Evidence for a Functional Role of Endothelial Transient Receptor Potential V4 in Shear Stress–Induced Vasodilatation

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Objective—Ca\textsuperscript{2+}-influx through transient receptor potential (TRP) channels was proposed to be important in endothelial function, although the precise role of specific TRP channels is unknown. Here, we investigated the role of the putatively mechanosensitive TRPV4 channel in the mechanisms of endothelium-dependent vasodilatation.

Methods and Results—Expression and function of TRPV4 was investigated in rat carotid artery endothelial cells (RCAECs) by using in situ patch-clamp techniques, single-cell RT-PCR, Ca\textsuperscript{2+} measurements, and pressure myography in conduit arteries (CAs) and Austroboletus gracilis. In RCAECs in situ, TRPV4 currents were activated by the selective TRPV4 opener 4\alpha-phorbol-12,13-didecanoate (4\alphaPDD), arachidonic acid, moderate warmth, and mechanically by hypotonic cell swelling. Single-cell RT-PCR in endothelial cells demonstrated mRNA expression of TRPV4. In FURA-II Ca\textsuperscript{2+} measurements, 4\alphaPDD increased [Ca\textsuperscript{2+}], by \approx 140 nmol/L above basal levels. In pressure myography experiments in CAs and A. gracilis, 4\alphaPDD caused robust endothelium-dependent and strictly endothelium-dependent vasodilatations by \approx 80\% (K\textsubscript{0}, 0.3 \mu mol/L), which were suppressed by the TRPV4 blocker ruthenium red (RuR). Wall shear stress–induced vasodilatation was similarly blocked by RuR and also by the phospholipase A\textsubscript{2} inhibitor AACOCF\textsubscript{3}. 4\alphaPDD produced endothelium-derived hyperpolarizing factor (EDHF)-type responses in A. gracilis but not in rat carotid artery. Shear stress did not produce EDHF-type vasodilatation in either vessel type.

Conclusions—Ca\textsuperscript{2+} entry through endothelial TRPV4 channels triggers NO- and EDHF-dependent vasodilatation. Moreover, TRPV4 appears to be mechanosensitively important in endothelial mechanosensing of shear stress. (Arterioscler Thromb Vasc Biol. 2006;21:000-000.)

Key Words: endothelium-dependent vasodilatation ■ transient receptor potential ■ TRPV4 ■ calcium ■ shear stress ■ nitric oxide ■ 4\alphaPDD ■ rat carotid artery

Ca\textsuperscript{2+}-influx in response to mechanical or humoral stimulation plays a significant role in a variety of endothelial functions and especially in the Ca\textsuperscript{2+}-dependent synthesis of endothelium-derived vasodilators such as NO, prostacyclin, or the endothelium-derived hyperpolarizing factor (EDHF).

Several members of the transient receptor potential (TRP) superfamily of cation channels have been identified in endothelial cells (ECs) of humans and other species and may provide Ca\textsuperscript{2+} influx pathways. Within the different subfamilies of TRP channels, ECs express members of the “canonical” TRP subfamily (TRPC), such as TRPC1, TRPC3, and TRPC4, which are primarily believed to serve as Ca\textsuperscript{2+} influx channels after receptor activation or store depletion. Regarding the other large TRP subfamilies, the “melastatin” (TRPM) and “vanilloid” (TRPV) subfamilies, TRPM4 and TRPM7, and TRPV4, respectively, are also endothelial TRPs. Although TRPV4 was proposed to contribute to EDHF signaling, the precise roles of TRPV4 and of other TRPs in the mechanism of endothelium-dependent vasodilatation are still undefined.

Within the endothelial TRP channels, TRPV4 might be of special interest because this channel provides a significant Ca\textsuperscript{2+} entry pathway because of its moderately high Ca\textsuperscript{2+} permeability. Moreover, TRPV4 channels have been shown to be opened by diverse physical and chemical stimuli such as cell swelling and shear stress, moderate warmth (>27°C), low pH, and pharmacologically by the non-PKC-activating phorbol ester 4\alpha-phorbol-12,13-didecanoate (4\alphaPDD). Recent studies suggested that arachidonic acid (AA) and its metabolite 5,6 epoxyeicosatrienoic acid may serve as endogenous activators of TRPV4. However, thus far, it is unknown how TRPV4 contributes to endothelium-dependent vasodilatation.

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The mechanosensitivity of TRPV4 may point to a role of the channel as an endothelial mechanosensor and thus in the mechanisms of flow- or wall shear stress–induced vasodilatation. It is noteworthy that a shear stress–induced TRPV4-mediated Ca\(^{2+}\) entry has been shown in heterologous expression systems\(^{2,12}\) and renal tubular epithelial cells.\(^{9}\) Such flow- and shear stress–induced Ca\(^{2+}\) signals have also been observed in ECs in vitro and in the endothelium in intact vessel preparations.\(^{16–18}\) Although shear stress–activated Ca\(^{2+}\)/H\(_{\text{ATPase}}\) preparations.\(^{16–18}\) Although shear stress–activated Ca\(^{2+}\)/H\(_{\text{ATPase}}\) combinations also reduced outward currents.\(^{20}\) s) also reduced outward currents. Exposure times (\(\leq 300\) s) also reduced outward currents. Exposure times (\(\geq 10\) ms) also reduced outward currents. Exposure times (\(\geq 100\) ms) also reduced outward currents. Exposure times (\(\geq 1\) s) also reduced outward currents. Exposure times (\(\geq 10\) s) also reduced outward currents. Exposure times (\(\geq 100\) s) also reduced outward currents. Exposure times (\(\geq 1\) min) also reduced outward currents.

