Bradykinin regulates calpain and proinflammatory signaling through TRPM7-sensitive pathways in vascular smooth muscle cells

Alvaro Yogi,1 Glauzia E. Callera,1 Rita Tostes,2 and Rhian M. Touyz1
1Kidney Research Centre, Ottawa Health Research Institute, University of Ottawa, Ontario, Canada, 2Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil

Submitted 15 July 2008; accepted in final form 10 September 2008

Bradykinin regulates calpain and proinflammatory signaling through TRPM7-sensitive pathways in vascular smooth muscle cells. Am J Physiol Regul Integr Comp Physiol 296: R201–R207, 2009. First published September 17, 2008; doi:10.1152/ajpregu.90602.2008.—Transient receptor potential melastatin-7 (TRPM7) channels have recently been identified to be regulated by vasoactive agents acting through G protein-coupled receptors in vascular smooth muscle cells (VSMC). However, downstream targets and functional responses remain unclear. We investigated the subcellular localization of TRPM7 in VSMCs and questioned the role of TRPM7 in proinflammatory signaling by bradykinin. VSMCs from Wistar-Kyoto rats were studied. Cell fractionation by sucrose gradient and differential centrifugation demonstrated that in bradykinin-stimulated cells, TRPM7 localized in fractions corresponding to caveolae. Immunofluorescence confocal microscopy revealed that TRPM7 distributes along the cell membrane, that it has a reticular-type intracellular distribution, and that it colocalizes with flotillin-2, a marker of lipid rafts. Bradykinin increased expression of calpain, a TRPM7 target, and stimulated its cytosol/membrane translocation, an effect blocked by 2-APB (TRPM7 inhibitor) and U-73122 (phospholipase C inhibitor), but not by chelerythrine (PKC inhibitor). Expression of proinflammatory mediators VCAM-1 and cyclooxygenase-2 (COX-2) was time-dependently increased by bradykinin. This effect was blocked by Hoe-140 (B2 receptor blocker) and 2-APB. Our data demonstrate that in bradykinin-stimulated VSMCs: 1) TRPM7 is upregulated, 2) TRPM7 associates with cholesterol-rich microdomains, and 3) calpain and proinflammatory mediators VCAM-1 and COX2 are regulated, in part, via TRPM7- and phospholipase C-dependent pathways through B2 receptors. These findings identify a novel signaling pathway for bradykinin, which involves TRPM7. Such phenomena may play a role in bradykinin/B2 receptor-mediated inflammatory responses in vascular cells.

TRP channels; inflammation; signal transduction; bradykinin receptors; vascular cells

The mammalian transient receptor potential (TRP) superfamily of cation channels contains over 20 genes, which are grouped into three major subfamilies according to their sequence homology: 1) vanilloid (TRPV), 2) canonical (TRPC), and 3) melastatin (TRPM) (6, 30). Most cell types, including vascular cells, express several TRPs. In vascular smooth muscle cells (VSMC), the most highly expressed TRPs are TRPC1, TRPC3, TRPC4, TRPC6, TRPM4, TRPV2, and TRPV4 (21, 44), involved primarily in the regulation of transmembrane Ca2+ transport and in the modulation of vasomotor tone. We recently demonstrated that TRPM7, a novel Mg2+ transporter, is also expressed in VSMCs (19, 26, 38, 43, 44).

Little is known about the functional significance of TRPM7 in VSMCs. We reported that TRPM7 is an important regulator of cellular Mg2+ homeostasis and is critically associated with cell viability, proliferation, and contraction/dilation (19, 38, 42, 43). Others have demonstrated that TRPM7 influences vascular cell adhesion (7) and actomyosin contractility (8) and that it may be a mechanotransducer (17, 28), processes important in maintaining vascular integrity. In spontaneously hypertensive rats and in aldosterone-infused mice, TRPM7 is downregulated, and this is associated with altered VSMC Mg2+ transport, decreased intracellular free Mg2+ concentrations ([Mg2+]i) and impaired vasodilation (37, 42, 43).

TRPM7, similar to other TRPs is activated by numerous stimuli, including pressure, shear stress, osmolarity, and intracellular cations, as well as by ligand-receptor interactions, indicating the physiological importance of TRPM7 in cellular function (2, 10, 29). Of relevance to the vascular system, we showed that TRPM7 is regulated by intracellular Mg2+, aldosterone, and ANG II. TRPM7 is also activated by bradykinin, as demonstrated in a neuronal cell line by Langeslag et al. (22).

