PKCα mediates acetylcholine-induced activation of TRPV4-dependent calcium influx in endothelial cells

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Endothelial cells regulate the vascular tone by releasing relaxing factors in response to both mechanical forces (shear stress and cyclic strain) and soluble agonists [bradykinin and acetylcholine (ACh)] (3, 36). It has been established that the calcium influx through the plasma membrane is one of the proximal signaling factors in the production of relaxing factors such as nitric oxide (NO) and prostaglandins in response to external stimuli (11), although the identity of this calcium channel in the endothelium remained elusive. Recently, transient receptor potential (TRP) channels of a family of nonselective cation channels were identified as store- and receptor-operated calcium entry channels (9, 12, 24). Among all TRP channels, transient receptor potential vanilloid channel 4 (TRPV4) is ubiquitously expressed in endothelial cells and has gained increased attention, since it has been implicated in mechanotransduction (16, 26). We and others have recently shown that TRPV4 is activated by cyclic strain and flow-induced shear stress in endothelial cells, and the resulting calcium influx is required for endothelial cell reorientation and NO production (10, 21, 33). Importantly, with the use of TRPV4 knockout mice, it was demonstrated that TRPV4 is essential for shear stress-induced vasodilation (8, 23). However, TRPV4 is a polymodally activated channel and is demonstrated to be activated by a variety of physical and chemical stimuli such as temperature, hypotonicity, phorbol esters, endocannabinoids, arachidonic acid (AA), and epoxy eicosatrienoic acids (EETs) (15, 25, 38, 40). Interestingly, the vasoactive agonist ACh was recently shown to activate TRPV4-dependent calcium influx and NO production in endothelium, which is required for vasodilation in mesenteric arteries (44). These findings clearly demonstrate that TRPV4 plays an important role in the mechanical force and agonist-induced regulation of vascular tone. However, the molecular mechanism by which ACh activates TRPV4-dependent calcium influx in endothelial cells is not yet known. Because ACh acts through a G protein-coupled receptor, which can activate protein kinase C (PKC), and PKC was recently implicated in this signal.

Materials and Methods

Materials. ACh, GSK-1016790A, ruthenium red, and atropine were purchased fromSigma; AB-159908 was obtained from ABCR. Fluo 4 and Alexa-conjugated secondary antibodies were from Invitrogen.

Cell culture. Mouse dermal vascular endothelial cells (MEC) were cultured on fibronectin-coated tissue culture dishes and grown in a defined medium composed of low-glucose DMEM, 10% FBS, 10% Nu Serum IV, basic fibroblast growth factor (6 ng/ml), heparin salt (0.1 mg/ml), 1% insulin-transferrin-selenium, and antibiotic/antimycotic mix as described previously (7). Cells were cultured in a 37°C, 5% CO2 incubator, split at ~90–95% confluence, and used between passages 11 and 22. HEK-293 cells were cultured in high-glucose DMEM, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO2 environment.

Animals. Male wild-type and TRPV4 null (TRPV4−/−) mice of 10–15 wk of age generated in a C57BL6 background were used in this study (32, 44). All experiments were performed according to guide-
lines and approval of the Institutional Animal Care and Use Committee of the Northeast Ohio Medical University. Mice were housed in a temperature-controlled room with a 12:12-h light-dark cycle and maintained with access to food and water ad libitum.

**Calcium imaging.** Cells cultured on MatTek glass-bottomed dishes were loaded with fluo 4-AM (4 μM) for 30 min, washed three times in calcium medium (in mM: 136 NaCl, 4.7 KCl, 1.2 MgSO4, 1.1 CaCl2, 1.2 KH2PO4, 5 NaHCO3, 5.5 glucose, and 20 HEPES, pH 7.4), and kept in this medium on an inverted Olympus IX 70 confocal microscope (FV300). Cells were stimulated with ACh (10 μM), and images were acquired every 3 s and analyzed using Olympus fluoview software and Microsoft Excel as previously described (21, 33). In some experiments, cells were pretreated with inhibitors for 20 min using the following concentrations: 10 μM atropine, 10 μM AB-159908, and 1 and 10 μM Go-6976. To distinguish calcium influx from the internal store calcium release, cells were first stimulated with ACh (10 μM) or thapsigargin (1 μM) in calcium-free medium, and the influx was initiated by adding calcium chloride (2 mM final concentration).