Inward currents were abolished after substitution of Na\(^{+}\) by \(100\) mmol/L, the selective TRPV4 opener, activated moderately in RCAECs and small-sized \(A\).\textit{gracilis}. We show that pharmacological activation of endothelial TRPV4 induces robust vasodilatations in a NO- and EDHF-dependent manner. Moreover, we provide evidence that Ca\(^{2+}\) influx through TRPV4 is functionally important in the mechanisms of shear-stress–induced vasodilatation.

To elucidate how endothelial TRPV4 channels contribute to endothelial-dependent and especially shear stress–induced vasodilatation, we characterized TRPV4 channels in rat carotid artery ECs (RCAECs) and rat aorta ECs (RAECs) by using in situ patch-clamp techniques and single-cell RT-PCR analysis, FURA-II measurements, and pressure myography in CAs and small-sized \(A\).\textit{gracilis}. We show that pharmacological activation of endothelial TRPV4 induces robust vasodilatations in a NO- and EDHF-dependent manner. Moreover, we provide evidence that Ca\(^{2+}\) influx through TRPV4 is functionally important in the mechanisms of shear-stress–induced vasodilatation.

**Methods**

In situ patch-clamp experiments in rat ECs, “multiplex” single-cell RT-PCR, \([\text{Ca}^{2+}]_{i}\), measurements, and pressure myography were performed as described previously.\(^{18–21}\) For detailed methods, please see the online supplement, available at http://atvb.ahajournals.org.

**Results**

**Electrophysiological Characterization of TRPV4 in Rat Carotid Endothelium**

In whole-cell patch-clamp experiments in electrically uncoupled RCAECs of the endothelium in situ, 4αPDD (10\(^{-6}\) mol/L), the selective TRPV4 opener, activated moderately outward-rectifying currents (Figure 1A through D and 1F). Outward rectification of the current was more pronounced in the presence of extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{out}}\); Figure 1A and 1F) than at strongly buffered [Ca\(^{2+}\)]\(_{i}\) (Figure 1B and 1F). Inward currents were abolished after substitution of Na\(^{+}\) by the large nonpermeable cation NMDG\(^{+}\) (Figure 1B, left and right panels), indicating cation selectivity of this current. 4αPDD-activated TRPV4 currents reversed at a potential of \(\approx 0\) mV with 1 mmol/L or 20 mmol/L [Ca\(^{2+}\)]\(_{\text{out}}\). At high [Ca\(^{2+}\)]\(_{i}\) of 20 mmol/L, the current reversed at a more positive membrane potential of \(\approx 10\) mV (Figure 1C), demonstrating moderate preference for Ca\(^{2+}\) over Na\(^{+}\). Calculation of the relative Ca\(^{2+}\)/permeability ratio \((P_{\text{Ca}}/P_{\text{Na}})_{\text{p}}\) gave a \(P_{\text{Ca}}/P_{\text{Na}}\) value of \(\approx 6\), which is similar to \(P_{\text{Ca}}/P_{\text{Na}}\) values for cloned human and murine TRPV4.\(^{6}\)

Ruthenium red (RuR; 1 \(\mu\)mol/L), a blocker of TRPV channels,\(^{6,22}\) almost completely suppressed inward currents in a voltage-dependent fashion (Figure 1D), although longer exposure times (\(>20\) s) also reduced outward currents. Currents were also blocked by Gd\(^{3+}\) (50 \(\mu\)mol/L), a non-selective blocker of TRPs (data not shown). A similar 4αPDD-inducible current was observed in freshly isolated RAECs (Figure 1F).

The time course of 4αPDD-activated cation currents depended on the presence of \([\text{Ca}^{2+}]_{i}\). Whereas 4αPDD-induced currents were stable over time with low \([\text{Ca}^{2+}]_{i}\) (20 mmol/L; Figure 1B, right panel), currents showed a rapid decay (within 10 to 20 s), with 1 mmol/L [Ca\(^{2+}\)]\(_{i}\) (Figure 1A, left and right panels). However, in some RCAECs (\(n=4\)), we could observe current oscillation in the continuing presence of 4αPDD and with 1 mmol/L [Ca\(^{2+}\)]\(_{i}\) (Figure 1E). These observations may indicate that Ca\(^{2+}\) entry through the channel and the resulting increase of [Ca\(^{2+}\)] lead to channel inactivation. Reactivation of the channel may occur when [Ca\(^{2+}\)] returns to basal levels. This Ca\(^{2+}\) inactivation may point to a “negative-feedback” mechanism that has been proposed for cloned TRPV4 previously.\(^{6,23}\) In contrast to 4αPDD, capsaicin (10 \(\mu\)mol/L), an opener of TRPV1, did not produce any currents in RCAECs (data not shown).