The biological consequences of bradykinin-stimulated TRPM7 in VSMCs are unclear, especially since little is known about downstream targets of TRPM7. To date, at least three TRPM7-sensitive substrates have been identified including annexin-1, myosin heavy chain, and calpain (9, 11, 12, 40). Annexin-1 has been implicated in many cellular functions (5, 24), and in VSMCs it may regulate cell cycle progression by inhibiting cyclin D1 and activating ERK1/2 (1). Myosin heavy chain is involved in VSMC cytoskeletal organization and regulation of contraction/dilation (37, 47). Calpain is a calcium-dependent thiol protease that has been implicated in basic cellular events including cell growth (31). It also plays a role in pathological processes associated with fibrosis and inflammation (23). Calpain inhibition reduces migration rates, reduces cell invasiveness, and inhibits expression of proinflammatory mediators, VCAM-1, ICAM-1, and cyclooxygenase-2 (COX-2) (39). Whether these effects are modulated by TRPM7 in VSMCs and...
whether they are linked to bradykinin, which has been implicated in COX-2 induction and inflammation in the vasculature, remain unknown.

The aims of the present study were to evaluate whether bradykinin regulates TRPM7 and its downstream target calpain and to assess whether this is associated with proinflammatory signaling responses in VSMCs. Moreover, we questioned whether these effects involve phospholipase C (PLC) and protein kinase C (PKC), previously shown to be implicated in TRPM7 activation (11, 22, 29).

Fig. 1. Bradykinin regulates transient receptor potential melastatin-7 (TRPM7) in vascular smooth muscle cells (VSMCs). A: effects of bradykinin (10⁻⁶ mol/l, 0–12 h) on TRPM7 expression in VSMCs. Top: representative immunoblot. Bottom: corresponding bar graphs. Results are expressed as the TRPM7 expression normalized for the housekeeping protein β-actin. Control is taken as 100% and changes are determined as %change relative to control taken as 100%. Data are presented as means ± SE of 4 experiments. **P < 0.01 vs. control.

Fig. 2. Bradykinin-induced activation of calpain involves TRPM7. Effects of bradykinin (BK) stimulation on calpain expression and translocation (cytosol/membrane) in VSMCs in the absence and presence of the TRPM7 inhibitor 2-aminoethoxydiphenyl borate (2-APB). Data are presented as means ± SE of 4–6 experiments. *P < 0.05 vs. control, +P < 0.05 vs. corresponding group unexposed to 2-APB.

Fig. 3. Bradykinin-induced activation of calpain involves phospholipase C (PLC) but not protein kinase C (PKC). Effects of bradykinin on calpain translocation (cytosol to membrane) in VSMCs in the absence and presence of chelerythrine (PKC inhibitor) and U-73122 (PLC inhibitor; 30 min pretreatment). Data are presented as means ± SE of 4–6 experiments. *P < 0.05 vs. corresponding control, +P < 0.05 vs. corresponding group unexposed to U-73122.
MATERIALS AND METHODS

Cell culture. This study was approved by the Animal Ethics Committee of the University of Ottawa. VSMCs from mesenteric arteries of Wistar-Kyoto rats were examined. Cells were isolated by enzymatic digestion and cultured as we detailed previously (4). Cells were maintained in DMEM containing 10% FCS. Low passaged cells (passages 2–7) were studied.

Protocols for cell stimulation. VSMCs were stimulated with bradykinin (10^{-6} mol/l, 10^{-5} mol/l) acutely (10–30 min) or chronically (1–24 h). In some experiments, cells were preexposed for 30 min to Hoe-140 (B2 receptor antagonist; 10^{-5} mol/l), chelerythrine (PKC inhibitor; 10^{-5} mol/l), U-73122 (PLC inhibitor; 10^{-5} mol/l) or 2-aminoethoxydiphenyl borate (2-APB; TRPM7 inhibitor; 10^{-5} mol/l, 2 h pretreatment) (18, 50, 52).

VSMC fractionation by sucrose gradient centrifugation. A detergent-free method to purify caveolae/lipid rafts was used (37). VSMCs were stimulated with bradykinin (10^{-6} mol/l, 10 min), and lysed in 500 mmol/l Na_{2}CO_{3} (plus protein inhibitors), pH 11, by scraping with a rubber policeman. The homogenate containing 2 g of protein was adjusted to 45% sucrose in 2-(N-morpholino)ethanesulfonic acid (MES)-buffered saline (MBS) (25 mmol/l MES and 0.15 mol/l NaCl, pH 6.5) and overlaid with 6 ml of 35% sucrose and 2 ml of 5% sucrose, both in MBS with 250 mmol/l Na_{2}CO_{3}. After centrifugation at 35,000 rpm for 18 h (SW 41; Beckman Instruments), 12 1-ml fractions were collected. Proteins were precipitated with 10% trichloro acetic acid, and resuspended in 0.1 mol/l NaOH and sample buffer.

