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Regulation of Cerebral Artery Smooth Muscle Membrane Potential by Ca²⁺-activated Cation Channels

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Abstract

Arterial tone is dependent on the depolarizing and hyperpolarizing currents regulating membrane potential and governing the influx of Ca²⁺ needed for smooth muscle contraction. Several ion channels have been proposed to contribute to membrane depolarization, but the underlying molecular mechanisms are not fully understood. In this review, we will discuss the historical and physiological significance of the Ca²⁺-activated cation channel, TRPM4, in regulating membrane potential of cerebral artery smooth muscle cells. As a member of the recently described transient receptor potential super family of ion channels, TRPM4 possesses the biophysical properties and upstream cellular signaling and regulatory pathways that establish it as a major physiological player in smooth muscle membrane depolarization.

Keywords

Cerebral Artery Smooth Muscle Cells; TRPM4; Membrane Potential

INTRODUCTION

The degree of arterial tone controlling blood flow within the microcirculation is dependent on the contractile state of smooth muscle cells encircling the vessel wall. Movement of ions across the plasma membrane determines resting membrane potential and myocyte contraction. The ion channels responsible for these responses are still being characterized. Depolarization of the plasma membrane triggers smooth muscle contraction by increasing Ca²⁺ influx through dihydropyridine-sensitive voltage-dependent (L-type) Ca²⁺ channels. The subsequent rise in intracellular [Ca²⁺] initiates calmodulin-dependent cross-bridge cycling, leading to myocyte shortening and arterial constriction. Due to the steep voltage sensitivity of L-type Ca²⁺ channels (45), small variations in membrane potential can elicit significant changes in smooth muscle intracellular Ca²⁺ and vessel diameter (7.5 nM mV⁻¹ and 7.5 μm mV⁻¹ respectively, for cerebral arteries) (45). Therefore, ion channels influencing resting membrane potential and governing the opening of L-type Ca²⁺ channels play an essential role in regulating the contractile state of vascular smooth muscle cells. Efflux of K⁺ has been established as the major mechanism leading to membrane hyperpolarization and myocyte relaxation (42, 46, 77, 90), but less is known of the ion channels physiologically responsible for membrane depolarization in vascular smooth muscle cells. In the present review, we will summarize the contribution of Ca²⁺-activated cation channels in establishing the resting membrane potential of cerebral artery myocytes

and discuss the upstream mechanisms of channel regulation that are essential for smooth muscle cell contraction.

SMOOTH MUSCLE MEMBRANE POTENTIAL

Under normal physiological ionic gradients, the K^+ equilibrium potential is approximately -80 mV, yet resting membrane potential is consistently reported in pressurized cerebral arteries to be more depolarized at around -45 mV (66). Therefore, K^+ channel activity cannot completely determine membrane excitability. Channels permeable to cations (Ca^{2+} and Na^+ influx) or anions (Cl^- efflux) are present in smooth muscle cells, but the ions triggering membrane depolarization in smooth muscle cells are still unclear. The predominant divalent cation current in smooth muscle cells are from voltage-dependent L-type Ca^{2+} channels, but these channels do not contribute to membrane excitability. Even though the inhibition of L-type Ca^{2+} channels and blocking Ca^{2+} influx by dihydropyridine compounds causes myocyte relaxation, it does not alter the resting membrane potential (46). Dihydropyridine-insensitive (T-type) Ca^{2+} channels have been reported in some, but not all, types of arterial smooth muscle cells (1, 4, 6, 67, 86, 91); however, due to their kinetic properties, including rapid inactivation and low active voltage range (-65 mV to -45 mV) (4), these channels are thought to have a more transient or specialized role. Thus, Ca^{2+} ions entering through L-type Ca^{2+} channels do not have a major role in regulation of membrane excitability preceding contraction.

Cl^- currents can be recorded from vascular smooth muscle cells (2, 10, 49, 74), but, because the molecular identities of these channels was only recently discovered and due to the lack of specific pharmacology the significance of these currents has been difficult to determine. Some Cl^- channel blockers, like DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid), SITS 4-acetamino-4'-isothiocyanatostilbene-2,2'-disulphonic acid), and IAA-94 are effective in inhibiting pressure-induced vasoconstriction (2), but others, including 9-AC (9-anthracene chloride) and niflumic acid, have no effect (65). It was later shown that many of the Cl^- channel blockers reported to reduce myogenic response also inhibited nonselective cation channels (103) and L-type Ca^{2+} channels (20). The strongest evidence for the involvement of Cl^- channels in smooth muscle cell regulation comes from recent reports examining the role of transmembrane protein 16A (TMEM16A), a Ca^{2+} activated Cl^- channel (10, 14). Hypotonic solution-induced cell swelling activated Cl^- currents in arterial smooth muscle cells and were diminished following inhibition of TMEM16A channels through siRNA-mediated downregulation and channel specific blocking antibodies (10). Ca^{2+} activated Cl^- currents contribute to membrane depolarization and pressure-dependent vasoconstriction of cerebral arteries (10). Additionally, the small molecular blocker of TMEM16A, T16A_{inh}-A01, reduced Ca^{2+} -activated Cl^- currents in isolated myocytes and relaxed pre-constricted mouse thoracic arteries (14). These experiments clearly show that Cl^- conductance can contribute to membrane excitability, but additional experiments are needed to further and more acutely elucidate the physiological contributions of these channels in cerebral arteries.