Similar to cloned human and murine TRPV4 channels,\(^{24}\) 4αPDD-like currents in RCAECs were activated by superfusion with a warmed (37°C) solution (\(n=4\); data not shown) and by hypotonic stress (HTS)-induced cell swelling (Figure 1G, left panel). AA (1 \(\mu\)mol/L), which was reported to mediate HTS-induced TRPV4 activation, but not that by heat or 4αPDD,\(^{24}\) effectively activated TRPV4-like current in rat ECs (Figure 1G, right panel). Similar to 4αPDD-induced currents, HTS-induced and AA-induced currents were largely inhibited by RuR (Figure 1G, left and right panels). Moreover, inhibition of phospholipase A\(_{2}\) (PLA\(_{2}\)) by AACOCF, (4 \(\mu\)mol/L), and thus the prevention of AA release, precluded the activation of HTS-inducible TRPV4-like currents (Figure 1G, middle panel).

Subsequent to in situ patch-clamp experiments, single RCAECs were harvested with the patch pipette, and mRNA expression of TRPV channels was verified by “multiplex” single-cell RT-PCR\(^{25}\) (Figure 2). To ensure the selective harvest of RCAECs, cell samples were tested for mRNA expression of endothelial NO synthase (eNOS) as EC marker. Expression of myosin heavy chain (MyHC), as vascular smooth muscle cell marker, was not detectable (data not shown). TRPV4 mRNA was detected in 7 of 10 eNOS-positive and MyHC-negative RCAEC samples. Expression of other closely related members of the TRPV subfamily (ie, TRPV1 through TRPV3) was not detected in any of the cell samples. TRPV4 transcripts were not detected in freshly dissociated and MyHC-positive vascular smooth muscle cell samples (\(n=14\); data not shown).

**[Ca\(^{2+}\)]\(_{i}\) Transients Elicited by TRPV4 Activation in Rat ECs**

To determine TRPV4-mediated Ca\(^{2+}\) entry in single rat ECs, we switched for technical reasons to freshly isolated RAECs. 4αPDD (1 \(\mu\)mol/L) increased [Ca\(^{2+}\)]\(_{i}\) by \(\approx 140\) mmol/L above basal levels (60±5 mmol/L; \(n=50\); Figure 3A). The increase in [Ca\(^{2+}\)] peaked within \(\approx 10\) s and returned thereafter to levels slightly above baseline. After the initial peak, we frequently observed additional peaks of smaller amplitude.
Figure 1. Electrophysiological properties of TRPV4-like currents in RCAECs in situ. A, Left panel, Representative recording of 4αPDD (1 μmol/L)-induced currents with 1 mmol/L [Ca²⁺]ₜₒᵤₜ, reversal potential (indicated by arrow) was 1±1 mV (n=5). Note that activation is transient. Right panel, Time course of transient 4αPDD (1 μmol/L)-induced TRPV4 currents in the presence of [Ca²⁺]ₜₒᵤₜ (1 mmol/L). B, Left panel, Weaker current rectification with low [Ca²⁺]ₜₒᵤₜ (20 nmol/L). Nonpermeable NMDG abolishes inward currents at negative potentials, proving cation selectivity of this current. Right panel, Stable 4αPDD (1 μmol/L)-induced TRPV4 currents in the presence of low [Ca²⁺]ₜₒᵤₜ (20 nmol/L). C, 4αPDD (1 μmol/L)-induced Ca²⁺ currents at 0 mV with high [Ca²⁺]ₜₒᵤₜ (20 mmol/L); reversal potential: 10±2 mV; n=4. D, Inhibition of TRPV4 currents by RuR (1 μmol/L). E, Current oscillations in the continuing presence of 4αPDD and with 1 mmol/L [Ca²⁺]ₜₒᵤₜ. F, Normalized 4αPDD-induced currents in RCAECs and RAECs (ΔI [pA/pF]) with 20 nmol/L or 1 mmol/L [Ca²⁺]ₜₒᵤₜ. Note the smaller amplitude of inward currents at 1 mmol/L [Ca²⁺]ₜₒᵤₜ, which indicates that outward rectification is more pronounced at physiological [Ca²⁺]ₜₒᵤₜ than at strongly buffered [Ca²⁺]ₜₒᵤₜ ([Ca²⁺]ₜₒᵤₜ; 20 nmol/L). G, Left panel, Representative recordings showing activation of TRPV4 currents by HTS (n=10) and effects of RuR (1 μmol/L; n=5). Middle panel, Suppression of HTS-inducible currents in the presence the PLA₂ inhibitor ACOOCF₃ (4 μmol/L; n=6; middle panel). Right panel, Activation of TRPV4 currents by AA (10 μmol/L; n=6) and inhibition by RuR (1 μmol/L; n=6). Experiments were conducted at very low [Ca²⁺]ₜₒᵤₜ (20 nmol/L) to avoid Ca²⁺-entry mediated coactivation of Ca²⁺-activated channels.
4αPDD-induced [Ca²⁺]i transients were not observed in the presence of RuR (1 μmol/L; Figure 3B) or with strongly buffered [Ca²⁺]out (20 nmol/L; Figure 3C), thus demonstrating that 4αPDD-induced increases in [Ca²⁺]i are elicited by Ca²⁺ entry. This was further supported by our observation that 4αPDD application results in Mn²⁺ quenching of the FURA-II signal (Figure 3D). When compared with TRPV4-mediated increases in [Ca²⁺]i, acetylcholine (Ach; 1 μmol/L) induced a more pronounced increase in [Ca²⁺]i, by ∼600 nmol/L above basal levels that peaked more rapidly (within 2 s) and was followed by a prolonged plateau phase (Figure 3E and 3F) as a consequence of inositol triphosphate–triggered Ca²⁺ release from internal stores and subsequent Ca²⁺ entry. Similar to 4αPDD, HTS-induced Ca²⁺ mobilization was greatly reduced in the presence of RuR. In contrast, Ach-induced Ca²⁺ responses were unaffected by RuR (Figure 3F).

