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Endogenous Cytosolic Ca²⁺ Buffering is Necessary for TRPM4 Activity in Cerebral Artery Smooth Muscle Cells

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Abstract

The melastatin transient receptor potential (TRP) channel, TRPM4, is a critical regulator of smooth muscle membrane potential and arterial tone. Activation of the channel is Ca²⁺-dependent, but prolonged exposures to high global Ca²⁺ causes rapid inactivation under conventional whole-cell patch clamp conditions. Using amphotericin B perforated whole cell patch clamp electrophysiology, which minimally disrupts cytosolic Ca²⁺ dynamics, we recently showed that Ca²⁺ released from inositol trisphosphate receptors (IP₃R) on the sarcoplasmic reticulum (SR) activates TRPM4 channels, producing sustained Transient Inward Cation Currents (TICCs). Thus, Ca²⁺-dependent inactivation of TRPM4 may not be inherent to the channel itself but rather is a result of the recording conditions. We hypothesized that under conventional whole-cell configurations, loss of intrinsic cytosolic Ca²⁺ buffering following cell dialysis contributes to inactivation of TRPM4 channels. With the inclusion of the Ca²⁺ buffers ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA, 10 mM) or bis-ethane-N,N,N',N'-tetraacetic acid (BAPTA, 0.1 mM) in the pipette solution, we mimic endogenous Ca²⁺ buffering and record novel, sustained whole-cell TICC activity from freshly-isolated cerebral artery myocytes. Biophysical properties of TICCs recorded under perforated and whole-cell patch clamp were nearly identical. Furthermore, whole-cell TICC activity was reduced by the selective TRPM4 inhibitor, 9-phenanthrol, and by siRNA-mediated knockdown of TRPM4. When a higher concentration (10 mM) of BAPTA was included in the pipette solution, TICC activity was disrupted, suggesting that TRPM4 channels on the plasma membrane and IP₃R on the SR are closely opposed but not physically coupled, and that endogenous Ca²⁺ buffer proteins play a critical role in maintaining TRPM4 channel activity in native cerebral artery smooth muscle cells.

1. INTRODUCTION

The melastatin (M) Transient Receptor Potential (TRP) channel TRPM4 is a crucial mediator of pressure-induced vascular smooth muscle membrane depolarization and vasoconstriction, and is essential for autoregulation of cerebral blood flow [1, 2]. High levels of intracellular Ca²⁺ (1–10 μM) are required for activation of TRPM4 [3], and under inside-out [1, 4, 5] or traditional whole cell patch configuration [3, 6], Ca²⁺ is introduced in order to activate and record TRPM4 currents. However, under these conditions TRPM4 also undergoes fast, Ca²⁺-dependent inactivation, and currents decay to baseline levels within 3 minutes [1, 4, 7–9]. TRPM4 channel activity can be rescued from inactivation by inhibition

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of phospholipase C (PLC) activity or by inclusion of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂) in the intracellular solution [10, 11]. These findings suggest that high global levels of Ca²⁺ used to record TRPM4 currents in traditional whole cell and inside-out patch clamp configurations activate a Ca²⁺-dependent PLC isoform [12] that inactivates the channel by depleting PIP₂. It is possible that Ca²⁺-dependent inactivation precludes observation of TRPM4 currents during patch clamp experiments, leading to under-estimation of channel activity under native conditions. We recently identified Transient Inward Cation Currents (TICCs) as sustained TRPM4 channel activity in freshly isolated smooth muscle cells [13]. These currents can be continuously recorded for as long as 30 minutes using the whole cell perforated patch clamp configuration [13], a method that restricts cell dialysis and causes minimal disruption of the intracellular environment, allowing global and local Ca²⁺ dynamics to function naturally. Thus, Ca²⁺-dependent inactivation of TRPM4 may not be an inherent property of the channel itself but is a consequence of recording methods. However, the mechanisms underlying this phenomenon are not clear. The goal of the current study is to determine how Ca²⁺-dependent activation of TRPM4 currents is maintained in cerebral artery smooth muscle cells under native conditions.

Subcellular regions with Ca²⁺ levels much greater than the global [Ca²⁺] result from Ca²⁺ influx from the extracellular space [14, 15] or from Ca²⁺ released from intracellular stores [16–19]. The temporal and spatial characteristics of these small Ca²⁺ domains are shaped by the magnitude and duration of the initial Ca²⁺ signal, and by Ca²⁺ removal and intrinsic Ca²⁺ buffering within the cytosol [20]. For example, the plasma membrane Ca²⁺-ATPase (PMCA), the Na⁺/Ca²⁺ exchange system, the sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA), and Ca²⁺ sequestering in the mitochondria and nucleus [20] all actively remove Ca²⁺ from the intracellular space. Additionally, cytosolic proteins, such as calmodulin, calpain, and troponin C, bind Ca²⁺ and limit the availability of free intracellular Ca²⁺ [21]. These Ca²⁺ buffering mechanisms are essential for insuring the transient nature of intracellular Ca²⁺ signaling events by limiting spatial spread and preventing prolonged high cytosolic Ca²⁺ levels.

Localized, transient increases in cytosolic Ca²⁺ can directly activate Ca²⁺-sensitive ion channels [17, 22] in vascular smooth muscle cells. Our laboratory recently reported loss of TRPM4 channel activity following specific inhibition of the SR inositol Ca²⁺ release channel, 1,4,5-trisphosphate receptor (IP₃R) [13], suggesting that subcellular cytosolic Ca²⁺ domains also activate TRPM4 channels in the plasma membrane in native smooth muscle cells. However, the role of endogenous Ca²⁺ buffering in regulation of TRPM4 activity has not been reported. We hypothesized that under conventional whole cell conditions, loss of intrinsic cytosolic Ca²⁺ buffering following cellular dialysis contributes to Ca²⁺-dependent inactivation of TRPM4 channels. To test this hypothesis, we examined the consequences of manipulating intracellular Ca²⁺ buffering on TRPM4 activity in freshly isolated cerebral myocytes. In the absence of cytosolic Ca²⁺ buffering, we found that TICC activity quickly dissipated with the same inactivation kinetics as recombinant TRPM4 activity recorded in the presence of high global Ca²⁺ under conventional whole cell conditions. Using conventional whole cell patch clamp electrophysiology, we recorded sustained, TICC-like currents (whole-cell TICCs) with ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) or 2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) added to the intracellular recording solution at concentrations (10 and 0.1 mM, respectively) that mimic the effects of endogenous Ca²⁺ buffering. These currents were pharmacologically and biophysically identical to TRPM4-dependent currents recorded under perforated patch clamp conditions. Higher concentrations of BAPTA abolished these currents, demonstrating that TRPM4 channels are present on the plasma membrane very near, but not physically coupled to, IP₃Rs. This conclusion is supported by TRPM4 and

IP₃R immunolabeling experiments. This study demonstrates that intact endogenous Ca²⁺ buffering is required to maintain TRPM4 channel activity in smooth muscle cells.