Immunofluorescence confocal microscopy. VSMCs, grown on glass coverslips, were washed, fixed, and incubated with primary antibody (monoclonal anti-flotillin-2, 1:100, monoclonal anti-TRPM7 1:150). Proteins were detected with secondary antibody (anti-mouse Alexa Fluor 488) or rhodamine. Alexa Fluor 488-TRPM7 was detected as green fluorescence and rhodamine-flotillin-2 as red fluorescence. Flotillin-2, a lipid raft-associated protein, was used as a marker of lipid rafts (36). Imaging was acquired with a ZEISS Axiovert S100TV LSM 510 laser scanning system. Slides were scanned to obtain digital images as Tagged image file format files. Composites were generated using Adobe Photoshop 7.0. Colocalization of flotillin-2 and TRPM7 was visualized as orange fluorescence.

Western blot analysis. Total or fractionated proteins (20 μg) from unstimulated or bradykinin-stimulated VSMCs were extracted, separated by electrophoresis on polyacrylamide gel (10% or 12%), and transferred onto a polyvinylidene difluoride membrane as previously described (4). Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween (TBS-T; 1 h, room temperature). Membranes were incubated with anti-TRPM7 antibody (1:750; Abcam, Cambridge, MA), anti-calpain antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-VCAM-1 antibody (1:500; Santa Cruz Biotechnology) and anti-COX-2 antibody (1:500; Cell Signaling) in TBS-T-milk at 4°C overnight with agitation. Washed membranes were incubated with horseradish peroxidase-conjugated second antibody (1:2,000) in TBS-T-milk (room temperature, 1 h). Membranes were washed, and immunoreactive proteins were detected by chemiluminescence. Blots were analyzed densitometrically (Image-Quant software; Molecular Dynamics, Sunnyvale, CA).

Fig. 4. Bradykinin-induced VCAM-1 actions involve B2 receptors and TRPM7. Effects of bradykinin on VCAM-1 expression in VSMCs in the absence and presence of Hoe-140 (B2 receptor inhibitor) and 2-APB (TRPM7 inhibitor). Data are presented as means ± SE of 4 experiments. Values are expressed as %change relative to control taken as 100%. β-actin was used as an internal control. *P < 0.01 vs. corresponding control.

Fig. 5. Bradykinin stimulation increases cyclooxygenase-2 (COX-2) expression through TRPM7-mediated pathways. Effects of bradykinin on COX-2 expression in VSMCs in the absence and presence of 2-APB (TRPM7 inhibitor). Data are presented as means ± SE of 4 experiments. Values are expressed as %change relative to control taken as 100%. β-actin was used as an internal control. *P < 0.01 vs. corresponding control, +P < 0.05 vs. corresponding group unexposed to 2-APB.
Measurement of \([\text{Mg}^{2+}]_i\) in VSMCs. The selective fluorescent probe, mag fura-2AM, was used to measure \([\text{Mg}^{2+}]_i\), as previously described (19). Briefly, VSMCs were loaded with mag fura-2AM (2 \(\mu\)mol/l in 0.01% pluronic) and incubated for 10 min at 37°C. \([\text{Mg}^{2+}]_i\) was measured in multiple cells simultaneously acquired by the Stallion Digital Hi-Speed Multi-Channel Imaging System (Zeiss, Germany) using an emission wavelength of 520 nm and alternating excitatory wavelengths of 343 nm and 380 nm. Coverslips containing mag fura 2-AM-loaded cells were placed in a temperature-regulated (37°C) chamber mounted on the stage of an inverted microscope and continuously superfused at 2 ml/min with Hanks’ buffer (in mmol/l: 137 NaCl, 4.2 NaHCO3, 3 NaHPO4, 5.4 KCl, 0.4 KH2PO4, 1.3 CaCl2, 1.17 MgSO4, 10 glucose, and 5 HEPES, pH 7.4). \([\text{Mg}^{2+}]_i\) responses to 10^{-6} mol/l bradykinin were measured in cells incubated in Hanks’ buffer. In experiments with the TRPM-7 inhibitor, VSMCs were preexposed to 2-APB (10 \(\mu\)M, for 1 h) prior to bradykinin stimulation. Results were expressed as the fluorescence ratio of 340 nm/380 nm.

RESULTS

TRPM7 regulation by bradykinin in VSMCs. Stimulation of cells with bradykinin for 4–24 h resulted in a significant increase in TRPM7 abundance as assessed by immunoblotting (Fig. 1A). Following short-term stimulation, TRPM7 localized in cholesterol-rich domains of VSMCs, as assessed by immunofluorescence and sucrose-gradient fractionation of cells. Figure 1B demonstrates cellular distribution of TRPM7 by immunofluorescence confocal microscopy and shows that TRPM7 localizes in or near the plasma membrane and that it is also expressed intracellularly in a reticular pattern. The protein

![Image](http://www.ajpregu.org)

Fig. 6. 2-APB effects on bradykinin-induced intracellular free Mg^{2+} concentrations ([Mg^{2+}]_i) in VSMCs. VSMCs were loaded with mag fura-2AM and exposed to bradykinin, in the absence and presence of 2-APB. A: representative fluorescence images of VSMCs in basal conditions and in cells stimulated with bradykinin in the absence and presence of 2-APB. B: tracings demonstrate effects of bradykinin on mag fura-2AM fluorescence, indicative of [Mg^{2+}], without 2-APB pretreatment (top) and in 2-APB pretreated cells (bottom). C: bar graphs demonstrate [Mg^{2+}], in VSMCs. Data are presented as mag fura-2AM fluorescence (340/380 nm). Data are means ± SE of 5–7 experiments with each experiment comprising multiple cells. *P < 0.05 vs. basal, +P < 0.05 vs. BK alone.
appeared punctuate, particularly in the membrane-associated regions. Moreover, it localized with flotillin-2, a specific marker of lipid rafts/caveolae, indicating association with cholesterol-rich domains. This was confirmed when cells were separated into different cholesterol-containing fractions as shown in Fig. 1C, where TRPM7 localizes in fractions 2 and 3, corresponding to lipid rafts/caveolae. In basal conditions, TRPM7 was almost undetectable in cholesterol-rich fractions.

**Bradykinin stimulates activation of calpain.** Calpain is a TRPM7 kinase-sensitive downstream target as demonstrated in TRPM7-overexpressing cell (21). Exposure of VSMCs to bradykinin resulted in a significant increase in calpain expression and in translocation from the cytosol to the membrane, essential for calpain activation (31) (Fig. 2). Calpain activation by bradykinin was inhibited by 2-APB (Fig. 2) and U-73122 (PLC inhibitor), but not by the PKC inhibitor chelerythrine (Fig. 3).

**Bradykinin increases expression of proinflammatory mediators via TRPM7.** To investigate in greater detail the functional significance of TRPM7 activation by bradykinin in VSMCs, we evaluated some molecular mediators of inflammation in 2-APB-pretreated cells. As shown in Figs. 4 and 5 bradykinin time dependently increased expression of VCAM-1 and COX-2, with maximal effects obtained within 12 h of stimulation. Effects were blocked by Hoe-140 and were significantly reduced by the TRPM7 inhibitor 2-APB.

**2-APB inhibits bradykinin-stimulated [Mg^{2+}]i responses in VSMCs.** To confirm that 2-APB inhibits transmembrane Mg^{2+} transport, VSMCs were preincubated with 2-APB prior to bradykinin addition. As shown in Fig. 6, bradykinin induced a sustained increase in [Mg^{2+}]i. This was significantly inhibited in cells preexposed to 2-APB.

**DISCUSSION**

Major findings from our study demonstrate that 1) TRPM7 abundance is increased in response to bradykinin and it localizes to caveolae/lipid rafts in stimulated VSMCs, 2) bradykinin induces activation of calpain through TRPM7- and PLC-dependent pathways, and 3) TRPM7 is involved in bradykinin/B_2 receptor-mediated upregulation of proinflammatory mediators. These results identify a novel signaling pathway whereby bradykinin, through B_2 receptors linked to TRPM7 influences molecular processes of inflammation in VSMCs (Fig. 7).

TRP channels, particularly of the TRPC family, are increasingly being recognized to play a role in the regulation of vascular tone and blood pressure regulation (14, 37). Here we extend these findings and show that TRPM7 is functionally active in VSMCs and that it is involved in molecular processes associated with vascular inflammation and remodeling. We examined effects of bradykinin on TRPM7 signaling because previous studies demonstrated that TRPM7 is bradykinin-sensitive (22) and because this vasoactive peptide signals through G protein-coupled receptors, which are critically involved in modulating VSMC function (34, 49, 51).