Functional Role of TRPV4 in Endothelium-Dependent Vasodilatation

To understand the functional role of TRPV4 in the mechanism of endothelium-dependent vasodilatation of CAs and small more resistance-like arteries, we conducted pressure myograph experiments in CAs and in small-sized A gracilis, respectively. Intraluminal application of 4αPDD caused robust and dose-dependent vasodilatations of CAs (80% at 1 μmol/L 4αPDD; KD of ∼0.3 μmol/L; Figure 4A and 4B) that were similar in magnitude to that induced by 1 μmol/L Ach (Figure 4A, left part). The vasodilatory responses to 4αPDD were strictly endothelium dependent because vasodilatation was abolished by endothelial inactivation, and extraluminal application of 4αPDD had no effect on vessel diameter (data not shown). Intraluminal application of capsaicin (10 μmol/L) or anandamide (10 μmol/L), known to activate TRPV1, did not induce vasodilatation (data not shown). 4αPDD-induced vasodilatation was completely antagonized by RuR (1 μmol/L; Figure 4A, left part), whereas Ach-induced vasodilatations were slightly reduced by RuR (Figure 4A, left part). Intraluminal application of RuR alone was without effect (data not shown). 4αPDD-induced vasodilatation was also eliminated by chelation of endothelial [Ca²⁺]i by BAPTA-AM (10 μmol/L; intraluminal preincubation for 10 minutes; 3±2% vasodilatation; n=3).
In the presence of inhibitors of both NO and prostacyclin synthesis or of the NO synthase inhibitor alone, 4aPDD induced only weak vasodilatation in CAs (Figure 4A, middle part), whereas ACh or pharmacological opening of endothelial IKCa1 and SKCa3 channel by 1-EBIO caused substantial EDHF-type vasodilatation in CAs (Figure 4A, middle part). The reported mechanosensitivity of TRPV4 channels may point to a putative role of TRPV4 in endothelial mechanosensing and thus in shear stress–induced vasodilatation. We therefore tested whether vasodilatation in response to an increase in fluid viscosity and thus shear stress is sensitive to the TRPV blocker RuR. As shown in Figure 4A

**Figure 3.** A. Increase in \([Ca^{2+}]_i\), after activation of TRPV4×4aPDD (1 \(\mu\)mol/L) in freshly isolated RAECs. B. RuR (1 \(\mu\)mol/L) in the bath solution. C. Strong buffering of \([Ca^{2+}]_o\) prevented 4aPDD-induced increases in \([Ca^{2+}]_i\). D. Application of 4aPDD evoked Mn\(^{2+}\) (1 mmol/L) quenching of FURA-II fluorescence (n=17), indicating that 4aPDD causes Ca\(^{2+}\) influx. As positive control, the Ca\(^{2+}\) ionophore ionomycin (1 \(\mu\)mol/L) caused additional strong quenching. E. ACh (1 \(\mu\)mol/L; n=10) induced increases in \([Ca^{2+}]_i\). F. Calculated mean increases in \([Ca^{2+}]_i\) (D\([Ca^{2+}]_i\), nmol/L) above basal levels after stimulation with 4aPDD in the absence (n=25) and in the presence of RuR (1 \(\mu\)mol/L; n=3) or at low \([Ca^{2+}]_o\) (n=10), with warmth (37°C; n=14), with HTS in the absence (n=10) and in the presence of RuR (1 \(\mu\)mol/L; n=22), with ACh in the absence (n=10) and in the presence of RuR (1 \(\mu\)mol/L; n=6).
The increase in shear stress by 3 dyne/cm² elicited substantial vasodilatation by H11015 17%. Similar to 4/PD-induced vasodilatation, the shear stress–induced vasodilatation was reduced greatly in the presence of 1/H9262 mol/L RuR (Figure 4A, right part) or by chelation of [Ca²⁺]i by BAPTA-AM (3/H11006; n=3). Similar to the increase in viscosity, a 2-fold increase in flow rate (n=4) caused vasodilatation by 16±2% (n=4), which was also suppressed by RuR (5±1%; n=4).