The Cl^- equilibrium potential (-30 mV) is slightly depolarized compared to resting membrane potential (-40 mV) of arterial myocytes, and due to the relatively small driving force the Cl^- conductance may yield only a small depolarization. In contrast, the ionic gradient for Na^+ is relatively large ($E_{Na} = +60$ mV), and replacement of Na^+ with the non-permeable cation N-methyl-D-glucamine (NMDG) causes myocyte hyperpolarization in rabbit posterior cerebral arteries (38). In addition, swelling- or pressure-induced depolarization of rat cerebral artery myocytes are reported to arise from activation of cation channels (15, 103). These findings strongly suggest that cation influx, in particular, Na^+ ions, is a major contributor to membrane depolarization, but molecular identity of the ion channels responsible was not determined. The presence of conventional voltage-gated Na^+

channels in arterial smooth muscle cells has been a point of controversy. Tetrodotoxin-sensitive Na^+ currents are present in various types of cultured arterial myocytes (11, 56, 76, 78) and portal vein smooth muscle cells (87), but were not detected from cerebral arteries (36) and freshly isolated smooth muscle cells (56, 78). The isolation protocol used to liberate smooth muscle cells from arteries may influence detection of Na^+ channels (8). In one study, when the serine protease elastase was used to enzymatically dissociated cells from mesenteric arteries, Na^+ channel activity was detected, but when the cysteine protease papain was used Na^+ currents were absent (8). Additional experiments examining the effects of cell culturing and enzymes digestion on voltagegated Na^+ channels are required to determine the presence or contributions of this family of ion channels in the resting membrane potential of arterial smooth muscle cells.

Ca^{2+} -activated non-selective cation channels (NSC_{Ca}) have been observed in numerous cell types (97), and until recent years the molecular identity and physiological significance of these channels were unknown. Since their first discovery by Hamill et al (1981), NSC_{Ca} currents have been recorded from numerous cell types, including; fibroblasts, mast cells, sensory neurons, cochlear outer hair cells, renal tubules, cardiac myocytes, and capillary endothelium (for review see (97)). The biophysical properties of NSC_{Ca} channels distinguish them from other channels (Figure 1). NSC_{Ca} channels have a unitary conductance ranging from 18–34 pS, are selective for monovalent cations with a low or undetectable Ca^{2+} permeability ($\text{P}_{\text{Ca}}/\text{P}_{\text{Na}} = 0\text{--}0.14$), and are activated by intracellular Ca^{2+} (97). Channels within this group exhibit a diverse range of Ca^{2+} sensitivities (100 nM to 100 mM) (97). In pancreatic acini, NSC_{Ca} channels required 100 nM intracellular Ca^{2+} for activation, but following exposure to Ca^{2+} currents rapidly inactivate (53). Sturgess et al. (1986) showed that NSC_{Ca} channels are also inhibited by intracellular ATP (92). NSC_{Ca} channels until recently have rarely been associated with a clear physiological function in any of the aforementioned cell types (97). In excitable cells, NSC_{Ca} channels were proposed to be a major contributor to long lasting membrane depolarization (75), but due to lack of pharmacology and molecular tools at the time of this study the channel's molecular nature was not resolved.