2. MATERIAL AND METHODS

2.1. 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 cold 3-(N-morpholino) propanesulfonic acid (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, 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.

2.2. A7r5 Cell Culture and Transient DNA Transfection

A7r5 cells (ATCC) were cultured in Dulbecco's 1X High Glucose Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 0.5% penicillin-streptomycin (Gibco). Cells were maintained at 37°C with 6% CO₂, media was changed every two to three days, and cells were sub-cultured when confluent using 0.25% trypsin-EDTA (Gibco). A7r5 cells were transiently transfected with a plasmid encoding a TRPM4-GFP fusion protein [23] with the aid of Effectene Transfection Reagent (Qiagen) according to manufacturer's instructions for adherent cells. Media was changed 24 hours after transfection, and cells were cultured for 1–2 days prior to electrophysiology experiments.

2.3. Isolation of Cerebral Artery Smooth Muscle Cells

Vessels were placed on ice in a magnesium-based physiological saline solution (Mg-PSS) containing (in mM) 5 KCl, 140 NaCl, 2 MgCl₂, 10 HEPES, and 10 glucose. Arteries were initially digested in 0.6 mg/mL papain (Worthington) and 1 mg/mL dithioerythritol at 37°C for 17 minutes, followed by a 15 minute incubation at 37°C in 1.0 mg/ml type II collagenase (Worthington). The digested segments were washed three times in ice-cold Mg-PSS solution and incubated on ice for 30 minutes. Following this incubation period, vessels were triturated to liberate smooth muscle cells and stored in ice-cold Mg-PSS for use. Smooth muscle cells were studied within 6 hours following isolation.

2.4. RNAi and Reversible Permeabilization

Small interfering RNAs (siRNA) against TRPM4 were used to down-regulate expression of the channel in isolated cerebral arteries [13]. siRNA molecules purchased from Qiagen (1027280 [AllStars Negative Control], SI02868292 [Rn_Trpm4_1], and SI02868313 [Rn_Trpm4_4]) 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) 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 these procedures, arteries were 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 then used for smooth muscle cell isolation.

2.4. Immunocytochemistry and Membrane Staining

Cells were enzymatically dissociated from cerebral vessels 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 antibodies overnight at 4°C. The following primary antibodies were used as appropriate: goat anti-TRPM4 at 1:50 (sc-27540, Santa Cruz) and rabbit anti-IP3R at 1:50 (AB1622, Chemicon). Cells were subsequently washed and incubated with appropriate fluorescent secondary antibody for 2 hours at room temperature. The following secondary antibodies were used as appropriate; anti-rabbit conjugated to Texas Red, (sc-2780, Santa Cruz) and anti-goat conjugated to FITC, (sc-2704, Santa Cruz). Membrane specific fluorescent staining was performed using 5 µg/mL ER-Tracker™ Green (Invitrogen), 5 µg/mL ER-Tracker™ Red (Invitrogen), or 5 µg/mL CellMask™ Deep Red (Invitrogen). For specific plasma membrane staining, cells were incubated in CellMask™ Deep Red in Mg-PSS for 10 minutes at 37°C. For specific SR membrane staining, cells were incubated in ER-Tracker™ Green or ER-Tracker™ Red in MgPSS for 30 minutes at 37°C. For dual membrane staining, the cells were incubated in ER membrane stain, washed, and incubated in plasma membrane stain. For simultaneous immunocytochemistry and membrane staining, fixed cells were first immunolabeled for the appropriate protein and then incubated in the specific membrane stain. Fluorescent images were obtained using a spinning disk confocal microscope (Andor) and a 100x oil immersion objective. Excitation of Texas Red and FITC was by illumination with the 543-nm line and 488-nm line, respectively. Immunofluorescence was not detected in cells probed with secondary antibody alone. All images were acquired at 1024×1024 pixels and were analyzed using Volocity 6.0 (PerkinElmer).

2.5. General Electrophysiological Recordings

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. Currents were filtered at 1 kHz, digitized at 40 kHz, and stored for subsequent analysis. Clampex and Clampfit versions 10.2 (Molecular Devices) were used for data acquisition and analysis, respectively. All experiments were performed at room temperature (22°C).

2.6. Electrophysiological Recording Solutions

Macroscopic whole cell currents from HEK 293 cells transfected with TRPM4-GFP were recorded in external bathing solution containing (in mM): 146 NaCl, 5 CaCl₂, 10 HEPES, and 10 Glucose (pH = 7.4). The bathing solution was supplemented with the potassium channel blocker TEA (10 mM). The pipette solution contained (in mM): 146 CsCl, 1 MgCl₂, 10 HEPES and 0.1CaCl₂. TICC activity recorded under perforated patch clamp conditions was performed in external bathing solution containing (in mM): 134 NaCl, 6 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 glucose at pH 7.4 (NaOH). The pipette solution contained (in mM): 110 K-aspartate, 1 MgCl₂, 30 KCl, 10 NaCl, 10 HEPES, and 5 µM EGTA at pH 7.2 (NaOH). TICC activity recorded under whole-cell patch clamp conditions was performed in external bathing solution containing (in mM): 140 NaCl, 5 CsCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose at pH of 7.4 (NaOH). The pipette solution contained (in mM): 20 CsCl, 87 K-aspartate, 1 MgCl₂, 5 MgATP, and 10 HEPES (pH 7.2, CsOH). External solution with the K⁺ channel blocker tetraethylammonium (TEA) contained (in mM): 130 NaCl, 10 TEA, 5 CsCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose at pH 7.4. Additional external solutions include, Na⁺-free external solution containing (in mM): 140 N-methyl-D-

glucamine (NMDG), 5 CsCl₂, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 10 glucose at pH 7.4 (HCl); and Ca²⁺ free external solution containing (in mM): 130 NaCl, 5 CsCl₂, 1 MgCl₂, 0.73 CaCl₂, 1 EGTA, 10 HEPES, and 10 glucose at pH 7.4 (NaOH).

2.7. Electrophysiological Recordings from TRPM4-Expressing A7r5 Cells

Conventional whole cell patch clamp studies were conducted using non-transfected A7r5 cells or cells transiently transfected with TRPM4-GFP [23]. Transfected cells were identified by the presence of green fluorescence. Cells were initially held at a membrane potential of 0 mV. Currents were measured during voltage ramps between -100 and +100 mV (time of ramp) repeated every 4 seconds.

