1:500 dilution) for 2 hours at room temperature. Immunofluorescence images were obtained using a Fluoview 1000 laser scanning confocal microscope (Olympus) and a 60x, 1.4 numerical aperture oil immersion objective, with the pinhole diameter set for 1 Airy Unit. Excitation of Texas Red was by illumination with the 543-nm line set at 74% transmission and emission collected using a variable bandpass filter set to 555–655 nm. All images were acquired at 1,024 x 1,024 pixels at 4.0 μs/pixel and were analyzed in ImageJ version 1.42q (NIH). Membrane Fluorescence (F₀) was determined using the mean fluorescence of a region of interest (ROI) isolating the membrane and Total Fluorescence was determined using the mean fluorescence of the ROI for the cytosol of the total cell.

Electrophysiological recordings. Isolated smooth muscle cells were placed into a recording chamber (Warner Instruments) and allowed to adhere to glass coverslips for 20 min at room temperature. Whole-cell currents were recorded using an Axopatch 200B amplifier equipped with an Axon CV 203BU headstage (Molecular Devices). Recording electrodes (1–3 MΩ) were pulled, polished and coated with wax to reduce capacitance. GΩ seals were obtained in a magnesium-based physiological saline solution (Mg-PSS) containing (in mM) 5 KCl, 140 NaCl, 2 MgCl₂, 10 HEPES and 10 glucose. Amphotericin B (40 μM) was included in the pipette solution to perforate the membrane. Perforation was deemed acceptable if series resistance was less than 50 MΩ. TICC activity was recorded in normal external bathing solution containing (in mM) 134 NaCl, 6 KCl, 1 MgSO₄, 2.5 CaCl₂, 1 KH₂PO₄, 0.02 EDTA, 2 pyruvate and 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 prior to further manipulation.

Isolated cerebral artery smooth muscle cell preparation. Vessels were placed in the following cell isolation solution (in mM): 60 NaCl, 80 Na-glutamate, 5 KCl, 2 MgCl₂, 10 glucose and 10 HEPES (pH 7.2). Arterial segments were initially incubated in 1.2 mg/ml papain (Worthington) and 2.0 mg/ml diithioerythritol for 17 min at 37°C, followed by 15 min incubation at 37°C in 1.0 mg/ml type II collagenase (Worthington). The digested segments were then washed three times in ice-cold cell isolation solution and incubated on ice for 30 min. Following this incubation period, vessels were triturated to liberate smooth muscle cells and stored in ice-cold cell isolation solution for use. Smooth muscle cells were studied within 6 hours following isolation.

RNAi and reverse permeabilization. Small interfering RNAs (siRNA) against TRPM4 were used to downregulate expression of the channel in isolated cerebral arteries. siRNA molecules purchased from QIagen were dissolved as instructed at a concentration of 20 μM in siRNA Suspension Buffer. Control siRNA or TRPM4 siRNA molecules were introduced into intact cerebral arteries using a reversible permeabilization procedure. To permeabilize the arteries, segments were first incubated for 20 minutes 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 hours at 4°C and then transferred to a third siRNA-containing solution with elevated MgCl₂ (10 mM) for 30 minutes 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 minutes 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 minute period. Following the reversible permeabilization procedures, arteries were organ cultured for two to three days in D-MEM/F-12 culture media supplemented with L-glutamine (2 mM) (Gibco) and 0.5% penicillin-streptomycin (Gibco). Arteries were used for smooth muscle cell isolation.
Isolated smooth muscle cells were held at a membrane potential (E_m) of -70 mV, and all recordings are performed at room temperature (22°C). In our recording solutions, the calculated reversal potential for total monovalent cations is -1.8 mV and -30.6 mV for monovalent anions (Cl^-). TICC activity at -70 mV was calculated as the sum of the open channel probability (NP_o) of multiple open states of 1.75 pA. This value was based on the reported unitary conductance of TRPM4 (25 pS). Channel open probability (NP_o) was calculated using the following equation:

\[ NP_o = \sum_{j=1}^{N} \frac{t_j}{T} \]

where \( t_j \) = time spent in seconds with \( j = 1, 2, \ldots, N \) channels open, \( N = \) max number of channels observed and \( T = \) duration of measurement.

Calculations and statistics. All data are means ± SE. Values of \( n \) refer to the number of cells for immnocytochemistry and patch clamp experiments. Patch clamp data were compared using student’s unpaired t-test. A level of \( p \leq 0.05 \) was accepted as statistically significant. Histograms were constructed using Origin 8.1 (OriginLab Corp.).

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