The shear stress–induced vasodilatation of CAs was mediated mainly by NO because shear stress–induced vasodilatation was suppressed greatly by either the combination of the NO synthase blocker L-NNA and the cyclooxygenase (COX) inhibitor indomethacin (INDO) or L-NNA alone (Figure 4A, right part).

In keeping with the idea that TRPV4 channels in RCAECs are activated by AA as a possible endogenous activator of the channel, especially after mechanical stimulation, we tested whether inhibition of PLA₂ or thus prevention of AA generation affect shear stress–induced vasodilatation. After intraluminal preincubation with the PLA₂ inhibitor AACOCF₃ (4 mol/L), shear stress–induced vasodilatation was almost completely abolished (Figure 4A, right part). Inhibition of PKC by calphostin-C (0.5 mol/L; Figure 4A, right part) or of COX by INDO (10 mol/L; data not shown) did not reduce shear stress–induced vasodilatations, indicating that activation of these pathways is not required for vasodilatation in response to increased shear stress. None of these compounds (at the concentrations used) blocked 4/PD-induced vasodilatation of CAs was mediated mainly by NO because shear stress–induced vasodilatation was suppressed greatly by either the combination of the NO synthase blocker L-NNA and the cyclooxygenase (COX) inhibitor indomethacin (INDO) or L-NNA alone (Figure 4A, right part).
latation (data not shown). These results indicate that shear stress–induced vasodilatations of CAs require PLA₂ activation. The release of AA may then lead to activation of TRPV4 as also shown in patch-clamp experiments in RCAECs.

In another set of experiments, we tested whether TRPV4-mediated vasodilatation is also present in small arteries that are considered more important in regulating blood pressure. We therefore conducted pressure myograph experiments in small A gracilis with spontaneous myogenic tone (diameter of ≈200 μm) using the same stimulation protocols. Similar to the vasodilatory response in CAs, intraluminal but not extraluminal application of 1 μmol/L 4αPDD induced a robust and almost complete vasodilatation in these small A gracilis (Figure 4C, left part). Furthermore, 1 μmol/L RuR antagonized this vasodilatation effectively. ACh-induced vasodilatations were not significantly reduced by RuR. In the presence of l-NNA or of l-NNA and INDO, 4αPDD was still able to cause almost complete vasodilatation in these small vessels (Figure 4C, left part).

Regarding shear stress–induced vasodilatation in these vessels, the increase in shear stress elicited by adding 5% dextran to the perfusion medium resulted in more pronounced vasodilatation by 73% than in CAs (15%). This difference in the magnitude of vasodilatation might be explained by the higher degree of shear stress exerted by 5% dextran (~15 dyne/cm²) in these small vessels than in large-sized CAs (~3 dyne/cm²). Similar to CAs, shear stress–induced vasodilatation in A gracilis was suppressed greatly by 1 μmol/L RuR or 100 μmol/L l-NNA (Figure 4C, right part).

Thus, these findings show that the presumed TRPV4-mediated vasodilatation, induced either pharmacologically or mechanically, is also present in small arteries. Moreover, shear stress–induced vasodilatation is exclusively mediated by NO, whereas pharmacological opening of TRPV4 is capable to produce considerable EDHF responses in small arteries.

Discussion

In the present study, we investigated the function and expression of the TRPV4 channel in rat endothelium and its functional role in the mechanisms of endothelium-dependent vasodilatation. We provide evidence that pharmacological activation of endothelial TRPV4 and subsequent Ca²⁺ influx triggers NO-mediated vasodilatation. Moreover, we propose that mechanical activation of TRPV4 by shear stress might be an important component of endothelial mechanotransduction and thus of shear stress–induced vasodilatation.

We obtained the following evidence that the cation current described here is mediated by TRPV4: (1) The current was activated by 4αPDD, which is considered a selective opener of the TRPV4 channels, and does not affect the function of any other TRP or other channel and enzymes as known so far. Moreover, in mice lacking TRPV4, 4αPDD-induced currents as well as Ca²⁺-entry is absent, which further supports the selectivity of 4αPDD. (2) The endothelial TRPV4-like current in RCAECs exhibited a Ca²⁺-dependent outward rectification and Ca²⁺-dependent inactivation similar to cloned TRPV4. (3) The endothelial TRPV4-like current exhibited moderate Ca²⁺ selectivity with a Pₐ/Pₙa of ≈6, similar to clon TRPV4. (4) Endothelial TRPV4-like current was sensitive to RuR, which is considered a fairly selective blocker of TRPV channels within the TRP gene family. (5) Similar to cloned human and murine TRPV4, the TRPV4-like current in RCAECs was activated by moderate warmth, mechanically by cell swelling, and directly by AA as the potential endogenous mediator of mechanical TRPV4 activation. (6) Activation of rat TRPV4 by either pharmacologically or other physical stimuli caused significant Ca²⁺ entry.

Moreover, our in situ single-cell RT-PCR analysis revealed mRNA expression of TRPV4 in RCAECs but of none of the other closely related members of this TRPV subfamily.