2.8. Electrophysiological Recordings from Isolated Smooth Muscle Cells

Freshly isolated cerebral artery smooth muscle cells were placed into a recording chamber (Warner Instruments) and allowed to adhere to glass coverslips for 20 minutes at room temperature. GΩ seals were obtained in Mg-PSS. For perforated whole cell patch clamp recordings, amphotericin B (40 μM) was included in the pipette solution. Perforation was deemed acceptable if series resistance was less than 50 MΩ. For conventional whole cell patch clamp recordings, isolated smooth muscle cells were voltage clamped at a membrane potential (E_m) of -70 mV. In our recording solutions, the calculated reversal potential for total monovalent cations is 7.7 mV and -48.9 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 with unitary amplitude of 1.75 pA (i.e. 1 open state). This value was based on the reported unitary conductance of TRPM4 (25 pS).

Channel open probability (NP_o) was calculated using the following equation:

$$NP_o = \sum_{j=1}^N \frac{(t_j \cdot j)}{T}$$

Where:

t_j = time spent in seconds with $j = 1, 2, \dots, N$ channels open

N = max number of channels observed

T = duration of measurement.

2.9. Calculations and Statistics

All data are means $\pm$ SE. Values of n refer to the number of cells for patch clamp experiments. Data were compared as indicated using paired t-tests or one-way repeated measures analysis of variance (ANOVA). A level of $P \leq 0.05$ was accepted as statistically significant for all experiments. All peak amplitude histograms were constructed using Origin 8.1 (OriginLab Corp.).

3. RESULTS

3.1. Disruption of the Intracellular Environment Leads to Ca²⁺-Dependent TRPM4 Inactivation

Under traditional whole cell patch clamp configurations, TRPM4 currents recorded from recombinant channels expressed in HEK 293 cells, or from native channels in freshly isolated cerebral artery myocytes, exhibit rapid, Ca²⁺-dependent inactivation [3, 6]. To better characterize the time course of TRPM4 inactivation we compared recombinant

macroscopic TRPM4 currents from smooth muscle-derived A7r5 cells and native TRPM4 channel activity in freshly-isolated smooth muscle cells. Currents were obtained from A7r5 cells transfected with TRPM4-GFP [23] using the conventional whole cell configuration with 100 μM Ca^{2+} included in the patch pipette solution. As expected, in the presence of high cytosolic Ca^{2+} levels, cation currents exhibited outward rectification and rapid inactivation (Figure 1A). Peak TRPM4-GFP currents (black arrowhead, $n=5$) decreased by $26 \pm 6\%$ at 8 seconds (grey arrowhead) and $60 \pm 5\%$ at 60 seconds (white arrowhead). These findings are consistent with previous reports of TRPM4 channel activity in HEK 293 cells [3, 6] and demonstrate that recombinant TRPM4 channel currents in A7r5 cells quickly dissipate following prolonged exposure to high global Ca^{2+} .

In native cerebral artery smooth muscle cells patch clamped under perforated patch conditions (leaving the intracellular environment undisturbed), TRPM4 currents manifest as TICC. TICC can be recorded under perforated patch conditions for as long as seal integrity can be maintained. We recorded TICC activity under perforated patch clamp conditions (PP, Figure 1B) and TICC-like currents under conventional whole cell patch clamp (WC, Figure 1C) configuration without the addition of Ca^{2+} to the pipette solution. TICC recorded under perforated patch conditions did not inactivate, while under whole cell conditions we observed time-dependent decay of TICC-like activity. Activity decreased by $16 \pm 10\%$ at 10 seconds and by $64 \pm 6\%$ at 60 seconds following cellular dialysis. The time course of inactivation of whole cell TRPM4 currents recorded from A7r5 cells (Figure 1A) and the decay of TICC activity in native cerebral artery smooth muscle cells (Figure 1B) are identical (Figure 1C), suggesting that inactivation results from a common mechanism. Furthermore, these findings are consistent with the hypothesis that in freshly isolated smooth muscle cells patch clamped under whole cell conditions, cytosolic Ca^{2+} levels are increased following cellular dialysis, disruption of the intracellular environment, and loss of intrinsic Ca^{2+} buffering.

3.2. EGTA Emulates Endogenous Cytosolic Ca^{2+} Buffering

To examine the hypothesis that loss of intracellular Ca^{2+} buffering capacity during cellular dialysis under conventional whole cell conditions results in TRPM4 inactivation, we used established software (CalC version 6.0, [24]) to model the effects of different concentrations of the slow Ca^{2+} buffer EGTA on the linear spread of Ca^{2+} released from a single IP_3R (Ca^{2+} puff). The current strength and on- and off-times of Ca^{2+} puffs were assumed to be 0.2 pA, 17 ms, and 100 ms, respectively, [25, 26] and resting, free global Ca^{2+} was assumed to be 200 nM with a diffusion coefficient of 200 $\mu\text{m}^2\text{s}^{-1}$ [27]. Ca^{2+} movements during three buffering states were modeled: no buffering, endogenous smooth muscle cell buffering, and buffering with EGTA. As a first order approximation, we modeled endogenous cytosolic Ca^{2+} buffering capacity of a cell with the mean of reported values (Table 1) for the most abundant Ca^{2+} binding protein calmodulin (CaM) at a reported concentration of 40 μM in smooth muscle cells [28]. Additionally, our model used the reported values for the diffusion coefficient, Ca^{2+} affinity, dissociation constant, and Ca^{2+} on- and off-rates for CaM and EGTA (Table 1) [27–31] and compared the diffusion of free Ca^{2+} within model cells containing different concentrations of EGTA with those of cells with endogenous buffering.

In cells modeled with endogenous Ca^{2+} buffering, Ca^{2+} levels decreased logarithmically with distance from the Ca^{2+} source (Figure 2). Cells modeled with no Ca^{2+} buffering elicit a nearly linear relationship between $[\text{Ca}^{2+}]$ and the distance traveled from the Ca^{2+} source (Figure 2). Thus, in the absence of Ca^{2+} buffering, Ca^{2+} levels are greater at any given point away from the Ca^{2+} source than what is predicted for native endogenous buffering conditions. The no buffer condition models the conventional whole-cell patch clamp conditions used for the recording shown in Figure 1B, resulting in time-dependent inactivation of TRPM4 currents. Thus, it is possible that TRPM4 inactivation under these

conditions results from loss of endogenous Ca^{2+} buffering. The effects of EGTA were also examined in our modeling experiments. These findings predict that at a concentration of 10 mM the buffering capacity of EGTA has the same effect on the spatial spread of Ca^{2+} , up to 100 nm, as projected for endogenous intracellular Ca^{2+} buffers (Figure 2).