TRPM4

The discovery of the mammalian Transient Receptor Potential (TRP) superfamily of cation channels in 1995 provided novel insight into the molecular identity of NSC_{Ca} channels and the ion channels responsible for smooth muscle membrane depolarization. The TRP superfamily consists of 28 gene products forming cation channels that respond to extracellular stimuli and mediate responses to chemical compounds and changes in temperature, osmolarity, light, and pressure (59). TRP channels are subdivided into six subfamilies: TRPA (ankyrin, 1), TRPC (canonical, 1–7), TRPM (melastatin, 1–8), TRPML (mucolipin, 1–3), TRPP (polycystin, 1–3), and TRPV (vanilloid, 1–6). Several Ca^{2+} -permeable members of the TRPC, TRPV, TRPM, TRPP channels are involved in smooth muscle cell intracellular Ca^{2+} homeostasis and dynamics, including receptor-activated and mechanosensitive calcium entry (for review see (22, 23)). Non-selective cation-conducting TRP channels, including TRPC3 and TRPC6, can conduct Na^+ as well as Ca^{2+} and have important roles in smooth muscle membrane excitability in cerebral arteries (81, 102). TRPC3 channels are involved in receptor-mediated membrane depolarization and constriction of arterial smooth muscle (81), whereas TRPC6 channels are intrinsically mechanosensitive (89) and contribute to pressure-dependent membrane depolarization of cerebral arteries (102). However, due to little, or no, ion selectivity of these channels, the downstream contribution of either Na^+ or Ca^{2+} ions is still debated. Additional work is needed to determine whether Na^+ influx via TRPC3 or TRPC6 initiates membrane depolarization, or if Ca^{2+} influx through these channels activate a downstream Ca^{2+}

sensitive mechanism influencing membrane depolarization. One member of the TRPM subfamily of TRP channels, TRPM4, is present in cerebral arteries and due to its ion selectivity it has become a prime candidate for the regulation of membrane depolarization and smooth muscle contraction.

TRPM4 and its closest relative TRPM5 have biophysical properties that differ from other members of the TRP channel superfamily, and are reminiscent of NSC_{Ca} channels (Figure 1). TRPM4 and TRPM5 channels do not conduct Ca²⁺ but are highly selective for monovalent cations (50). As determined by ion substitution experiments in HEK 293 expression systems, TRPM4 is equally permeable for Na⁺ and K⁺, and to a lesser extent, Cs⁺ and Li⁺ (Na⁺ ≈ K⁺ > Cs⁺ > Li⁺) (69). Additionally, replacement of extracellular Na⁺ with the non-permeable cation NMDG⁺ has little effect on outward currents at depolarizing potentials, but completely abolished inward currents at hyperpolarizing potentials, suggesting that Na⁺ is the major ion conducted at physiological membrane potentials (69). The current-voltage (IV) relationship from single-channel recordings of inside-out patches in symmetrical cation solutions exhibits a non-rectifying conductance of ~25 pS (50). Conventional whole-cell macroscopic currents recorded from TRPM4-expressing HEK 293 cells exhibit a large outward and smaller inward rectification (50, 69). TRPM4 is not considered to be classically voltage-dependent. Although shifts in the resting membrane potential can increase or decrease the total open probability of the channel, changes in voltage do not gate it, with channel activation being primarily dependent on intracellular Ca²⁺ concentrations.

TRPM4 has an unusual relationship with Ca²⁺, as Ca²⁺ is required to stimulate the channel but also leads to channel inactivation. TRPM4 channel activity is dependent upon a rise in intracellular Ca²⁺ above global resting levels. Under whole-cell conditions, channels are more sensitive to Ca²⁺ (EC₅₀ = 400 nM) (50) compared with recordings from the inside-out configuration (EC₅₀ = 200 μM) (26, 69). This difference in Ca²⁺ sensitivity suggests the presence of a Ca²⁺-sensitive intracellular channel modulator under whole cell conditions that is lost when membrane patches are excised. In addition to requiring Ca²⁺ for activation, TRPM4 exhibits a rapid Ca²⁺-dependent inactivation, leading to loss of current within two minutes following exposure to high global Ca²⁺ (25, 26, 50, 68, 71). The channel's Ca²⁺ sensitivity may provide insight to regulatory mechanisms, where high Ca²⁺ is required for activation, but prolonged exposure or improper removal of Ca²⁺ leads to loss of channel activity.

Ca²⁺ sensitivity of TRPM4 is regulated by several intracellular pathways. The channel contains a putative phosphatidylinositol 4, 5-bisphosphate (PIP₂) binding site, five calmodulin binding sites, several Protein Kinase C (PKC) phosphorylation sites, and six possible ATP-binding sites (68, 70, 71). PIP₂ modulates channel activity by shifting the IV relationship resulting in an increase in TRPM4 current magnitude at negative holding potentials (68). Blocking of phospholipase C (PLC) activity (68) or including PIP₂ in the recording pipette solution (68, 105) can prevent Ca²⁺-dependent inactivation. Maintaining channel activity by supplementing the cells with a phospholipid source or inhibiting phosphoinositide 3-kinase activity suggests the involvement of Ca²⁺-dependent PLC isoforms (82) in regulating channel activity. With five possible binding sites, two on the N-terminus and three on the C-terminus, the Ca²⁺ binding protein calmodulin can directly influence the Ca²⁺ sensitivity of TRPM4 (71). Overexpression of a truncated C-terminus mutant for TRPM4 or expression with dominant negative calmodulin mutants drastically decreases the amplitude of Ca²⁺-activated TRPM4 currents (71). TRPM4 also contains multiple possible interaction sites for PKC, suggesting phosphorylation-dependent modulation of the channel. The PKC activating compound phorbol 12-myristate 13- acetate (PMA) increased the incidence of occurrence and current density of TRPM4 channels by