Bradykinin exerts its actions via binding to B_1 and B_2 receptors, both of which are G protein-coupled (13). B_2 receptors are constitutively expressed in various cell types, including VSMCs, whereas B_1 receptors are weakly expressed in physiological conditions, but strongly expressed in pathological states. Activation of endothelial B_2 receptors induces vasorelaxation and growth inhibition, whereas VSMC B_2 receptors mediate vasoconstriction and proliferation (13, 42). In addition, recent findings indicate that bradykinin, through induction of COX-2 expression and upregulation of cell adhesion molecules, is proinflammatory (33). Bradykinin has also been shown to influence calpain (20, 25), a TRPM7-sensitive target, in inflammation. Considering these interactions, we questioned whether TRPM7 plays a role in proinflammatory signaling by bradykinin in VSMCs.

TRPM7 has been shown to be regulated by various receptor agonists, including ANG II, aldosterone, estrogen, bradykinin, and thrombin by mechanical factors such as stretch and flow, by cations (specifically Mg^{2+}), and by biochemical factors such as pH and osmolarity (3, 19, 38, 40, 43, 44). TRPM7 is rapidly phosphorylated through G protein-coupled receptors, and it accumulates at the cell membrane in response to bradykinin stimulation (22). These changes may reflect a change in compartmentalization of TRPM7 as suggested previously (22) and are supported here where we show that TRPM7 interacts with caveolae/lipid rafts in activated cells, as shown by immunofluorescence and by localization in flotillin-2-containing cholesterol-rich cell fractions. TRPM7/caveolae/lipid raft association may facilitate TRPM7 scaffolding to cell membrane receptors, such as G protein-coupled receptors and to signaling molecules, such as calpain, which has been shown to be a caveolae-associated protein (15, 37).

Bradykinin-induced TRPM7 activation was associated with increased translocation of calpain, an effect that was attenuated by 2-APB. 2-APB has been shown to inhibit TRPM7 activity (18). To confirm this in our system, we examined effects of 2-APB on bradykinin-induced Mg^{2+} transients in VSMCs. Using siRNA technology, we previously demonstrated that transmembrane Mg^{2+} transport involves TRPM7 (43, 44), an effect that is inhibited by 2-APB as observed here. Hence, our
data suggest that bradykinin-induced calpain actions involve, at least in part, TRPM7/Mg\(^{2+}\). However, we cannot exclude the possibility that other 2-APB-sensitive targets, such as gap junctions and Cu\(^{2+}\) channels, may also play a role (33, 43). We also found that bradykinin-mediated calpain effects are inhibited by U-73343 but not by chelerythrine, suggesting that PLC but not PKC is involved in calpain regulation by bradykinin.

Previous studies in TRPM7-overexpressing cells demonstrated that TRPM7 activates calpain, which plays an important role in regulating cell adhesion (40). Here we extend these findings to show that bradykinin, through TRPM7, induces expression of proinflammatory molecules VCAM-1 and COX-2, which are known downstream targets of calpain (24, 37, 45). Previous studies showed that bradykinin influences inflammatory mediators and cellular adhesion molecules (35). Such effects may contribute to bradykinin-mediated vascular inflammation, fibrosis, and remodeling. Whereas 2-APB abrogated bradykinin VCAM-1 actions, it only partially reduced effects on COX-2, indicating the importance of other signaling pathways in bradykinin regulation of COX-2. To our knowledge, these are the first data demonstrating a link between B\(_2\) receptors, TRPM7, and proinflammatory signaling.

In summary, the present study provides new data whereby bradykinin may influence VSMC function. We demonstrate that bradykinin induces TRPM7 localization in caveolae/lipid rafts, that it stimulates, in part, activation of calpain, and that it increases expression of VCAM-1 and COX-2. Such effects are mediated via B\(_2\) receptors. Our findings identify a novel signaling pathway whereby bradykinin, through TRPM7, may influence proinflammatory responses in VSMCs.

**GRANTS**

This study was supported by grants from the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Canada. Dr. Touyz is supported through a Canada Research Chair/Canadian Foundation for Innovation Award.

**REFERENCES**


35. Sainz IM, Uknis AB, Isordia-Salas I, Dela Cadena RA, Pixley RA, Colman RW. Interactions between bradykinin (BK) and cell adhesion