Regarding the functional role of TRPV4 in endothelium-dependent vasodilatation, our myograph experiments revealed that pharmacological opening of TRPV4 caused a robust vasodilatation in both small-sized (A gracilis) and large CAs. It is noteworthy that this 4αPDD-induced vasodilatation was almost as large as that achieved by physiological relevant concentrations of ACh. The 4αPDD-induced vasodilatation required a functionally intact endothelium, which indicates that the 4αPDD-induced vasodilatation is indeed caused by opening of endothelial TRPV4. Moreover, 4αPDD elicited vasodilatation with a Kᵣ of ≈0.3 μmol/L, which is comparable to the Kᵣ reported for TRPV4 activation. 4αPDD-induced vasodilatation was prevented by buffering endothelial [Ca²⁺], and by the TRPV4 channel blocker RuR, indicating that 4αPDD exerts its vasodilating effect by inducing Ca²⁺ influx and subsequently synthesis of endothelial vasodilators. RuR modestly reduced ACh-induced vasodilatation, indicating that endothelial TRPV4 does not contribute substantially to agonist-induced Ca²⁺ signaling and vasodilatation. Perfusion with RuR was without effect on basal vessel diameter, which suggests that TRPV4 is not involved in the basal control of vascular tone. This may also indicate that the rather nonselective blocker RuR does not exert gross unspecific effects or other effects caused by blocking ryanodine-sensitive Ca²⁺ release channels in smooth muscle.

In CAs with a functionally active endothelium, inhibition of NO synthase alone or in combination with blockade of prostacyclin synthesis almost completely suppressed TRPV4-mediated vasodilatation, suggesting that this type of vasodilatation largely relies on the Ca²⁺-dependent synthesis and action of NO after TRPV4-mediated Ca²⁺ influx, whereas the other 2 major vasodilator systems (ie, the prostacyclin system or the EDHF system) do not seem to make a significant contribution in this CA. Regarding EDHF-mediated vasodilatation, Ca²⁺-dependent activation of endothelial Kᵥα₇ channels of the IKCa1 and SKCa3 type and subsequent endothelial hyperpolarization have been considered a prerequisite for the generation of the EDHF signal in many vessels and species including rat CAs. Moreover, EDHF-type vasodilatations have been shown to become more important when vessel size decreases. In the large CAs, in which the EDHF system is apparently less important than the NO-system, 4αPDD-induced TRPV4 activation did not cause major EDHF-mediated vasodilatation. In contrast, 4αPDD was able to produce EDHF-mediated vasodilatation in small-sized A gracilis. Therefore, pharmacological opening of TRPV4 ap-
pears to be sufficient to induce EDHF-type vasodilatation in small-sized arteries, in which EDHF plays a significant role.

In keeping with the proposed mechanosensitivity of TRPV4,1,7-9 we speculated that TRPV4 activation and Ca2+ entry may occur by mechanical stimulation of the endothelium by increased fluid viscosity and thus shear stress. In this regard, shear stress– or flow-induced increases in [Ca2+], attributable to both Ca2+ influx and Ca2+ release from internal stores have been observed in cultured ECs17,18 as well as in endothelium of perfused vessels.16 It is noteworthy that such a shear stress–induced increase in [Ca2+] is prevented by strongly buffering extracellular Ca2+ or by the MSC and TRP blocker Gd3+/H11001, indicating that an increase in shear stress activates a “directly” or “indirectly” mechanosensitive Ca2+ entry channel.

In the present study, we found that an increase in shear stress caused vasodilatation of rat CAs and of small-sized A. gracilis in a strictly NO-dependent fashion, which is in agreement with findings in arteries of humans20 and other species,30 whereas in mice, both prostaglandins as well as NO mediate this type of vasodilatation.31 Similar to 4αPDD-induced vasodilatation, shear stress–induced vasodilatation in rat CAs was greatly blocked by the TRPV4 inhibitor RuR as well as by buffering endothelial [Ca2+] with BAPTA-AM, suggesting an involvement of TRPV4 in this response. Inhibition of PKC and of TK was without effect, suggesting that protein phosphorylation or potential TRPV4 phosphorylation by one of these kinases does not seem to play a major role in shear stress–induced vasodilatation or activation.

Importantly, shear stress–induced vasodilatation was prevented by inhibition of PLA2 and thus AA release in rat CAs. Release of AA and production of AA metabolites in response to flow is well documented in cultured ECs,32 and a role of AA metabolites in flow-induced vasodilatation has been proposed previously.31 With respect to TRPV4, exogenous- and endogenously applied AA have been shown to activate rat TRPV4, as shown here, and cloned TRPV4 previously,5 and endogenously produced AA mediates mechanical activation of cell swelling.24 These roles of AA in both shear stress–induced vasodilatation and TRPV4 activation tempted us to speculate that PLA2-mediated AA release after shear stress stimulation mediates TRPV4 activation. This interpretation also implies that TRPV4 is unlikely to be the mechanosensor per se. Nonetheless, AA-dependent activation of TRPV4 might be an essential component in the signal transduction mechanism of endothelial mechanotransduction.