enhancing the Ca^{2+} sensitivity of the channel (71). Intracellular ATP inhibits TRPM4 currents with a half maximal inhibitory concentration (IC_{50}) around 2–19 μM (70). TRPM4 is also modulated by adenine nucleotides with the highest sensitivity to ADP and least to adenosine ($\text{ADP} > \text{ATP} > \text{AMP} \gg \text{adenosine}$) (72). The nucleotide triphosphates GTP, UTP, and CTP have no effect at concentrations lower than 1 mM (72). In addition to the inhibition of TRPM4 by free ATP, higher concentrations (in the millimolar range) can prevent Ca^{2+} -dependent inactivation of the channel (71). Mutations of putative ATP binding sites on TRPM4 abolish this effect, indicating that prevention of Ca^{2+} -dependent inactivation is associated with direct binding of ATP to the channel (71). In a similar manner, heat shifts the voltage-dependent activation curve for TRPM4 ($Q_{10} = -5.6 \pm 0.5 \text{ mV } ^\circ\text{C}^{-1}$) (95), a phenomenon also observed in other thermosensitive TRP channels (17, 24). Modulation of the monovalent cation conductance through TRPM4 by voltage, adenine nucleotides, phospholipids, and heat and dependence on intracellular Ca^{2+} for activation are consistent with NSC_{Ca} channels previously described in many cell types, suggesting the identity of these channels as TRPM4.

The pharmacology for TRPM4 is limited. The most commonly used TRPM4 blockers include flufenamic acid, spermine, Gd^{3+} , and sulfonylurea glibenclamide (16, 69, 72, 99) are poorly selective. Recently, a hydroxytricyclic derivative, 9-phenanthrol, has been shown to inhibit TRPM4 current with no effect on its closest relative TRPM5 (33). Additionally, at a concentration that nearly abolished TRPM4 channel activity (33), 9-phenanthrol did not alter TRPC3, TRPC6, voltage-gated K^+ Channels (K_v), large-conductance Ca^{2+} -activated K^+ channels (BK_{Ca}), inwardly rectifying K^+ channels (K_{IR}), or L-type Ca^{2+} channel currents (32). These data suggest that 9-phenanthrol may be a specific inhibitor of TRPM4. Pharmacological activators for TRPM4 include decavanadate (70) and the pyrazole derivative BTP2 (N-[4–3, 5-bis(trifluoromethyl)pyrazol-1-yl]-4-methyl-1,2,3-thiadiazole-5-carboxamide) (94), however, these agents also lack specificity. Decavanadate interferes with ATP-dependent inhibition of TRPM4 channel activity but can also interact with other ion channels, including IP_3R and purinoreceptors (P2X) (57). BTP2 increases TRPM4 channel activity and reduces Ca^{2+} -dependent inactivation, but can also inhibit TRPC3 and TRPC5 channels (37). 9-phenanthrol is currently the most useful pharmacological tool for unmasking the functional significance of TRPM4.

With the advancements of molecular techniques, previous observations of NSC_{Ca} currents now can be identified as TRPM4 channels. Similar to the broad distribution reported for NSC_{Ca} channels, TRPM4 channels have been detected at high levels in the heart, pancreas, placenta, and prostate (for review see (35)), and with lower levels of TRPM4 detected in kidney, skeletal muscle, liver, intestines, thymus, and spleen (35). The functional significance and expression of TRPM4 have been extensively characterized in four cell types. TRPM4 regulates cytokine secretion in lymphocytes and insulin secretion in pancreatic β -cells (for review see (88)). TRPM4 plays a role in controlling respiratory rhythmogenesis in neurons of the pre-Bötzinger complex (for review see (34)). Gain of function mutations within the TRPM4 gene in Purkinje fibers suggests it plays a role in isolated cardiac conduction disease in human heart (for review see (34)). Lastly, and as the focus of the current review, TRPM4 is essential for smooth muscle membrane depolarization and vasoconstriction in cerebral arteries.