3.3. Sustained TRPM4 Currents Under Conventional Whole Cell Patch Clamp Conditions

To test the hypothesis that depletion of Ca^{2+} buffering contributes to Ca^{2+} -dependent inactivation of TRPM4, we compared channel activity recorded in cerebral arterial myocytes under perforated and conventional whole cell configuration with EGTA (10 mM) included in the pipette. Under perforated patch clamp conditions, TICC_s have a frequency of 20.1 ± 1.6 Hz ($n = 5$) (Figure 3A). To better characterize the channel activity, a peak amplitude histogram was constructed (Figure 3B) and 5 peaks (solid line) were identified at -1.70 , -3.43 , -5.02 , -6.5 , and -8.49 pA and are consistent with the calculated unitary current amplitude for 1 (-1.7 pA), 2 (-3.4 pA), 3 (-5.1 pA), 4 (-6.8 pA), and 5 (-8.5 pA) TRPM4 channel openings as well as our previous findings [13]. These currents were compared to those recorded using the whole cell configuration with 10 mM EGTA added to the pipette solutions. Currents recorded under these conditions (Figure 3C) are reminiscent of TICC_s recorded under perforated patch clamp conditions (Figure 3A). For clarity, we refer to TICC-like currents recorded using the conventional whole cell configuration as “whole-cell TICC_s”. Whole-cell TICC_s have a frequency of 20.1 ± 2.0 Hz ($n = 5$) (Figure 3C). A peak amplitude histogram was constructed (Figure 3D) and 4 peaks (solid line) were identified with the calculated current amplitude for multiple TRPM4 channel openings. The peaks in the histogram occur at -1.7 , -3.3 , -5.1 , and -6.8 pA and are consistent with the calculated unitary current amplitude for TRPM4 and peaks we observed under perforated patch clamp conditions (Figure 3B). The correspondence of event frequency and peak amplitude histograms between the two methods suggests that whole-cell TICC_s are identical to TICC_s recorded under the perforated patch clamp conditions.

Biophysical properties of whole-cell TICC_s were compared with those of TICC_s recorded under perforated patch clamp conditions and TRPM4 currents recorded using conventional whole cell and inside-out configurations. To rule out the influence of K^+ channels, recordings were made in the presence of the broad-spectrum K^+ channel blocker tetraethylammonium (TEA, 10 mM). TEA had no effect on the total open probability (NP_{O}) of current activity ($n=4$), demonstrating that these cation currents are independent of K^+ channel activity (Figure 4A). Whole-cell TICC_s were eliminated when Na^+ in the bathing solution was replaced with the non-permeable cation N-methyl-D-glucamine (NMDG) (Figure 4B), indicating that currents are conducted by Na^+ ions, consistent with the selectivity of TRPM4 for monovalent cations. To examine the voltage dependence of whole-cell TICC_s, currents were recorded in the presence of TEA and at different holding potentials, with the mean current amplitude (I) plotted as a function of membrane potential (V). Similar to the I/V relationships reported for TRPM4 obtained under conventional conditions [3, 4], whole-cell TICC_s displayed modest inward and outward rectification, and reversed at 5–10 mV, consistent with the reversal potential for monovalent cations (7.7 mV) calculated for solutions used in these experiments (Figure 4C).

Pharmacological TRPM4 inhibitors were employed to provide further evidence that whole-cell TICC_s result from TRPM4 channel activity. Flufenamic acid reportedly inhibits TRPM4 ($\text{EC}_{50} = 2.8 \mu\text{M}$) and TRPM5 ($\text{EC}_{50} = 24.5 \mu\text{M}$) currents [32]. Whole-cell TICC activity was diminished following the administration of flufenamic acid (10 μM) (Figure 5A). The selective TRPM4 blocker 9-phenanthrol (30 μM) also attenuated whole-cell TICC activity (Figure 5B). At the concentration used for this study, 9-phenanthrol inhibits TRPM4 and not TRPM5 [33], TRPC3, TRPC6, BK_{Ca} , K_{IR} , K_{V} , and L-type Ca^{2+} channels [2]. These

findings demonstrate that whole-cell TICC activity is suppressed by TRPM4 blockers, suggesting that these currents are carried by TRPM4 channels.

To provide additional evidence for a link between TRPM4 and whole-cell TICC, TRPM4 expression was silenced using siRNA. We previously reported that TRPM4 siRNA specifically downregulates TRPM4 mRNA and protein expression but had no effect on TRPC3 or TRPC6 expression [13]. We found that whole-cell TICC were present in arterial smooth muscle cells isolated from arteries treated with control siRNA, whereas treatment with TRPM4-specific siRNA nearly abolished these currents (Figure 5C). Thus, whole-cell TICC have properties similar to TICC recorded under perforated patch clamp conditions and TRPM4 currents recorded using conventional methods. Furthermore, whole-cell TICC activity is attenuated by TRPM4 blockers and diminished in cells treated with TRPM4 siRNA. These findings indicate that the molecular identity of the channel responsible for whole-cell TICC in native smooth muscle cells is TRPM4. More importantly, our findings indicate that restoration of intracellular Ca^{2+} buffering to endogenous levels is sufficient to prevent Ca^{2+} -dependent inactivation of TRPM4, suggesting that endogenous Ca^{2+} buffers are essential for preventing TRPM4 channel inactivation in smooth muscle cells under native conditions.

3.4. Whole-Cell TICC are Activated by IP_3R -dependent SR Ca^{2+} Release

A prior study from our lab showed that in cerebral artery smooth muscle cells patch clamped in the perforated patch configuration, TRPM4 is activated by Ca^{2+} release from IP_3R [13]. To examine Ca^{2+} -dependent activation of TRPM4 channels under cytosolic EGTA-buffered conditions, we recorded whole-cell TICC activity following the removal of extracellular Ca^{2+} and during pharmacological manipulation of SR Ca^{2+} stores. Removal of extracellular Ca^{2+} did not acutely disrupt whole-cell TICC activity, but extended exposure (3 minutes) to Ca^{2+} -free bathing solution resulted in decreased activity (Figure 6A). Following disruption of SR Ca^{2+} stores by the inhibition of SERCA pumps with cyclopiazonic acid (CPA, 30 μM), we observed a decrease in whole-cell TICC activity (Figure 6B), suggesting that SR Ca^{2+} release is still necessary for activation of TRPM4 channels under these conditions. Consistent with our previous report [13], inhibition of ryanodine receptors by ryanodine (50 μM) had no effect (Figure 6D), while blocking IP_3R with Xestospongin C (1 μM) greatly attenuated whole-cell TICC activity (Figure 6C). To further characterize the role of IP_3R , we employed the membrane permeable IP_3 analog, Bt- IP_3 [34] to activate IP_3R . We observed a dose-dependent increase in TICC activity in the presence of Bt- IP_3 (Figure 6E), which was blocked in the presence of Xestospongin C. These data provide additional evidence for the activation of TRPM4 by Ca^{2+} released from IP_3R . Overall, these findings suggest that inclusion of EGTA (10 mM) in the pipette solution does not disrupt activation of TRPM4 by IP_3R -mediated Ca^{2+} release but does provide sufficient buffering capacity to prevent rapid Ca^{2+} -dependent inactivation of the channel.