**Transfection.** Cells were transfected with expression vectors containing red fluorescent protein (RFP)-TRPV4 (1), PKCα-kininase-dead (PKCa-KD)-enhanced green fluorescent protein (EGFP) (30), or PKCε-kinase-dead (PKCe-KD)-EGFP (17, 31) using either Effectene (Qiagen) for HEK-293 cells and Targeting (Targeting systems) for MEC. We found that the transfection efficiencies were around 90% for HEK-293 and 60–80% for MECs.

**Immunofluorescence of phospho-endothelial nitric oxide synthase.** MEC cultured on glass cover slips were washed with PBS (3 times) and fixed for 20 min at room temperature in PBS containing 4% paraformaldehyde. Cells were then rinsed and permeabilized with PBS containing 0.25% Triton X-100, washed with PBS, and blocked with serum-containing media for 20 min. Afterward, cells were incubated with the primary antibodies [phospho-endothelial nitric oxide synthase (eNOS) antibody from Cell Signaling; 1:100] at room temperature for 1 h. The cells were then washed with PBS and incubated with Alexa Fluor-conjugated secondary antibodies (1:500). Cells were mounted on glass slides using fluoromount containing DAPI (Vector labs). Images were obtained using an Olympus fluorescence microscope with a 40 objective and processed using Image J software. For 12-membered GTPase assay, cells were transfected with RFP-TRPV4 and anti-TRPV4 (Alomone), anti-PKCε (BD Biosciences), and anti-α-tubulin (Abcam). The enhanced chemiluminescence method was used with anti-rabbit or -mouse IgG-conjugated horseradish peroxidase (Pierce West Pico) at a dilution of 1:20,000 and developed with Kodak XAR film. Results were quantified using Image J software. For 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced downregulation of PKC isoforms, cells were exposed to chronic concentrations of TPA (1 μM) from 0 to 24 h, and the lysates were subjected to SDS-PAGE and Western blotting and probed with isoform-specific PKC antibodies.

**Isolation of endothelial cells.** Endothelial cells were isolated using the aortic explant method as described (19). Briefly, aortic rings were placed in Matrigel for 7 days. The aortic rings were carefully removed, and endothelial cells were isolated, washed, and plated on gelatin (0.1%)-coated dishes.

**RT-PCR analysis.** RT-PCR analysis of endothelial cells was performed using a Qiagen One-Step RT-PCR Kit and specific primers for mouse TRPV4 and CD31 as described previously (44).