TRPM4 IN CEREBRAL ARTERY SMOOTH MUSCLE CELLS

TRPM4 channels are present and functionally critical for the regulation of cerebral artery tone (26). Inside-out patch clamp recordings from freshly isolated cerebral artery smooth muscle cells in symmetrical cation solutions demonstrate a monovalent cation-selective and Ca^{2+} -dependent ($\text{EC}_{50} = 200 \mu\text{M}$) current with unitary conductance suggestive for TRPM4

(~24 pS) (26). Single channel activity exhibited a linear voltage dependency, with inward currents at negative potentials, reversal at 0 mV, and outward currents at positive potentials (26). Ion substitution experiments revealed the channel to be selective for Na⁺ ions (26), and suppression of TRPM4 expression via antisense technology decreased channel activity, establishing the molecular identity of these currents as TRPM4 (26). Downregulation of TRPM4 impaired smooth muscle depolarization and myogenic constriction of isolated cerebral arteries (26). In addition, *in vivo* knockdown of TRPM4 expression impaired autoregulation of cerebral blood flow in living animals (80). Following suppression of the channel by infusion of antisense oligodeoxynucleotides targeting TRPM4 into rat cerebral spinal fluid, blood flow was recorded to be higher at resting and elevated mean arterial pressures (80). These were the first findings to demonstrate the functional significance of TRPM4 as a major contributor to cerebral blood flow regulation. The channel blocker 9-phenanthrol was employed to further and more acutely examine the role of TRPM4 in cerebral arteries. Blocking of TRPM4 currents with 9-phenanthrol reversibly hyperpolarizes smooth muscle cells leading to dilation of cerebral arteries (32). Consistent with these findings, 9-phenanthrol was used to pharmacologically identify TRPM4 as the major contributor to resting membrane potential in human and monkey colonic smooth muscle cells (21). These findings demonstrate the functional significance of TRPM4 channels in the regulation of smooth muscle cell membrane potential.

In contrast with the findings discussed above, recent studies using TRPM4 knockout (TRPM4^{-/-}) mice reported no difference in myogenic tone in arteries from mouse hind limbs (54). One explanation is that the relationship between membrane potential and myogenic tone can vary between arteries of different vascular beds. In cerebral arteries, vascular tone as a function of membrane potential exhibits a near linear relationship (45), while in skeletal muscle arterioles this relationship is reported to be sigmoidal or non-linear (47). This difference between arteries of two vascular beds may suggest variations in the mechanism that regulate arterial tone. Alternatively, compensatory mechanisms may exist within knockout mouse systems. For example, TRPC6 deficient (TRPC6^{-/-}) mice exhibited a higher mean arterial blood pressure compared to controls, but this was associated with an upregulation of TRPC3 channels (19). Changes in expression levels of other TRP channels in TRPM4^{-/-} mice were not reported, so possible compensation by other channels cannot be ruled out. Further experiments are warranted to resolve these issues.

Elucidation of cellular pathways that regulate TRPM4 channels in cerebral artery smooth muscle cells have been hampered by the channel's intrinsic and rapid Ca²⁺-dependent inactivation (26, 31, 50). Micromolar concentrations of Ca²⁺ traditionally have been included in the recording pipette to activate TRPM4 channels following cellular dialysis under conventional whole-cell recording conditions. Within a few minutes following exposure to high Ca²⁺, TRPM4 activity rapidly inactivates (50, 69). Block of PLC activity or including PIP₂ in the recording pipette solution can rescue the channel from inactivation (68, 105). These observations suggest that loss of channel activity could be an artifact of conventional whole cell recording conditions. High intracellular concentrations of Ca²⁺ maintained following dialysis may over-stimulate Ca²⁺-dependent PLC isoforms leading to depletion of local PIP₂ stores. To test this possibility, an amphotericin B perforated patch-clamp configuration was employed in which whole cell currents were recorded with minimal disruption of subcellular Ca²⁺ signaling pathways (39). This patch-clamp method allowed novel sustained inward cation currents to be recorded from native cerebral artery smooth muscle cells for as long as seal viability could be maintained (> 30 min). These currents are referred to as "transient inward cation currents" (TICCs) (30). TICCs have an apparent single channel conductance of ~25 pS, reverse near 0 mV in symmetrical cation solutions, and channel activity is lost following substitution of extracellular Na⁺ with impermeant cation NMDG (30). TICC activity was inhibited by TRPM4-blockers

flufenamic acid and 9-phenanthrol, and is attenuated by siRNA-mediated downregulation of TRPM4 expression in cerebral artery myocytes (30). These findings demonstrate that the molecular identity of the channel responsible for TICC activity is TRPM4 (Figure 1). Recording of sustained TRPM4 currents under the perforated patch-clamp technique provides a novel method for further characterization of channel activity under near-physiological conditions in smooth muscle cells.