3.5. Whole-Cell TICC Activity is Modulated by the Cytosolic Buffering with BAPTA

Our findings show that the activation of plasma membrane TRPM4 channels by Ca^{2+} released from IP_3Rs on the SR membrane is not blocked when EGTA (10 mM) is included in the intracellular solution, suggesting that the two channels are in close proximity. The effectiveness of Ca^{2+} buffers within a given volume is dependent on the Ca^{2+} affinity, binding rates, and mobility of the buffer [29]. EGTA and BAPTA have similar mobility and steady-state binding affinities for Ca^{2+} (Table 1) and differ only in the binding kinetics, i.e. BAPTA binds Ca^{2+} approximately 100 times faster than EGTA [35]. Comparing the buffering effectiveness of BAPTA versus an identical concentration of EGTA has previously been used to estimate the volume buffered within the range of tens of nanometers [35, 36]. To further characterize the Ca^{2+} domains between IP_3Rs and TRPM4, we modeled

the effects of different concentrations of BAPTA on the linear spread of Ca^{2+} released from a single IP_3R , as described above. We used buffering parameters previously reported for the diffusion coefficient, Ca^{2+} affinity, dissociation constant, and Ca^{2+} on- and off-rates for BAPTA (Table 1) [29]. With an intracellular [BAPTA] of 0.1 mM, our model predicts that the spatial spread of Ca^{2+} from a point source will be nearly identical to that of an endogenous buffering environment, whereas when [BAPTA] was increased to 10 mM, our model predicts that the spatial spread of Ca^{2+} is much more restricted (Figure 7).

To test the predictions of our cytosolic Ca^{2+} buffering model we examined Ca^{2+} -dependent activation and inactivation of TRPM4 channel activity. Different concentrations of EGTA and BAPTA were included in the pipette solution, and whole-cell TICC activity was recorded from freshly isolated cerebral artery myocytes. A graphical representation of the hypothesized effects of these buffering conditions on Ca^{2+} levels at the plasma membrane and TRPM4 activity is shown in Figure 8B. We found that when EGTA (10 mM) and BAPTA (0.1 mM), which emulate the effects of endogenous Ca^{2+} buffering (Figure 8B), were included in the pipette solution, whole-cell TICC activity did not differ from that observed when cells were patch clamped under perforated patch conditions (Figure 8A). When EGTA was included in the patch pipette at concentrations lower than 10 mM, whole-cell TICC activity was significantly less than that recorded under perforated patch conditions (Figure 8A), suggesting that buffering under these conditions was insufficient to prevent Ca^{2+} -dependent inactivation of TRPM4 channel activity (Figure 8B). Inclusion of BAPTA at 1 or 10mM diminished channel activity (Figure 8A), suggesting that TRPM4 channels are located very near to, but are not physically coupled with IP_3Rs [37, 38]. Our model predicts that the spatial spread of Ca^{2+} is restricted under these conditions (Figure 8B), suggesting that Ca^{2+} levels within the plasma membrane and SR junctions are insufficient to activate channel activity. These data demonstrate, in agreement with our modeling predictions, that EGTA (10 mM) and BAPTA (0.1 mM) are sufficient to restore endogenous Ca^{2+} buffering lost during cellular dialysis under whole cell patch clamp conditions and can prevent rapid Ca^{2+} -dependent inactivation of the channel.

3.6. TRPM4 and IP_3R are Proximate

Our functional electrophysiological data suggest that the activation of plasma membrane TRPM4 channels by Ca^{2+} released from IP_3Rs on the SR membrane requires that the two channels and membranes be in close proximity. To test this idea and assess the relative localization of TRPM4 and IP_3R in cerebral artery myocytes, isolated cells were immunolabeled with TRPM4 and IP_3R -specific antibodies, and the SR and plasma membranes were fluorescently stained with selective dyes. In cells stained with selective dyes, the SR membrane is plainly visible and is clearly separated from the plasma membrane, demonstrating dye specificity and the close proximity of the two membranes (Figure 9A, insert a). Separation of the SR membrane from the plasma membrane is apparent in intensity plots along a line segment as distinct fluorescence peaks corresponding to the plasma (red) and SR (green) membranes (Figure 9A, insert b). When immunolabeling and membrane staining are combined, we observe that TRPM4 channels are localized to the plasma membrane, and IP_3Rs are expressed in the SR membrane approaching the plasma membrane (Figure 9B–E). Dual immunolabeling experiments confirm the proximal localization of TRPM4 channels and IP_3R at distinct junction sites (Figure 9F, inserts a and b). These findings demonstrate the presence of SR to plasma membrane (SR/PM) junctions where IP_3R channels are present in the SR membrane proximal to TRPM4 channels localized in the plasma membrane.

4. DISCUSSION

The current study demonstrates the importance of endogenous intracellular Ca^{2+} buffering for maintaining TRPM4 activity in cerebral artery myocytes. Our major findings are: (1) restoration of endogenous Ca^{2+} buffering capacity lost when native smooth muscle cells are dialyzed under conventional whole cell conditions prevents time-dependent inactivation of whole-cell TICC; (2) the biophysical properties of whole-cell TICC are consistent with those of TICC recorded using the perforated patch clamp configuration and TRPM4 currents recorded using conventional methods; (3) the pharmacological and molecular profile of currents recorded under whole-cell conditions indicates that these novel currents are conducted by TRPM4 channels; and (4) high concentrations of BAPTA can extinguish the Ca^{2+} signaling required for the activation of TRPM4, suggesting that TRPM4 channels are not physically coupled to IP_3R but are located within 50 nm. These findings demonstrate for the first time that cytosolic Ca^{2+} buffering influences membrane excitability by maintaining and modulating TRPM4 channel activity in native cerebral artery smooth muscle cells.

Many of the biophysical and pharmacological characteristics of the novel whole-cell TICC reported here are identical to those we previously reported for TICC recorded under perforated patch clamp conditions [13], and those described for TRPM4 channels recorded under conventional patch clamp conditions. For example, whole-cell TICC recorded from native cerebral artery myocytes have the same frequency of occurrence (20.1 ± 2.0 Hz, $n = 5$) as TICC recorded under perforated patch clamp conditions (20.1 ± 1.7 Hz, $n = 5$) and are in agreement with the reported unitary conductance of TRPM4 [39–41]. Whole-cell TICC reversed at a membrane potential near 0 mV in symmetrical cationic solutions and substitution of Na^+ with the impermeant cation NMDG in the bathing solution completely blocked channel activity, consistent with TRPM4's selectivity for monovalent cations [3, 4]. We also found that whole-cell TICC activity was significantly less following administration of TRPM4 blockers, including the selective compound 9-phenanthrol [2, 13, 33], and in cells isolated from arteries treated with TRPM4-specific siRNA. Thus, we conclude that TICC recorded under our whole cell conditions represent authentic TRPM4 activity in native cerebral artery myocytes.