**RESULTS**

**ACh induces TRPV4-dependent rapid calcium signal in endothelial cells.** ACh was previously shown to induce a TRPV4-dependent calcium influx in endothelial cells of mouse mesenteric arteries (44). To determine whether ACh induces TRPV4-dependent calcium signal in cultured endothelial cells, we first measured TRPV4 expression in MECs. Western blot analysis revealed that MEC express TRPV4 with typical two bands corresponding to the molecular masses of 90 and 110 kDa, which represent nonglycosylated and glycosylated forms of mouse TRPV4 (Fig. 1A). RT-PCR analysis showed a band at 760 bp corresponding to TRPV4 message, further confirming that MEC express TRPV4 (Fig. 1A). Next, we measured calcium influx in response to the known TRPV4 activator GSK-1016790A in cells loaded with the calcium indicator fluo 4 (4 μM). Stimulation of MEC with GSK-1016790A [100 and 300 nM (35, 42)] induced robust calcium influx that was abolished when calcium was removed from the medium (Fig. 1B). We also checked the specificity of GSK-1016790A-induced calcium influx in HEK-293 cells (which do not express endogenous TRPV4) by overexpressing RFP-TRPV4. We found that GSK-1016790A induced a robust calcium signal in TRPV4-expressing, but not in control, HEK-293 cells, further confirming the specific activation of TRPV4 by GSK-1016790A (data not shown). ACh (10 μM) induced a rapid calcium signal in MEC that peaked at 20–25 s and slowly declined by 200 s, suggesting that ACh caused both the calcium release from the stores (peak) and calcium influx (sustained phase) through the plasma membrane (Fig. 1C). Pretreatment of the cells with the ACh receptor antagonist atropine completely abolished the calcium signal elicited by ACh (Fig. 1C). In contrast, pretreatment of the cells with the specific TRPV4 inhibitor AB-159908 (37) abolished the sustained phase of the ACh-induced calcium signal with a small inhibition of the peak calcium signal (Fig. 1, C and D), indicating that TRPV4 channels are required for the ACh-induced calcium influx but not for release from the internal stores.
TRPV4-mediated calcium signal is required for eNOS activation by ACh. The ACh-induced calcium signal was previously shown to result in the production of NO, which is responsible for the vasodilatory effects on the endothelium (44). Therefore, we investigated the possibility that ACh-induced TRPV4-dependent calcium influx activates eNOS in the MEC by using phosphospecific antibodies against eNOS Ser1177. MEC cultured on cover glass were stimulated with ACh in the presence or absence of TRPV4 inhibitors and subsequently stained for phospho-eNOS. Immunofluorescence analysis revealed that cells stimulated with ACh showed robust staining of phospho-eNOS, which was inhibited by pretreatment of the cells with AB-159908 (Fig. 2A). Quantification of the fluorescence intensity revealed significant reduction in the eNOS phosphorylation (Fig. 2B). Western blotting analysis also showed increased phosphorylation of eNOS in response to ACh, which was inhibited in the presence of atropine and ruthenium red, independently confirming that ACh induces eNOS activation in these cells (data not shown). Taken together, these findings suggest that eNOS activation by ACh in endothelial cells requires TRPV4-dependent calcium influx.

TRPV4 channels mediate ACh-induced calcium influx but not release from the internal stores. Although the sustained phase in ACh-induced calcium signal in Fig. 1 represents the calcium influx mediated by TRPV4, the slight reduction in the calcium peak suggests that TRPV4 may also contribute to the release of calcium from stores. To distinguish the calcium influx from the internal store calcium release, we performed calcium-imaging experiments using the calcium-free protocol. To achieve this, MEC were loaded with fluo 4 and stimulated with ACh in calcium-free medium followed by the addition of external CaCl₂ (2 mM final concentration). As shown in Fig. 3, ACh induced a rapid calcium release in MEC, and addition of external calcium (after the signal reached baseline) induced a robust increase in the calcium, indicating the calcium influx through the plasma membrane. Importantly, pretreatment of the cells with a specific TRPV4 antagonist, AB-159908, has no effect on the release of calcium from the stores but significantly inhibited the calcium influx (Fig. 3, A and B). To unequivocally confirm that the ACh-induced calcium influx is mediated by TRPV4, we measured calcium influx in endothelial cells isolated from the TRPV4 null mice. As shown in Fig. 4A, RT-PCR analysis confirmed that TRPV4 expression is absent in TRPV4 null endothelial cells isolated from the TRPV4 null mice. As shown in Fig. 4A, RT-PCR analysis confirmed that TRPV4 expression is absent in TRPV4 null endothelial cells (KOEC) compared with wild type (WTEC) and MEC. However, KOEC expressed the endothelial marker platelet endothelial cell adhesion molecule-1. The specific TRPV4 activator GSK-106790A induced robust calcium influx in WTEC but failed to induce calcium influx in
KOEC, further confirming that TRPV4 is absent in these cells (Fig. 4, B and C). Notably, we found that ACh-induced calcium influx was completely abolished in TRPV4 null endothelial cells (KOEC) (Fig. 5C), with no effect on calcium release from the internal stores, which was comparable to MEC (Fig. 5C) and wild-type endothelial cells (Fig. 4D). These findings clearly demonstrate that ACh-induced calcium influx in endothelial cells is dependent on TRPV4.

ACh-induced TRPV4-dependent calcium influx is mediated by a receptor-operated pathway. We next asked if the calcium influx induced by ACh is mediated by either the receptor-operated or store-operated pathway. We thus used our calcium-free protocol and measured calcium influx in wild-type and TRPV4 null endothelial cells after emptying the stores with the sarco(endo)plasmic reticulum Ca2+-ATPase inhibitor thapsigargin (TG), which is a widely accepted method to induce store-operated calcium influx. As shown in Fig. 5A, TG induced significant release of calcium from the internal stores in MEC and wild-type endothelial cells (data not shown), and addition of external calcium triggered robust calcium influx, indicating the activation of the store-operated channel pathway. Interestingly, depletion of stores with TG in TRPV4 null

Fig. 2. TRPV4-mediated calcium signal is required for endothelial nitric oxide synthase (eNOS) activation by ACh. A: fluorescent images of endothelial cells showing eNOS phosphorylation (p) by ACh. MEC were stimulated with ACh for 15 min in the presence or absence of the TRPV4 antagonist AB-159908. eNOS phosphorylation was measured by incubation with the phosphospecific antibody Ser1177 followed by Alexa-594-conjugated secondary antibody (red) and DAPI (blue). B: quantitative analysis of the eNOS phosphorylation. The results shown are means ± SE from 3 independent experiments (*P < 0.05).

Fig. 3. TRPV4 channels mediate ACh-induced calcium influx but not release from the internal stores. A: MEC were loaded with fluo 4, and calcium release (first peak) was measured in calcium-free media in response to ACh. The calcium influx (second peak) was initiated by the addition of calcium chloride (2 mM final concentration) to the media. Note that the TRPV4 antagonist AB-155908 reduced the calcium influx but not the release of calcium from the internal stores. Arrow indicates addition of the stimulator. B: quantitative analysis of the calcium influx. The results shown are means ± SE from 3 independent experiments (*P < 0.05).
endothelial cells also induced a robust calcium influx (approximately two times that of MEC and WTEC), indicating that the store-operated channel pathway is intact in the absence of TRPV4 (Fig. 5A). In contrast, release of calcium from internal stores by ACh induced calcium influx only in MEC and WTEC (Figs. 5C and 4D), which was significantly decreased in TRPV4 null endothelial cells (Fig. 5C). These findings clearly demonstrate that ACh-induced TRPV4-dependent calcium influx is mediated through a receptor-operated pathway.

PKCα activity is required for TRPV4-mediated calcium influx by ACh. We next asked which signaling molecule is required for receptor-operated activation of TRPV4-dependent calcium influx.

Fig. 4. A: RT-PCR analysis showing the expression of the endothelial marker platelet endothelial cell adhesion molecule (PECAM)-1 and the absence of TRPV4 in endothelial cells isolated from TRPV4 null [knockout (KO)] mice. The wild type (WT) and MEC express both PECAM-1 and TRPV4. B: calcium influx is absent in endothelial cells isolated from TRPV4 KO mice (KOEC) stimulated with the specific TRPV4 activator GSK-1016790A (GSK). WTEC, wild-type endothelial cells. Arrow indicates addition of the stimulator. C. quantitative analysis of the calcium influx (*P < 0.05). D. representative traces showing calcium influx measured by using a calcium-free protocol in WT endothelial cells in response to ACh. Arrow indicates addition of the stimulator.

Fig. 5. ACh-induced TRPV4-dependent calcium influx is mediated by a receptor-operated pathway. Representative traces showing calcium influx measured by using a calcium-free protocol in MEC and TRPV4 null endothelial cells (KOEC) in response to thapsigargin (TG; A) or ACh (C). Arrow indicates addition of the stimulator. Note that the calcium influx induced by ACh but not TG is inhibited in KOEC, indicating that TRPV4-dependent calcium influx is mediated through a receptor-operated pathway. Quantitative analysis of the calcium influx from the store-operated pathway induced by TG (B) or the receptor-operated pathway induced by ACh (D) in MEC and KOEC. The results shown are means ± SE from 3 independent experiments (*P < 0.05).
PKCα REGULATES AGONIST-INDUCED TRPV4 SIGNALING