The ability to record sustained channel activity from native cells without major disruption of intracellular Ca^{2+} dynamics has allowed TRPM4 channel regulation to be characterized. TRPM4 requires $[\text{Ca}^{2+}]$ above resting cytosolic levels for activation. In freshly isolated cerebral artery smooth muscle cells, removal of extracellular Ca^{2+} did not acutely affect TRPM4 channel activity (30, 31). Blocking of the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) rapidly inhibited TICC activity, suggesting that Ca^{2+} released from internal stores activated the channel (30, 31). Inhibition of inositol trisphosphate receptors (IP_3R), but not ryanodine receptors, diminished TICC activity, suggesting that TRPM4 channels are activated by Ca^{2+} released from the sarcoplasmic reticulum (SR) via IP_3R (30, 31). In agreement with these findings, the membrane permeable inositol trisphosphate (IP_3) analog Bt- IP_3 increased TICC activity (31). This effect was lost when cells were pretreated with the IP_3R blocker xestospongine C (31), providing further evidence to the involvement of IP_3R in the activation of TRPM4 channels. The coupling between TRPM4 and IP_3R was also reported for the neonatal mouse preBötzinger complex, where the channels contribute to neuronal control of respiratory rhythms (13). These data suggest that a Ca^{2+} release event culminating from IP_3R within the SR membrane activates TRPM4 channels located in the plasma membrane.

Changes in cytosolic Ca^{2+} dynamics influence IP_3R -dependent activation of TRPM4. Using immunocytochemistry and membrane specific staining, IP_3Rs in the SR membrane and TRPM4 channels located on the plasma membrane were found to be proximal to each other (31). These findings were complemented by conventional whole cell patch clamp experiments where the Ca^{2+} buffers ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) or bis-ethane-N,N,N',N'-tetraacetic acid (BAPTA) were included in the pipette solution and dialyzed into the cell under the whole cell configuration. BAPTA and EGTA have identical steady-state kinetics and Ca^{2+} binding affinities, but significantly differ in their binding rate constants (3). BAPTA can bind free Ca^{2+} at a rate that is two orders of magnitude faster than EGTA (3). IP_3R -mediated activation of TRPM4 was observed when 10 mM EGTA but not 10 mM BAPTA (31) was included in the recording pipette. This difference in channel activity by equal concentrations of BAPTA or EGTA suggests that the IP_3R -mediated activation of TRPM4 occurs within spatially restricted domains. 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 (62). 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 (62). Therefore, the difference in channel activity in the presence of equal concentrations of BAPTA vs. EGTA, suggests that TRPM4 channels and IP_3Rs are located no more than 50 nm apart within Ca^{2+} nanodomains (Figure 2). Additionally, loss of TRPM4 channel activity following Ca^{2+} chelation with BAPTA provides electrophysiological evidence that the IP_3R -mediated activation of TRPM4 channels occurs via a Ca^{2+} release event and that these signaling events occur within nanodomains where the SR and plasma membranes are in close proximity.

Ca^{2+} is an important second messenger for multiple overlapping intracellular signaling pathways. TRPM4 channels are activated by micromolar levels of cytosolic Ca^{2+} , and recent

work suggests that localized and transient release of Ca^{2+} from intracellular stores through IP_3R activates TRPM4 channels. IP_3Rs are also influenced by intracellular Ca^{2+} , but at much lower concentrations and exhibit a biphasic sensitivity where Ca^{2+} activates and inactivates the channel within a nanomolar range (300 nM) (40, 41). Ca^{2+} can excite or inhibit IP_3Rs directly at several distinct Ca^{2+} binding sites within the channel (for review see (63)) or indirectly by interfering with binding of IP_3 to the channel (5). Additionally, binding of IP_3 to IP_3R increases the Ca^{2+} sensitivity of channel (52) and the interplay between intracellular Ca^{2+} and IP_3 binding to IP_3R contributes to the versatility and hierarchical recruitment of observed Ca^{2+} signaling events. In response to increased levels of cytosolic IP_3 , individual IP_3Rs are activated giving rise to unitary events (Ca^{2+} blips) (9, 93), then clusters of IP_3Rs open together (Ca^{2+} puff) (79, 93, 96), and ultimately these events propagate from one cluster to another (Ca^{2+} waves) (61). The particular IP_3R -mediated Ca^{2+} release event physiologically important for activation of TRPM4 in cerebral artery myocytes is still not known, but recordings of sustained TRPM4 channel activity from smooth muscle cells suggest that PLC-dependent generation of IP_3 and intracellular Ca^{2+} required for IP_3R activation are continuously present. Separation of the IP_3R -dependent Ca^{2+} release event from the Ca^{2+} signaling pathways initially regulating IP_3R function can be achieved through the difference of Ca^{2+} sensitivity between TRPM4 and IP_3R . Low levels of intracellular Ca^{2+} can modulate communication between IP_3Rs without affecting TRPM4 channel activity and smooth muscle membrane depolarization. Additionally, signaling events initiated distal to SR/plasma membrane junctions can propagate between neighboring IP_3Rs towards nanodomains where IP_3R -dependent activation of TRPM4 channels occurs. These two tiers of Ca^{2+} sensitivity, with low nanomolar concentrations of Ca^{2+} stimulating IP_3Rs and higher micromolar concentrations activating TRPM4 channels, provides a separation and prevention of crosstalk between Ca^{2+} signaling events. This arrangement allows for multiple localized Ca^{2+} events to occur concurrently without stimulating TRPM4-mediated membrane depolarization and myogenic constriction.

Generation of myogenic tone requires PLC activity (73), but the mechanism behind PLC-dependent membrane depolarization and myocyte contraction is still not clear. Recent findings showing that TRPM4-mediated membrane depolarization requires Ca^{2+} released from IP_3Rs in the SR which may provide insight to PLC-dependent vasoconstriction. Production of the endogenous IP_3R activator IP_3 occurs through the hydrolysis of PIP_2 by PLC, and, in arterial smooth muscle cells, the intracellular concentration of IP_3 (64) and PLC activity (73) are positively correlated with changes in arterial intraluminal pressure. Additionally, pharmacological inhibition of PLC results in smooth muscle membrane hyperpolarization and disruption of sustained pressure-induced constriction of arteries (44, 73). Therefore, tonic PLC activity could generate intracellular IP_3 levels required for IP_3R activation and TRPM4-mediated smooth muscle membrane depolarization and maintenance of cerebral vascular tone. Multiple isoforms belonging to three families (β , γ , and δ) of PLC have been detected in vascular smooth muscle cells (51). Extracellular agonists bind to G protein-coupled receptors located on the plasma membrane and may activate $\text{PLC}\beta$ isoforms leading to receptor-mediated vasoconstriction. Early reports observed a considerable suppression of pressure- and agonist induced tone following the inhibition of tyrosine kinase, an upstream activator of $\text{PLC}\gamma$ isoforms (18, 73). The direct influence of $\text{PLC}\beta$ or $\text{PLC}\gamma$ isoforms on TRPM4 channel activity has not been shown, but generation of IP_3 and activation of sustained IP_3R -mediated TRPM4 currents could provide the depolarizing stimulus for PLC-dependent contractions. In addition, PLC has several upstream and downstream effects, expanding the possible interactions and molecular pathways that may influence TRPM4-mediated smooth muscle membrane depolarization and vasoconstriction.

PKC plays an important role in regulating basal arterial tone in cerebral arteries (25, 43, 84). Stimulation of PKC activity increases the Ca^{2+} -sensitive activation of TRPM4 currents (71),

and in cerebral artery myocytes PMA elicits TRPM4-dependent membrane depolarization and arterial constriction (25). To further elucidate the mechanism of PKC-dependent increases in cation current, cell surface biotinylation and TIRF microscopy were used to examine the expression of TRPM4 protein in the plasma membrane (12). Following PKC stimulation, TRPM4 channels rapidly translocated (~5 minutes) to the plasma membrane in A7r5 cells, primary cerebral artery myocytes, and intact arteries (12). This effect was lost following selective inhibition of PKC δ , but not following blocking of PKC α and PKC β (12). RNAi-mediated downregulation or pharmacological inhibition of PKC δ caused TRPM4 channels to translocate from the plasma membrane into the cytosol (29), suggesting that tonic PKC δ activity is required to maintain surface expression of TRPM4. Channel activity was inhibited following the administration of the PKC δ blocker rottlerin (29), and suppression of PKC δ expression hyperpolarized smooth muscle cells and impaired vessel constriction in response to both PMA and increases in intraluminal pressure in intact cerebral arteries (12). These findings provide a novel mechanism for the regulation of smooth muscle excitability through the dynamic trafficking of TRPM4 channels to and from the plasma membrane, and indicate that PKC-dependent increases in Ca²⁺ sensitivity of the channel may result from elevated levels of TRPM4 protein at the cell surface.

TRPM4 IN HYPERTENSION

Smooth muscle hyperexcitability leading to increased peripheral resistance within the microvasculature contributes to the development of essential hypertension (28). In vascular smooth muscle cells, the regulation and permeability of K⁺ and Ca²⁺ are altered during this disease, but involvement of TRPM4 channel activity has not been reported. In many hypertensive human and animal models, elevated vascular tone has been reported and is associated with a more depolarized resting membrane potential and disrupted Ca²⁺ handling (101). L-type Ca²⁺ current density in vascular smooth muscle cells from spontaneous hypertensive rats (SHR) is increased compared to their normotensive Wistar-Kyoto (WKY) controls (86, 101), leading to an increase in basal intracellular Ca²⁺ concentrations. In addition, BK_{Ca} channel activity is increased under hypertensive conditions (27, 85). The open probability of BK_{Ca} channels increases at more positive potentials, suggesting that increased channel activity during hypertension results from amplified depolarizing currents. TRPM4 is an intriguing potential contributor to this pathophysiological condition, as several of the molecular pathways that regulate its activity have also been shown to be altered under these conditions. For example, PLC activity and the generation of IP₃ increase in response to intraluminal pressures (64), and basal intracellular IP₃ concentrations are elevated in genetically hypertensive rats (7, 58, 83, 98, 100, 104). Although no difference in IP₃R expression or IP₃R-mediated Ca²⁺ release has been reported, an increase in intracellular IP₃ level together with elevated cytosolic Ca²⁺ levels could increase tonic IP₃R-mediated TRPM4 channel activity and membrane depolarization. PKC activity has also been linked with pressure-dependent vasoconstriction and PMA-mediated activation of PKC enhances myogenic activity (60). Inhibition of PKC in cerebral arteries has a significantly greater effect on myogenic tone in cerebral arteries isolated from SHR rats compared to WKY controls (43), suggesting an increase in PKC activity during hypertension. Surface expression of TRPM4 channels is dynamically regulated by PKC (12) and the observed increase in PKC activity in SHR rats can lead to more channels localized in the plasma membrane. Interestingly, a missense mutation of TRPM4 impaired the endocytosis of channels leading to elevated channel density at the plasma membrane, contributing to the development of progressive familial heart block type 1 in humans (48). Mutations with similar effects on TRPM4 distribution in vascular smooth muscle cells could lead to higher tonic TRPM4 channel activity, increased membrane depolarization, and ultimately contribute to smooth muscle hyperexcitability, thereby contributing to hypertension.

Additional studies investigating the involvement of TRPM4 in hypertension are needed to test this hypothesis.

CONCLUSION

The discovery of TRPM4 has unmasked the molecular identity of one type of NSC_{Ca} channel that was first described in 1981. TRPM4 processes biophysical properties and broad distribution reminiscent of NSC_{Ca} channels. In addition, TRPM4 channels are selective for monovalent cations, activated by, but impermeable to, Ca²⁺, and are regulated by components of the PLC and PKC pathways (Figure 3). Advancements in the ability to record TRPM4 channel activity in native smooth muscle cells has allowed for further insight to the signaling complexes that regulate the channel. It is apparent that TRPM4 is an essential ion channel for cerebral artery myocyte depolarization leading to contraction, but additional studies are needed to fully elucidate the possible complementary and integrative role it may play with other ion channels proposed to influence smooth muscle depolarization. More importantly, the pathophysiological role of TRPM4 in vascular dysfunction associated with hypertension, stroke, and other cardiovascular diseases warrants further investigation.

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Figure 1. Comparison of Nonselective Ca^{2+} -activated cation channels (NSC_{Ca}), recombinant human TRPM4 channels expressed in HEK 293 cells, and native TRPM4 channels in vascular smooth muscle cells (VSMC)

ATP, adenosine triphosphate; PM, plasma membrane; SR, sarcoplasmic reticulum; 9-phen, 9-phenanthrol; PKC, Protein Kinase C; and PIP_2 , phosphatidylinositol 4, 5-bisphosphate.

Figure 2. Simulation of TRPM4 by Ca²⁺ Nanodomain Created by Ca²⁺ released from the Sarcoplasmic Reticulum via IP₃R

In freshly isolated smooth muscles, TRPM4 activation requires Ca²⁺ released from the SR through proximal IP₃R. The Ca²⁺ concentration profile comparing the buffering capacity of the slow and fast Ca²⁺ chelators EGTA and BAPTA on [Ca²⁺]_i as a function of distance from a single IP₃R Ca²⁺ release site. Simulation data using CalC software v. 6.0 (55) was re-plotted from Gonzales and Earley (31).

Figure 3. Regulation of TRPM4 Activity in Contractile Cerebral Artery Smooth Muscle Cells
 PM, plasma membrane; SR, sarcoplasmic reticulum; PKCδ, protein kinase C; PIP₂, phosphatidylinositol 4, 5-bisphosphate; PLC, phospholipase C; IP₃, inositol triphosphate; IP₃R, inositol triphosphate receptor; V_M, membrane potential; and VGCC, voltage gated calcium channels.