Collectively, this set of data strongly suggests that Ca2+ entry through endothelial TRPV4 channels triggers NO-dependent vasodilatation in endothelium of rat CAs and NO- and EDHF-dependent vasodilatation of small-sized A. gracilis (more resistance-like artery). Moreover, we provide evidence that endothelial TRPV4 channels are involved in endothelial mechanosensing of shear stress–induced vasodilatation. Thus, among the numerous TRP channels expressed in endothelium, a role of TRPV4 might be specifically assigned to endothelial mechanotransduction. Moreover, because pharmacological opening of TRPV4 causes robust vasodilatation, endothelial TRPV4 may represent a novel pharmacological target for the treatment of hypertension.

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Disclosure(s)

None.

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Methods

Rat carotid artery and aortic endothelial cells

Freshly isolated CA from male Sprague-Dawley rats (350-400 g) were cut open longitudinally and fixed on a holding capillary to give direct access to the luminal surface. For \textit{in situ} harvesting of EC and whole-cell patch-clamp experiments, vessel slices were pre-incubated with 0.05 % trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS) without Ca\textsuperscript{2+}/Mg\textsuperscript{2+} for up to 15 min\textsuperscript{1}. For wash out, CA were superfused with PBS for 5 min. Under microscopic control, a single EC of carotid artery (RCAEC) was selectively fixed with the patch pipette and mechanically detached from the vessel wall. Whole-cell patch-clamp experiments were directly performed in these cells. For Ca\textsuperscript{2+} measurements and additional whole-cell patch-clamp experiments, rat aortic endothelial cells (RAEC) were freshly isolated as described previously\textsuperscript{2}. Cells were allowed to attach for 2-3 h and were immediately used for Ca\textsuperscript{2+} measurements thereafter. VSMC of CA were isolated as described previously\textsuperscript{1}.

\textit{In situ} Patch-clamp Experiments

Membrane currents in RCAEC of CA slices were recorded with an EPC-9 (HEKA) patch-clamp amplifier using voltage ramps (duration: 1000 ms) from -130 or -100 mV to +100 mV as described previously\textsuperscript{3}. RCAEC cells used for patch clamp measurements were mechanically detached and thus electrically “isolated” from the surrounding and tightly electrically coupled EC of the endothelial layer. This “in-situ patch clamp approach” allows the direct measurement of currents in the EC of the intact carotid artery without the necessity of standard cell isolation procedures which bear a risk of measuring artifacts due to cell culturing. Patch pipettes had a tip resistance of 2-4 MΩ in symmetrical KCl solution. If not otherwise stated, the standard pipette solution was composed of (in mmol/L): 20 CsCl, 100
cesium aspartate, 1 MgCl₂, 4 Na₂ATP, 10 EGTA, 0.9 CaCl₂, 10 HEPES, pH adjusted to 7.2 with CsOH; calculated free [Ca²⁺] was 0.02 µmol/L. The standard NaCl bath solution contained (mmol/L): 137 NaCl, 4.5 Na₂HPO₄, 3 KCl, 1.5 KH₂PO₄, 0.4 MgCl₂, 10 glucose, and 1 CaCl₂ (pH 7.4). In other sets of experiments, the bath solution was prepared with (mmol/L): 10 EGTA and 1 CaCl₂, or with 20 CaCl₂, 110 NaCl and 10 HEPES instead of phosphates; for cation substitution protocols, N-methyl-D-glucamine chloride (140 mmol/L) substituted for NaCl. In experiments employing hypotonic stress, isotonic and hypotonic bath solutions consisted of (mmol/L): 110 NaCl, 10 glucose, 10 HEPES, pH 7.4. The isotonic solution contained additionally 95 mmol/L mannitol. All experiments were performed at RT. Data analysis was performed as described previously. Relative cation selectivity of currents and the relative Ca²⁺ permeability (P_Ca/P_Na) were calculated as described elsewhere.

Reverse Transcription and single-cell RT-PCR

Reverse transcription of mRNA from single cell samples and "multiplex" single cell RT-PCR were performed as described previously. An additional reason to choose this approach was that a selective antibody against rat TRPV4 is not available so far. Primer pairs for endothelial nitric oxide synthase (reNOS) as endothelial cell marker were stated elsewhere. First and ‘nested’ primer pairs for all rTRPV spanned intronic sequences and identity of PCR products was verified by sequencing. Functionality of primer pairs was proven by amplification of TRPV1-4 from brain and liver cDNAs. Primer:

*rTRPV1*: F5’-AGCTGAAAAACACCGTGGGG-3’; R5’-GCTGGAATCCTCGGGTATAGTAGAG-3’; nested: F5’-CTTCTTCTCCGGATTCA-3’; R5’-GAGAACACCATGGAAGCCACAT-3’; (GenBank™ accession: NM_031982);

*rTRPV2*: F5’-ACAGTCCTGCTGCTCTGGTAA-3’; R5’-TGTCCACAGGGACAGGCTGTA-3’; nested: F5’-TTGCAGATAACTCGCCTGAGA-
3'; R5'-CCGCACAGGACCAGTAACAC-3' (GenBank accession: NM_017207).