calcium influx. PKC-dependent phosphorylation was recently shown to sensitize and activate TRPV4 (28); however, neither the natural agonist nor the specific PKC isoform involved in TRPV4 activation is known. To identify the molecular mechanism of ACh-induced activation of TRPV4 and dependent calcium influx, we first assessed the expression of PKC isoforms in MEC by using Western blot analysis. We found that these cells express PKCα, -γ, and -ε isoforms (Fig. 6A) but not PKCβ (data not shown). To identify the specific PKC isoform involved in ACh-induced TRPV4-dependent calcium influx, we used a TPA downregulation protocol. We and others (22, 27, 34) have previously shown that acute TPA (1 μM) treatment downregulates specific PKC isoforms at different time points. We found that TPA treatment significantly downregulated the expression of the PKCα isoform around 8 h that is sustained until 24 h (Fig. 6, A and B). In contrast, TPA treatment did not downregulate the expression of either PKCε or PKCγ (Fig. 6A), suggesting that it specifically downregulates PKCα expression in MECs. Next, we measured calcium influx following 8–12 h TPA treatment in the MECs (PKCα downregulated) in response to ACh. As shown in Fig. 6D, ACh-induced calcium influx was inhibited significantly in PKCα-downregulated cells compared with untreated cells, suggesting that PKCα is required for ACh-induced TRPV4-dependent calcium influx in MEC. Next, we asked if ACh activates PKCα in MEC. We found that ACh indeed activates PKCα in the endothelial cells as evidenced by increased phosphorylation of PKCα, which was reduced by the specific PKCα inhibitor Go-6976 (20) (Fig. 6C). Furthermore, pretreatment with Go-6976 significantly inhibited calcium influx in these cells at two different concentrations tested (1 and 10 μM) (Fig. 6D).

Next, to avoid any nonspecific effects of pharmacological reagents (TPA or Go-6976), we investigated the role of PKCα using PKCα KD-EGFP. When expressed in endothelial cells (EGFP expression in Fig. 7B, inset), we found that PKCα KD but not PKCε-KD significantly inhibited the calcium influx induced by ACh (Fig. 7, A and B). Taken together, these results confirm that PKCα activity is required for ACh-induced TRPV4-dependent calcium influx in endothelial cells.

Inhibition of PKCα activity reduces in situ ACh-induced vasodilation in isolated vessels. To investigate the physiological significance of the above results, we measured ACh-induced vasodilation in wild-type and TRPV4 knockout mice in the presence or absence of the PKCα inhibitor Go-6976 (20) using pressure myography of isolated mesenteric microvessels. Mesenteric arteries isolated from the mice were preconstricted with U-46619, and vasodilation was measured in response to ACh. We found no difference in the baseline vessel diameter between wild-type and TRPV4 knockout mice (113 ± 11 for wild type vs. 116 ± 18 for KO) or their response to U-46619 (39 ± 17 for wild type vs. 42 ± 9 for KO). We also found that ACh induced a dose-dependent vasorelaxation (data not shown) with a maximum dilation at 10 μM of ACh, which was used throughout the study. As shown in Fig. 8, ACh induced extensive dilation of mesenteric arteries in wild-type mice, which was significantly inhibited in TRPV4 null mice. Pretreatment with the PKCα inhibitor Go-6976 significantly inhibited ACh-induced vasodilation of mesenteric arteries in wild-type mice (Fig. 8) to the levels equivalent to that of

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**Fig. 6.** Protein kinase C (PKC) α activity is required for TRPV4-mediated calcium influx by ACh. Representative Western blot showing the downregulation of the expression of PKCα (A) but not PKCε or PKCγ in MEC by chronic 12-O-tetradecanoylphorbol-13-acetate (TPA, 1 μM) treatment. B: quantitative analysis of the downregulation of PKCα by TPA. C: representative Western blot showing the activation of PKCα by ACh, which is inhibited by the PKCα specific inhibitor Go-6976 (Go). Con, Control. Bar graph shows quantitative analysis of PKCα activation. D: quantitative analysis of the calcium influx induced by ACh in MEC either treated with chronic TPA for 8–12 h (ACh + TPA) or the specific PKCα inhibitor Go-6976 at two concentrations (ACh + Go 1 μM and ACh + Go 10 μM). The results shown are means ± SE from 3 independent experiments (*P < 0.05).
TRPV4 KO mice. Taken together, these results clearly demonstrate that PKCα activity is required for ACh-induced TRPV4-dependent vasodilation in mesenteric arteries.

DISCUSSION

In the present study, we elucidated the molecular mechanism of agonist-induced calcium influx through TRPV4 and demonstrated, for the first time, that the PKCα mediates the ACh-induced activation of TRPV4-dependent calcium influx in endothelial cells. Using a TRPV4-specific antagonist and TRPV4 null mice, we demonstrated that the TRPV4-dependent calcium influx is mediated through the ACh-induced receptor-operated pathway but not its associated store-operated pathway and is required for eNOS activation and vasodilation.

A role for TRPV4 in the ACh-induced NO-dependent vasodilation was confirmed using the mesenteric arteries from TRPV4 null mice (23, 44). In contrast, the ACh-induced vasodilation was unchanged in conduit arteries of these mice (8) and suggested that TRPV4 is a critical regulator of resistant vascular tone but not the conduit vessels. Although the present study focuses on the molecular mechanisms of TRPV4/NO-dependent vasodilation, TRPV4 has also been implicated in ACh-induced endothelial-derived hyperpolarizing factor (EDHF)-dependent vasodilation. In fact, Earley et al. (4) demonstrated that, in TRPV4-KO mice, ACh-induced vasodilation of resistance arteries can be reduced as much as 75% in the presence of nitric oxide synthase and cyclooxygenase inhibitors (4). This observation demonstrated that a significant portion of the ACh-induced TRPV4-dependent vasodilation is mediated through an NO-independent mechanism. Furthermore, it was demonstrated that activation of small, intermediate, and large-K⁺ channels by endothelial and smooth muscle cells is required for the observed EDHF-mediated hyperpolarization and vasodilation (4). Additionally, Saliez et al. (29) demonstrated the EDHF-mediated vasodilation was dependent on caveolin-1-mediated localization of TRPV4 to the plasma membrane and connexins to the myoendothelial gap junctions. This observation is consistent with the work by Ledoux et al. (14) in which calcium pulsars were activated by ACh in the myoendothelial projections of mesenteric arteries. Furthermore, these pulsars were demonstrated to be critical for the activation of endothelial calcium ion-induced K⁺ channels triggering the hyperpolarization of smooth muscle (14). Recently, the expression of TRPV4 channels has been shown to be enriched in the myoendothelial projections of rat posterior cerebral and superior cerebellar arteries (S. Earley, unpublished observation). Based on these findings, it is conceivable that ACh-induced vasodilation is dependent on TRPV4 calcium influx at myoendothelial projections.

Previous work has implicated TRPV4 in the ACh-induced vasodilation in both NO-dependent and -independent mechanisms (4, 29, 44); however, the molecular mechanism through which ACh activates TRPV4 is not known. The well-known mechanisms of TRPV4 activation are 1) direct binding of agonists such as synthetic phorbol ester, 4-α-PDD, which binds to the 3 and 4 transmembrane domains of TRPV4 (39) and 2) indirect activation by shear stress and osmotic stretch through the production of cytosolic phospholipase A2 (cPLA2)-dependent synthesis of AA and EETs (8, 38, 40). However, it is not known whether EETs bind directly to TRPV4 or act through a receptor. In contrast to these mechanisms, we and others (18, 21, 33) have shown that TRPV4 is directly activated by mechanical stretch.

![Fig. 7](image_url)  
Fig. 7. Expression of a kinase-dead PKCα inhibits ACh-induced calcium influx. A: representative traces showing calcium influx measured by using the calcium-free protocol in MEC transfected with PKCα-kinase dead (KD)-enhanced green fluorescent protein (EGFP) or PKCε-KD-EGFP expression plasmid in response to ACh. Note that the calcium influx induced by ACh is inhibited in PKCα-KD-expressing cells but not in PKCε-KD-expressing cells. Arrow indicates addition of the stimulator. B: quantitative analysis of the calcium influx induced by ACh in MEC-expressing PKCα-KD-EGFP or PKCε-KD-EGFP (inset shows EGFP fluorescence, confirming the expression of kinase-dead constructs). The results shown are means ± SE from 3 independent experiments (*P < 0.05).

![Fig. 8](image_url)  
Fig. 8. Inhibition of PKCα activity reduces ACh-induced vasodilation in situ in isolated vessels. Quantitative analysis of the vasodilation induced by ACh in mesenteric arteries from WT C57BL6 in the absence or the presence of the specific PKCα inhibitor Go-6976 (WT + Go-6976) and TRPV4 null mice (KO) (n = 5 vessels; *P < 0.05). NS, not significant.
The phosphorylation of TRPV4 at tyrosine residues by Src kinase or at serine residues by PKC/protein kinase A (PKA) have been implicated downstream of physiological stimuli, including hypotonicity. For example, TRPV4 activity can be regulated by the phosphorylation of Tyr253, which is thought to be required for hypotonicity-induced activation of TRPV4 (43). Recently, an additional tyrosine phosphorylation site, Tyr110, was found to be critical for the modulation of TRPV4 hypotonicity and phorbol 12-myristate 13-acetate activity (5). In fact, it was suggested that Tyr110 phosphorylation modulates TRPV4 activity but is not required for its activation. In addition, the physiological agonist bradykinin has also been implicated in the modulation of TRPV4 activity through PKC- and PKA-mediated phosphorylation (5). Notably, PKC-induced phosphorylation at Ser824 was found to be involved in the hypotonicity-induced TRPV4-dependent calcium influx (28).

Taken together, these findings suggest that the majority of stimuli appear to modulate TRPV4 activation through intracellular signaling involving cPLA2 or kinases such as PKC, PKA, and Src with 4α-PDD being an exception. The major finding of this study supports this notion and illustrates that the G protein-coupled receptor agonist ACh activates TRPV4-dependent calcium influx through PKC and further supports phosphorylation as an important regulator of TRPV4 activity in response to physiological stimuli.

This study presents evidence for the participation of PKCα in the regulation of ACh-induced TRPV4-dependent calcium influx. In contrast, the activation of another TRPV family member, TRPV1, is regulated by PKCε in response to bradykinin (2). The bradykinin-induced calcium signal of TRPV4 has also been established through a PKC-dependent phosphorylation pathway (41); however, the specific PKC isoform mediating this signal is not known. Additionally, it is not known whether bradykinin/ACh-induced calcium influx is mediated through store-operated or receptor-operated pathways. However, our results demonstrate that ACh-induced calcium influx is mediated through a receptor-operated pathway rather than a store-operated pathway. We additionally demonstrate that PKCα activity is physiologically relevant for ACh to induce a significant dilatation of mesenteric arteries in WT mice, which was reduced in TRPV4 null animals. The inhibition of PKCα activity by Go-6976 suppressed the ACh-induced mesenteric vasodilation from WT mice equal to that achieved in TRPV4 KO mice, suggesting that PKC-α is a major regulator of TRPV4 activity.

TRPV4 was also implicated in salt-induced regulation of blood pressure. Gao and coworkers (6) reported TRPV4 activation by 4α-PDD decreased the mean arterial blood pressure in normal salt-fed rats and was further suppressed in high-salt-fed animals. A high-salt diet is known to increase TRPV4 expression in mesenteric arteries, suggesting TRPV4 as a compensatory mechanism to high-salt-induced hypertension (6). This is of clinical significance and identifies TRPV4 as an important regulator of hypertension. However, the molecular mechanisms involved in salt-induced TRPV4 expression and activity are not known. In this study, we not only confirm the role of TRPV4 in agonist-induced vasodilation but also identify PKCα as an ACh-activated signaling molecule to mediate TRPV4-dependent calcium influx and vasodilation. We envisage these findings will provide new therapeutic targets for intervention in hypertension and other TRPV4-associated vascular disorders.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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