The current findings indicate that the I/V relationship of TRPM4 is strongly influenced by the patch clamp configuration. Single channel recordings for TRPM4 obtained from inside-out patches in symmetrical cation solutions have a linear current to voltage relationship [1], while currents obtained using the conventional whole-cell configuration with similar solutions are dually rectifying [3, 4]. In our previous work, we reported that under perforated patch clamp conditions with physiological bathing solution, TICC exhibited modest inward rectification at negative potentials and a small non-rectifying outward current at positive potentials [13]. In the current study, whole-cell TICC activity exhibits dual rectification, similar to that observed under conventional whole cell recording conditions. These discrepancies are likely a result of cellular dialysis. TRPM4 currents are blocked by the endogenous polyamine spermine [42, 43], which is removed by dialysis under whole cell conditions. Polyamine block is responsible for rectification of inwardly-rectifying K^+ (K_{IR}) channels in vascular smooth muscle cells [44, 45], and we propose that this effect accounts for TRPM4 current rectification under native conditions.

TRPM4 activity is necessary for pressure-induced smooth muscle vasoconstriction *in vitro* [1, 2, 4] and autoregulation of cerebral blood flow *in vivo* [46], demonstrating that the channel is a critical mediator of vascular function. However, rapid Ca^{2+} -dependent desensitization of TRPM4 currents when recorded under conventional whole cell and inside-out patch clamp conditions hindered our understanding of the regulatory mechanisms of the

channel under native conditions. TRPM4 activity can be prolonged by inhibition of PLC or by inclusion of the PIP₂ in the intracellular solution [7, 10]. These findings suggest that high intracellular Ca²⁺ levels (10–100 μM) typically used to initiate and record TRPM4 current in conventional whole cell and inside-out configurations activates a Ca²⁺-dependent PLC isoform, leading to TRPM4 inactivation by depleting PIP₂ levels. Additionally, TRPM4 activity does not inactivate when recorded under perforated patch clamp conditions when the cytosolic environment is maintained [13, 47]. In the current study, we investigated the role of endogenous Ca²⁺ buffering in this process and found that when Ca²⁺ buffers are not present in the intracellular solution, whole-cell TICC exhibits time-dependent inactivation with kinetics similar to those observed for TRPM4-expressing cultured cells patch clamped using the whole cell configuration and with 100 μM free Ca²⁺ included in the intracellular solution (Figure 1). We hypothesized that when endogenous free Ca²⁺ buffering mechanisms are disrupted during cell dialysis under conventional whole cell conditions, increases in cytosolic [Ca²⁺] resulting from intrinsic Ca²⁺ influx or release from the SR can also cause Ca²⁺-dependent inactivation of TRPM4. To test this possibility, we modeled the effects of endogenous Ca²⁺ buffers on the spatial spread of Ca²⁺ from a point source. The endogenous and exogenous buffering parameters used for our model are based on values reported in the literature, which have a wide range and may over- or under-estimate buffering capacity. Additionally, the model is based on buffering parameters for whole cell conditions, which may differ from those within specific subcellular nano or microdomains. Lastly, as a first order approximation, we only considered the endogenous buffering parameters of the most abundant Ca²⁺ binding protein, calmodulin, at a total intracellular concentration of 40 μM [28, 48]. However, even with the possible inaccuracies of our model, we were able to accurately predict that addition of 10 mM EGTA or 0.1 mM BAPTA to the intracellular solution restores TRPM4 activity to that observed under native Ca²⁺ buffering conditions in the perforated patch configuration. Thus, these findings suggest that levels of calmodulin, and perhaps other Ca²⁺-binding proteins like calpain and troponin C, together would equal a buffering capacity similar to 10 mM EGTA. Furthermore, if only free Ca²⁺ binding proteins are lost during cellular dialysis under whole cell conditions, our findings suggest that anchored Ca²⁺ homeostasis-important ion channels like PMCA, Na⁺/Ca²⁺ exchangers, and SERCA pumps play a minor role in preventing the Ca²⁺-dependent inactivation of TRPM4 channels under physiological conditions. Additionally, physical interaction of calmodulin with the c-terminus of TRPM4 is essential for Ca²⁺ sensitivity and activation of the channel in physiological range of intracellular Ca²⁺ concentrations [49]. Our ability to rescue channel activity following cell dialysis suggests that calmodulin required for channel activation is constitutively bound to the channel itself and not free within the cytoplasm. This is consistent with work from Nilius *et al.*, reporting five possible Ca²⁺-dependent calmodulin interacting domains on TRPM4, two at the n-terminus and three at the c-terminus [49]. Our findings clearly demonstrate that maintenance of TRPM4 activity is a novel role for free endogenous Ca²⁺ buffering proteins, a property that may regulate critical features of smooth muscle cell excitability and contractility.

Proper maintenance of SR Ca²⁺ stores is necessary for many cellular functions. SERCA pumps actively siphon Ca²⁺ from the cytoplasm back into the SR lumen, refilling internal Ca²⁺ stores. This process of recycling Ca²⁺ is important for maintaining the transmembrane Ca²⁺ gradient of low cytosolic and high SR lumen Ca²⁺ levels. Inhibition of the SERCA pumps by CPA disrupts the Ca²⁺ refilling of the SR, and Ca²⁺ is leaked from internal stores into the cytoplasm, disrupting the Ca²⁺ gradient. In many types of cells, SR Ca²⁺ store depletion activates store operated Ca²⁺ entry channels (SOCs) [56]. However, in the current study, we did not record SOC activity following the depletion of internal Ca²⁺ stores with CPA. This observation is consistent with prior studies that failed to demonstrate SOC activity in contractile cerebral artery smooth muscle cells [34, 57–60]. Consistent with our previous reports using perforated patch clamp [13], whole-cell TICC activity quickly

decreased following the pharmacological inhibition of SERCA pumps by CPA (30 μ M). It seems likely that depletion of SR Ca^{2+} stores translates to a loss of Ca^{2+} release and decreased activation of Ca^{2+} -sensitive ion channels. For example, the large conductance Ca^{2+} -activated potassium channel (BK_{Ca}) is stimulated by Ca^{2+} release from ryanodine receptors located on the SR membrane to produce spontaneous transient outward currents (STOCs) [17, 50]. Store depletion significantly decreases Ca^{2+} dependent activation of BK_{Ca} channels and STOC activity is abolished within 40–120 seconds following CPA administration [51–53]. Thus, the time required for BK_{Ca} inactivation in response to CPA is similar to that observed for TRPM4 inactivation following this treatment in the current study, suggesting that loss of TRPM4 activity results from diminished SR Ca^{2+} -release. Alternatively, it is possible that CPA-induced Ca^{2+} leak from internal stores elevates $[\text{Ca}^{2+}]$ within small cytoplasmic spaces proximal to the plasma membrane to levels that lead to Ca^{2+} -dependent inactivation of TRPM4 channels. Consistent with this possibility, reports using fluorescent Ca^{2+} indicators in smooth muscle cells show an immediate increase in cytosolic Ca^{2+} , presumably due to Ca^{2+} leak from the SR, reaching maximum $[\text{Ca}^{2+}]_i$ within 40 – 300 seconds [54, 55]. This subsequent increase in global calcium may lead to TRPM4 channel inactivation and the relative rapid loss of TICC activity. Regardless of the exact mechanisms, our findings clearly show that intact SR Ca^{2+} stores are required for TRPM4 activity in native cerebral artery myocytes.

Differential modulation of TRPM4 channel activity by the slow Ca^{2+} buffer EGTA and the fast Ca^{2+} buffer BAPTA provides strong evidence that TRPM4 channels are proximal and functionally coupled with IP_3R through Ca^{2+} signaling events. BAPTA and EGTA have similar steady-state Ca^{2+} binding affinities, but significantly different binding rate constants (Table 1) [27]. Based on this divergence, comparison of BAPTA- and EGTA-dependent disruptions of Ca^{2+} -dependent processes can be used to classify localized regions of elevated Ca^{2+} as “ Ca^{2+} nanodomains” or “ Ca^{2+} microdomains” [35, 36, 61]. Ca^{2+} microdomains, with a Ca^{2+} source-to-sensor distance of greater than 50 nm, are defined by Ca^{2+} -dependent responses that are equally disrupted by identical concentrations of BAPTA and EGTA [35, 61]. In contrast, Ca^{2+} nanodomains, with a Ca^{2+} source-to-sensor distance less than 50 nm, are defined by Ca^{2+} -dependent responses that are significantly interfered with by BAPTA, but not by equal concentrations of EGTA [35, 61]. Our findings demonstrating that whole-cell TICC activity can be recorded in the presence of 10 mM EGTA but not in 10 mM BAPTA suggest that IP_3R -mediated activation of TRPM4 channels occur in Ca^{2+} nanodomains, and that TRPM4 channels and IP_3R are less than 50 nm apart. The current estimate of the junctional spaces between plasma membrane caveolae and SR membrane projections is 15 ± 7 nm [62, 63]. Therefore, our findings suggest that IP_3R and TRPM4 channels are localized within these small spaces. Additionally, recent studies have reported physical coupling of IP_3R and the canonical TRP channel, TRPC3, through the caveolae scaffolding protein caveolin-1 in smooth muscle cells [34, 37, 38]. Inhibition of whole-cell TICC by Ca^{2+} chelation with BAPTA provides electrophysiological evidence that plasma membrane TRPM4 channels are not physically coupled to IP_3R located in the SR, but rather are activated by Ca^{2+} release events from IP_3R located less than 50 nm away from TRPM4 channels on the plasma membrane.

In conclusion, our findings suggest that endogenous intracellular Ca^{2+} buffering and SR Ca^{2+} recycling are essential for maintenance of TRPM4 channels activity in cerebral artery smooth muscle cells. Furthermore, we demonstrate that IP_3R -mediated activation of TRPM4 channels occur within Ca^{2+} nanodomains created by SR/plasma membrane junctions. Lastly, the regulation of smooth muscle cytosolic Ca^{2+} buffering and the SR/plasma membrane architecture play integral roles in excitability and arterial function through modulation of TRPM4 channel activity.

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SUMMARY

In smooth muscle cells, regulation of intracellular Ca^{2+} concentration by endogenous buffering is vital for the maintenance of many basic cellular functions. The melastatin transient receptor potential (TRP) channel, TRPM4, is essential for pressure-induced membrane depolarization and vasoconstriction. The channel is activated by Ca^{2+} released from internal stores, but prolonged exposure to high global Ca^{2+} causes the channel to quickly inactivate. Endogenous buffering insures that Ca^{2+} release events are short and localized. However, the role of endogenous Ca^{2+} buffering in regulation of TRPM4 activity has not been reported. Using computer modeling and patch clamp electrophysiology, we examined the effects of manipulating intracellular Ca^{2+} buffering on TRPM4 channel activity. We demonstrate that TRPM4 channels are activated by small Ca^{2+} domains created from Ca^{2+} release sites found in close proximity to the plasma membrane, and that intracellular Ca^{2+} buffering influences smooth muscle excitability and arterial function through modulation of TRPM4 channel activity.

Figure 1. The Kinetics of Ca^{2+} -Dependent TRPM4 Desensitization are Similar in A7r5 and Freshly Isolated Smooth Muscle Cells

A: Current/Voltage (I/V) relationship showing total current at 8 (grey arrowhead) and 60 seconds (white arrowhead) after peak (black arrowhead) in A7r5 cells expressing TRPM4-GFP and non-transfected cells (*). Cells were recorded under conventional whole cell patch clamp conditions (WC) with $100 \mu M$ Ca^{2+} added to the pipette solution. B: Representative trace of Transient Inward Cation Currents (TICC) activity recorded from a freshly isolated cerebral artery smooth muscle cell (SMC) under perforated whole cell patch clamp conditions (PP). Membrane potential = -70 mV. C: Representative trace of TICC-like activity recorded from a freshly isolated cerebral artery smooth muscle cell under

conventional whole cell patch clamp conditions when no Ca^{2+} buffer is present in the intracellular solution. Membrane potential = -70 mV. D: Time course of inactivation of TRPM4 currents (at $+70$ mV) in A7r5 cells ($n=6$), TICC activity recorded under perforated patch clamp conditions ($n=8$), and TICC-like currents recorded under whole-cell conditions in native myocytes ($n=5$) as a fraction of the peak current following cellular dialysis.

Figure 2. Endogenous Ca^{2+} Buffering Can be Mimicked with EGTA

Modeling the effects of endogenous buffering capacity and different concentrations (1 and 10 mM) of the slow Ca^{2+} chelator EGTA on $[\text{Ca}^{2+}]$ as a function of distance from a single IP_3R Ca^{2+} release site.

Figure 3. Sustained Transient Cation Channel Activity in Freshly Isolated Smooth Muscle Cells

A: Representative perforated patch clamp recordings of Transient Inward Cation Currents (TICC) obtained using the amphotericin B perforated patch configuration. Representative of 5 cells. Insert shows expanded time scale. B: Peak amplitude histogram of TICC activity recorded from freshly isolated smooth muscle (0.1 pA bins) under perforated patch clamp conditions. Data are fitted with multiple Gaussian functions. The solid-line peaks match calculated current amplitude for TRPM4 channel openings. The dash-line peaks do not match with calculated current amplitude for TRPM4 channel openings. C: Representative conventional whole cell patch clamp recording of whole-cell TICC activity with 10 mM EGTA included in the pipette solution. D: Peak amplitude histogram of whole-cell TICC activity recorded from freshly isolated smooth muscle (0.1 pA bins) with 10 mM EGTA in the pipette solution. Data are fitted with multiple Gaussian functions, with the solid-line and dash-line peaks matching and not matching, respectively, with the calculated current amplitude for TRPM4 channel openings.

Figure 4. Biophysical Properties of Whole-Cell TICC Activity

A: Representative trace and summary data of whole-cell TICC activity in the presence of the tetraethylammonium (TEA) (10 mM, $n=4$). B: Representative trace and summary data of whole-cell TICC activity with Na^+ replaced with the non-permeable cation N-methyl-D-glucamine (NMDG), $n=9$, $*P \leq 0.05$ vs. Control. C: Sample traces of whole-cell TICC recordings obtained at holding potentials (V_H) from +80 to -70 mV in the presence of TEA. Average current amplitude to voltage relationship (I/V) for cells ($n=3-5$) with symmetrical total cations solutions under whole cell patch clamp conditions.

Figure 5. Whole-Cell TICC Represent TRPM4 Channel Activity

A: Representative trace and summary data of whole-cell TICC activity in the presence of fluflenamic acid (10 μ M, n=3), a blocker of TRPM4 activity. B: Representative traces and summary data of whole-cell TICC activity in the presence of the selective TRPM4 blocker 9-phenanthrol (30 μ M, n=3). *P $\leq$ 0.05 vs. control. C: Representative recordings and summary data of whole-cell TICC activity from a cell isolated from arteries treated with control (top; n=10) and TRPM4 (bottom n=10) siRNA. *P $\leq$ 0.05 vs. control siRNA.

Figure 6. Whole-Cell TICC_s are Activated by IP₃R-dependent SR Ca²⁺ Release

A: Representative trace and summary data of whole-cell TICC activity in the presence of extracellular Ca²⁺ (n=4) and at 45 sec [1] (n=4) and ~3 minutes [2] (n=3) after extracellular Ca²⁺ is removed. B: Representative trace and summary data of whole-cell TICC activity in the presence of the SERCA pump blocker cyclopiazonic acid (CPA, 30 μM, n=3). C: Representative trace and summary data of whole-cell TICC activity in the presence of the specific ryanodine receptor blocker ryanodine (50 μM, n=3). D: Representative trace and summary data of whole-cell TICC activity in the presence of the specific IP₃R blocker Xestospongine C (1 μM, n=3). *P≤0.05 vs. control. E: Representative trace and dose response of the effects of the membrane permeable IP₃ analog, Bt-IP₃ (10 μM) on whole-

cell TICC activity ($EC_{50} = 1.6 \mu M$); $n = 3-5$ per dose. F: Representative trace and summary data of whole-cell TICC activity in the presence of Xestospongin C ($1 \mu M$, $n=3$), and Bt-IP₃ ($10 \mu M$, $n=4$). * $P \leq 0.05$ vs. control.

Figure 7. Endogenous Ca^{2+} Buffering Can be Mimicked with BAPTA

Modeling the effects of endogenous buffering capacity and different concentrations (0.1 and 10 mM) of the fast Ca^{2+} chelator BAPTA on $[\text{Ca}^{2+}]$ as a function of distance from a single IP_3R Ca^{2+} release site.

Figure 8. Effects of Buffer Concentration on Whole-Cell TICC Activity

A: Representative traces of channel activity from recordings under perforated patch clamp (PP) and whole-cell configuration with different concentration of EGTA or BAPTA added to the pipette solution. B: Summary data showing average total open probabilities (NP_o) of whole-cell TICC activity at different concentrations of Ca^{2+} buffers; $n = 5-7$ per group.

* $P \leq 0.05$ vs. PP, # $P \leq 0.05$ vs. 10 mM EGTA, and † $P \leq 0.05$ vs. 0.1 mM BAPTA. C: Schematic representation of the Ca^{2+} spread and hypothetical activation and inactivation of TRPM4 following the Ca^{2+} release from a single IP_3R .

Figure 9. Localization of TRPM4 and IP3R in Freshly Isolated Smooth Muscle Cells

A: Representative images demonstrate the specific fluorescent staining of the sarcoplasmic reticulum (SR, green) and plasma membrane (red) in a freshly isolated smooth muscle cell. Merged image shows little overlap of the SR and plasma membrane. Bar = 5 μ m. (a) Higher magnification of insert illustrating specific staining of SR and plasma membrane. (b) Intensity profile of SR and plasma membrane fluorescence along the line segment. B: Representative images of an isolated smooth muscle cell immunolabeled for TRPM4 (green) and stained for the plasma membrane (red). Bar = 10 μ m. Bottom: High magnification insert demonstrating the co-localization (yellow) of TRPM4 with the plasma membrane. C: Representative images of an isolated smooth muscle cell immunolabeled for TRPM4 (green) and stained for the SR membrane (red). Bar = 10 μ m. Bottom: High magnification insert demonstrating separation of TRPM4 and the SR membrane. D: Representative images of an isolated smooth muscle cell immunolabeled for IP₃R (green) and stained for the plasma membrane (red). Bar = 10 μ m. Bottom: High magnification insert demonstrating separation of IP₃R and the plasma membrane. E: Representative images of an isolated smooth muscle cell immunolabeled for IP₃R (green) and stained for the SR membrane (red) Bar = 10 μ m. Bottom: High magnification insert demonstrating the co-localization of IP₃R with the SR membrane (yellow). F: Representative images of an isolated smooth muscle cell immunolabeled for IP₃R (green) and TRPM4 (red). Bar = 10 μ m. (a and b) High magnification of inserts illustrating specific localization of TRPM4 (red, outside) and IP₃R (green, inside). Notice the distinct points of co-localization (arrowhead).

Table 1**Buffer Parameters**

<u>Parameters</u>	<u>Endogenous</u>	<u>BAPTA</u>	<u>EGTA</u>
Diffusion Coefficient ($\mu\text{m}^2\text{s}^{-1}$)	20	220	200
Ca^{2+} affinity (K_d) (nM)	200	150	150
Dissociation constant β_F/α_F (μM)	10	0.22	0.15
Ca^{2+} On-rate α_F ($\mu\text{M}^{-1}\text{s}^{-1}$)	1	400	5
Ca^{2+} Off-rate β_F (s^{-1})	10	80	0.75

References: [27, 29–31, 64–66]