*rTRPV3*: F5'-GGCGAACATGCTCTACTACACG-3'; R5'-GTAGGTGGAGTTCTGCTGGATG-3'; nested: F5'-CTTCCAGTCTATGGGCATGTACAG-3'; R5'-CACCAGCATCGCTGAAGCT-3' (GenBank accession: XM_573134);

*rTRPV4*: F5'-ACAACACCCGAGAGAACACCAA-3'; R5'-AAGAATACACAGGCCCGTAGGC-3'; nested: F5'-TGACCTGTTGCTTCTCAAGTGC-3'; R5'-TTGAACCTTTGAGACAGGTGCT-3' (GenBank accession: NM_023970.1).

**[Ca\(^{2+}\)]** \(_i\) Measurements

\([Ca^{2+}]_i\) was measured with a monochromator-based imaging system consisting of a Polychrome IV monochromator and a PCO SensiCam CCD-camera (Till Photonics, Martinsried, Germany) connected to an Axiovert 135 inverted microscope (Zeiss, Göttingen, Germany). RAEC were loaded with the Ca\(^{2+}\)-sensitive indicator fura-2-acetoxymethylester (5 \(\mu\)mol/l) for 15 min at 37°C. As a measure of \([Ca^{2+}]_i\), the fluorescence emission ratio at 340/380 nm excitation wavelength was calculated after subtraction of the background and auto fluorescence. Fura-2 signals were calibrated and the calculation of absolute changes in \([Ca^{2+}]_i\) was done as described previously\(^2\).

**Pressure Myography**

Pressure myography in CA were performed as described previously\(^1\). Bath and perfusion solution contained (in mmol/L): 145 NaCl, 1.2 NaH\(_2\)PO\(_4\), 4.7 KCl, 1.2 MgSO\(_4\), 2 CaCl\(_2\), 5 glucose, 2 pyruvate, and 3 MOPS buffer (pH 7.4 at 37°C). CA were pressurized to 80 mmHg and were lengthened up to their in vivo length, and continuously perfused at a flow rate of 0.6 ml/min and at a constant intraluminal pressure. After an equilibration period for 30 min, CA were pre-constricted with 1 \(\mu\)mol/L phenylephrine in the bath solution. After
development of stable tone, CA were perfused with 4αPDD (10 nmol/L - 10 µmol/L), 1-ethyl-2-benzimidazolinone (1-EBIO, 100 µmol/L; both from TOCRIS (Köln, Germany), or acetylcholine (ACh, 1 µmol/L) in the presence and absence of the NO-synthase-inhibitor N\textsuperscript{G}-nitro-L-arginine (L-NNA, 100 µmol/L) and the cyclooxygenase inhibitor indomethacin (INDO, 10 µmol/L). For functional inactivation of the endothelium, the vessel lumen was exposed to air for 10 min and endothelial inactivation was confirmed by the absence of ACh-inducible vasodilatation.

For induction of WSS-mediated vasodilatation, the viscosity of the perfusion medium was increased (from 0.7 to 2.9 mPa*s) by adding 5% dextran which enhanced WSS in CA from 1 to 3 dyne/cm\(^2\) as mathematically estimated according to the law of Hagen-Poiseuille: \(\tau=4\eta Q/\pi r^3;\) \(\tau=\)shear stress; \(\eta=\)viscosity; \(Q=\)flow, and \(r=\)radius. Diameter changes were expressed as a percentage of the maximal dilatation induced by 1 µmol/L sodium nitroprusside (SNP). Standard chemicals were obtained from Sigma-Aldrich (München, Germany). Inhibitors of phospholipase A2 (PLA\(_2\)), AACOCF\(_3\) (4 µmol/L), of PKC, calphostin-C (Cal-C, 0.5 µmol/L), and of tyrosine kinases (TK), genistein (10 µmol/L) were obtained from TOCRIS. BAPTA-AM (10 µmol/L) was obtained from Invitrogen (Karlsruhe, Germany). CA were pre-incubated with the respective compound for 10 min before stimulation. At the indicated concentrations none of these substances caused any non-specific vasodilating or vasocontracting effects.

Pressure myography in rat A. gracilis were performed as described previously in more detail \(^5\). In brief, A. gracilis were pressurized to 80 mmHg, were lengthened up to their \textit{in vivo} length, perfused at a flow rate of \(\approx35\ \mu l/min\), and allowed to develop spontaneous (basal) myogenic tone for 30 min (\(\approx30\%\) reduction in vessel diameter, 210 - 290 µm in the fully relaxed state). Bath and perfusion solution consisted of (mmol/L): 120 NaCl, 4.5 KCl, 1.2 NaH\(_2\)PO\(_4\), 1 MgSO\(_4\), 1.6 CaCl\(_2\), 0.025 EDTA, 5.5 glucose, 26 NaHCO\(_3\), 5 HEPES, at pH 7.4. Vasodilatory responses to 4αPDD (1 µmol/L) and 5% dextran were determined as stated
above. The increase in fluid viscosity enhanced WSS in A. gracilis from ≈ 9 to 18 dyne/cm².

The involvement of PLA₂ in shear stress-induced vasodilatation in A. gracilis could not be tested in these vessels since intraluminal application AACOCF₃ (1 - 4 μmol/L) caused a loss of myogenic tone.

Statistical analysis

Data are given as mean ± SE. The unpaired Students t-Test was used to assess differences between groups. P-values of <0.05 were considered significant.

References: