# Modulation of the Ca2 Permeable Cation Channel TRPV4 by Cytochrome P450 Epoxygenases in Vascular Endothelium

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*Circ. Res.* 2005;97;908-915; originally published online Sep 22, 2005;
DOI: 10.1161/01.RES.0000187474.47805.30
Circulation Research is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7330. Online ISSN: 1524-4571

# Modulation of the $Ca^{2+}$ Permeable Cation Channel TRPV4 by Cytochrome P450 Epoxygenases in Vascular Endothelium

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***Abstract***—TRPV4 is a broadly expressed $Ca^{2+}$-permeable cation channel in the vanilloid subfamily of transient receptor potential channels. TRPV4 gates in response to a large variety of stimuli, including cell swelling, warm temperatures, the synthetic phorbol ester 4$\alpha$-phorbol 12,13-didecanoate (4$\alpha$-PDD), and the endogenous lipid arachidonic acid (AA). Activation by cell swelling and AA requires cytochrome P450 (CYP) epoxygenase activity to convert AA to epoxyeicosatrienoic acids (EETs) such as 5,6-EET, 8,9-EET, which both act as direct TRPV4 agonists. To evaluate the role of TRPV4 and its modulation by the CYP pathway in vascular endothelial cells, we performed $Ca^{2+}$ imaging and patch-clamp measurements on mouse aortic endothelial cells (MAECs) isolated from wild-type and TRPV4$^{-/-}$ mice. All TRPV4-activating stimuli induced robust $Ca^{2+}$ responses in wild-type MAECs but not in MAECs isolated from TRPV4$^{-/-}$ mice. Upregulation of CYP2C expression by preincubation with nifedipine enhanced the responses to AA and cell swelling in wild-type MAECs, whereas responses to other stimuli remained unaffected. Conversely, inhibition of CYP2C9 activity with sulfaphenazole abolished the responses to AA and hypotonic solution (HTS). Moreover, suppression of EET hydrolysis using 1-adamantyl-3-cyclo-hexylurea or indomethacin, inhibitors of soluble epoxide hydrolases (sEHs), and cyclooxygenases, respectively, enhanced the TRPV4-dependent responses to AA, HTS, and EETs but not those to 4$\alpha$-PDD or heat. Together, our data establish that CYP-derived EETs modulate the activity of TRPV4 channels in endothelial cells and shows the unraveling of novel modulatory pathways via CYP2C modulation and sEH inhibition. **(*Circ Res*. 2005;97:908-915.)**

**Key Words:** TRP channels ■ endothelium ■ epoxygenases ■ epoxide hydrolases

TRPV4 is a $Ca^{2+}$ entry channel belonging to the vanilloid subfamily of the transient receptor potential (TRP) channels.[1,2] It is expressed in a broad range of tissues, including lung, spleen, kidney, testis, fat, brain, cochlea, skin, smooth muscle, liver, and vascular endothelium.[3–5] The physiological role of TRPV4 in these tissues may be highly divergent because TRPV4 is able to respond to a wide variety of physical, thermal, and chemical stimuli.

Initially, TRPV4 was put forward as a mechanosensor or osmosensor. This was based on the finding that the channel opens in response to hypotonic cell swelling[3–8] as well as shear stress.[9] Indeed, mice lacking the TRPV4 gene show a disturbance of osmoregulation and an increased mechanical nociceptive threshold.[10,11] Furthermore, TRPV4 functions as a transducer of hypo-osmotic stimuli in primary afferent nociceptors[7] and plays an essential role in taxol-induced nociceptive behavioral responses to mechanical and hypotonic stimulation of the hind paw.[12]

More recently, it was found that TRPV4 can also be activated by heating,[13,14] and that it is required for normal thermal responsiveness in vivo. The TRPV4$^{-/-}$ mice "select" a warmer floor temperature in a gradient apparatus than their wild-type littermates and exhibit prolonged withdrawal latencies during acute tail heating.[15] Moreover, TRPV4 can be activated by a large variety of chemical stimuli, including low pH and citrate[11] and the synthetic phorbol-derivative 4$\alpha$-phorbol 12,13-didecanoate (4$\alpha$-PDD), which can be considered the most robust and direct channel activator.[16] Recently, we have shown that arachidonic acid (AA) and the endocannabinoid anandamide (arachidonoylethanolamide) can act as endogenous activators of TRPV4. AA and anandamide act via the cytochrome P450 (CYP) epoxygenase-dependent formation of epoxyeicosatrienoic acids (EETs; 5,6-EET and 8,9-EET), which act as direct channel activators.[17]

EETs are known to have antihypertensive and anti-inflammatory properties and play a role in the maintenance of

Original received June 8, 2005; revision received September 7, 2005; accepted September 12, 2005.

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This manuscript was sent to Hans Michael Piper, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

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*Circulation Research* **is available at http://circres.ahajournals.org** **DOI: 10.1161/01.RES.0000187474.47805.30**

renal vascular function.[18] They have been identified as endothelium-derived hyperpolarizing factors[19,20] and elicit vasodilatation attributable to hyperpolarization by activating smooth muscle $Ca^{2+}$-activated potassium channels.[21,22] Although $Ca^{2+}$-activated $K^+$ channels play a central role in mediating many of the effects of EETs in vascular tissue, other molecular targets for EETs may exist. Therefore, the main objective of our work was to evaluate TRPV4 as a possible EET-regulated $Ca^{2+}$ entry channel in mouse aorta endothelium. We report here that pharmacological agents that affect CYP epoxygenase activity and EET metabolism can efficiently modulate TRPV4, a target for EETs in endothelial cells.

## Materials and Methods

### Cell Culture, Transfection, and TRPV4$^{-/-}$ Mouse

Mouse aortic endothelial cells (MAECs) were isolated using the "primary explant technique" described in detail previously.[23] For all manipulations and measurements, only MAECs from the first and the second passage were used. The TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice[11] were maintained on ad labium diet chow and tap water for drinking and used at 16 weeks of age for isolation of endothelial cells. For the CYP2C expression experiments, mice aortas were isolated, cleaned of adherent connective tissue, and cut into rings, which were incubated in culture medium containing either solvent or nifedipine (0.1 $\mu$mol/L) for 18 hours. Thereafter, the rings were recovered and endothelial cell proteins harvested by shaking in Triton X-100 lysis buffer for 30 minutes at 4°C. EA.hyb926 cells,[24] which are derived from human umbilical vein endothelial cells (HUVECs), were transiently transfected with the murine TRPV4 (mouse mTRP12; accession number CAC20703) vector, and cloned as a *Bam*HI fragment into the *Bcl*I site of the pCAGGS/IRES-GFP vector, which allows detection of transfected cells based on their green fluorescence when illuminated at 475 nm. For transfection, we used GeneJuice Transfection Reagent (Novagen). Green fluorescent negative cells from the same batch were used as controls.

### RNA Extraction, cDNA Synthesis, and Analysis

Detection of TRPV4 mRNA expression in MAECs was performed as described.[25] Briefly, total RNA from MAECs was isolated with the RNeasy kit (Qiagen) and cDNA was synthesized using Ready-to-Go You-Prime First-Strand Beads (Amersham Biosciences), according to the manufacturer recommendations. Specific TRPV4 and $\beta$-actin fragments were amplified in a 50-$\mu$L polymerase chain reaction (PCR) with 25 pmol/L of each of the primers 5′-GAT GGA GGA GAA AGG TCG TG and 5′-GAG AAC TGT CTC CAG GTT GC (for amplification of a 733-bp TRPV4 fragment) or 25 pmol/L of each of the primers 5′-GTC GTC GAC AAC GGC TCC GGC and 5′-CAC AGC CTG GAT GGC TAC GTA C (for amplification of a 350-bp $\beta$-actin fragment), one tenth of cDNA synthesis reactions, and Taq DNA polymerase (Amersham Biosciences), according to manufacturer protocol.

### Protein Extraction and Immunodetection

Isolated MAECs grown in standard medium on Matrigel (Becton Dickinson) were collected by centrifugation at 500*g* for 5 minutes at 4°C (all subsequent steps are performed at 4°C), resuspended in 3 mL ice-cold lysis buffer (50 mmol/L Tris, pH 7.5, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride [PMSF]), and a protease inhibitor cocktail (10 $\mu$g/mL leupeptin and antipain and 2 $\mu$g/mL chymostatin and pepstatin). After rotation on an end-over-end stirrer for 30 minutes, cells were subjected for 3 freeze–thaw cycles and then were passed 10 times through a 26-gauge needle. Normal osmolarity of resulted lysates were restored by supplementation with 150 mmol/L NaCl and then they were centrifuged at 4000*g* for 15 minutes to remove nuclei, mitochondria, and any remaining large cellular fragments. Obtained supernatants were centrifuged at 20 000*g* for 20 minutes and then 100 000*g* for 1 hour, yielding low-speed ($P_{20/20}$) and high-speed ($P_{100/60}$) pellets of membrane fractions, which were solubilized in a cold PBS (10 mmol/L phosphate buffer, pH 7.4, 137 mmol/L NaCl, and 2.7 mmol/L KCl,) containing 1% Triton X-100, 0.2% sodium dodecyl sulfate (SDS), 1 mmol/L PMSF, and a protease inhibitor cocktail. Protein concentrations were determined by the bicinchoninic acid assay method, using BSA as a standard. Samples (2 $\mu$g) were solubilized in 5 $\mu$L of 3-fold concentrated sample buffer (240 mmol/L Tris, pH 6.8, 30% glycerol, 6% SDS, 3% dithiothreitol, and 0.015% bromophenol blue) by heating to 56°C for 15 minutes and then subjected to 8% SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) and probed with purified polyclonal rabbit anti-TRPV4 (1:1000 dilution)[13] or anti–secretory pathway $Ca^{2+}$-ATPase 1 (SPCA1; 1:3000 dilution)[26] antibodies for 1 hour at room temperature. Immunoreactive complexes were visualized by chemiluminescence using anti-rabbit IgG antibodies conjugated to horseradish peroxidase (1:4000 dilution; Amersham Biosciences).

For immunodetection of CYP2C, MAECs were lysed in a buffer containing 50 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl, 100 mmol/L NaF, 15 mmol/L $Na_4P_2O_7$, 2 mmol/L $Na_3VO_4$, 1% (v/v) Triton X-100, 44 $\mu$g/mL PMSF and the protease inhibitor cocktail, left on ice for 10 minutes and centrifuged at 10 000*g* for 10 minutes. Proteins (50 $\mu$g) were subjected to SDS-PAGE as described above, and the CYP2C specific bands were visualized using a polyclonal antibody raised in rabbit against CYP2C9 (Abcam). To ensure equal protein loading, the blots were reprobed with polyclonal anti-platelet/endothelial cell adhesion molecule-1 (PECAM-1) antibody raised in goat (Santa Cruz Biotechnology). The detection of the specifically bound antibodies was performed as described above.

### Solutions

For electrophysiological measurements, the standard extracellular solution contained (in mmol/L): 150 NaCl, 6 CsCl, 1 $MgCl_2$, 5 $CaCl_2$, 10 glucose, and 10 HEPES, buffered at pH 7.4 with NaOH. The osmolarity of this solution, as measured with a vapor pressure osmometer (5 Wescor 5500; Schlag), was 320±5 mosmol/L. When measuring swelling-activated responses, we used an isotonic solution containing (in mmol/L): 105 NaCl, 6 CsCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 90 D-mannitol, 10 glucose, and buffered pH 7.4 with NaOH (320±5 mosmol/L). Cell swelling was induced by omitting mannitol from this solution (240 mosmol/L; 25% reduction of osmolarity).

The pipette solution was composed of (in mmol/L): 20 CsCl, 100 Asp, 1 $MgCl_2$, 10 HEPES, 4 $Na_2ATP$, 10 BAPTA, and 5.56 $CaCl_2$. The free $Ca^{2+}$ concentration of this solution is ≈200 nmol/L. The non-protein kinase C–activating phorbol ester (Sigma Aldrich) was applied at a 1-$\mu$mol/L concentration from a 10-mmol/L stock solution in ethanol. AA (Sigma) was used at a concentration of 3 $\mu$mol/L from a 10-mmol/L stock solution in ethanol, and 5,6-EET and 8,9-EET (Biomol) were applied at a concentration of 500 nmol/L and 1 $\mu$mol/L, respectively. Nifedipine (2 $\mu$mol/L; Sigma Aldrich), sulfaphenazole (1 $\mu$mol/L; Sigma Aldrich), 1-adamantyl-3-cyclohexylurea (ACU; 2 $\mu$mol/L), and indomethacin (2 $\mu$mol/L, Sigma Aldrich) were applied to the extracellular medium for 18 hours before the application of the different activation stimuli. ACU was synthesized by standard procedures, and high purity was supported by proton and carbon nuclear magnetic resonance, high-performance liquid chromatography (HPLC)–mass spectrometry, HPLC-time of flight, and other means.[27] Indomethacin was dissolved in 1% Na bicarbonate to an end concentration of 10 $\mu$mol/L. In the case of activation by heat, the perfusate was warmed using a water jacket device. We changed the temperature in the bath from 22°C to 43°C in 50 seconds. Other experiments were performed at room temperature (22°C to 25°C).

### Electrophysiological Recordings and $Ca^{2+}$ Measurements

Whole-cell membrane currents and $Ca^{2+}$ measurements were measured as described in detail previously.[8] For every condition, at least

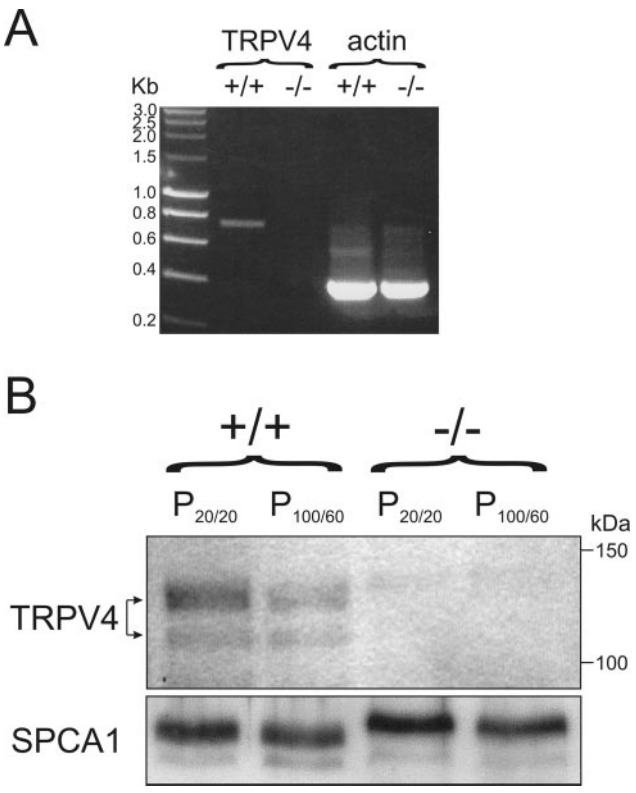


**Figure 1.** Expression of TRPV4 in MAECs. A, RT-PCR analysis of TRPV4 transcripts in MAECs isolated from TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice. Amplification of actin was used as positive control for the experiment. B, Immunodetection of TRPV4 protein in low- ($P_{20/20}$) and high-speed pellet ($P_{100/60}$) fractions (2 $\mu$g/lane) obtained by a differential centrifugation from MAEC lysates isolated from TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice. Proteins were separated by SDS-PAGE and TRPV4 was detected by Western blotting with an antibody raised against the C-terminus of mouse TRPV4. Equal loading of proteins was assessed by reblotting with an antibody against SPCA1.

20 cells from at least 3 independent experiments were assayed. The calibration procedure is described previously.[8]

### Data Analysis

Electrophysiological data were analyzed using WinASCD software (Guy Droogmans). The Origin 6.1 software package (OriginLab Corporation) was used for statistical analysis and data display. Data are expressed as the mean±SEM. Statistics on $Ca^{2+}$ increases are done on peak responses. Significance between individual groups was tested using Student's *t* test for "normal" data and the nonparametric and distribution-free Kolmogorov–Smirnoff Test for "non-normal" data.

## Results

### TRPV4 Is Expressed in MAECs

To confirm the expression of TRPV4, we first performed RT-PCR analysis on mRNA extracted from MAECs isolated from wild-type (TRPV4$^{+/+}$) and TRPV4-deficient (TRPV4$^{-/-}$) mice. In the TRPV4-deficient mouse, a PKG-neo cassette has been inserted into exon 4 of the *TRPV4* gene, resulting in the premature termination and the loss of TRPV4 expression.[11] As shown in Figure 1A, the TRPV4 fragment was amplified from TRPV4$^{+/+}$ MAECs but not from TRPV4$^{-/-}$ MAECs. Subsequently, we examined TRPV4 expression in MAECs at the protein level. Using a differential centrifugation protocol, we prepared low-speed ($P_{20/20}$) and high-speed ($P_{100/60}$) membrane fractions from MAECs isolated from TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice. The $P_{20/20}$ fraction comprised the plasma membrane together with a part of large intracellular membrane fragments (the plasma membrane–enriched fraction), whereas other intracellular membranes were present in the $P_{100/60}$ fraction (data not shown). Specific anti-TRPV4 antibodies[13] recognized 2 bands of ≈110 and 120 kDa in both fractions isolated from TRPV4$^{+/+}$ MAECs (Figure 1B). The TRPV4 band intensity was higher in the $P_{20/20}$ fraction, suggesting that the majority of TRPV4 is most likely present in the plasma membrane. In contrast, neither of these 2 bands were present in isolates from TRPV4$^{-/-}$ mice.

Next, we performed intracellular $Ca^{2+}$ measurements to evaluate functional expression of TRPV4 channels in MAECs. We compared the response to 6 different TRPV4-activating stimuli in wild-type and TRPV4$^{-/-}$ MAECs: 4$\alpha$-PDD (1 $\mu$mol/L), a 25% hypotonic solution (HTS), AA (3 $\mu$mol/L), 5,6-EET (500 nmol/L), 8,9-EET (1 $\mu$mol/L), and heating to 42°C (Figure 2A through 2F). All 6 stimuli evoked robust increases in $[Ca^{2+}]_i$ in TRPV4$^{+/+}$ MAECs. In TRPV4$^{-/-}$ MAECs, responses to 4$\alpha$-PDD, 5,6-EET, and 8,9-EET were fully abolished (Figure 2A, 2D, and 2E), whereas the responses to HTS, AA, and heat were reduced but still detectable (Figure 2B, 2C, and 2F). As a control stimulus, we used ATP (100 nmol/L), which causes an increase in $[Ca^{2+}]_i$ in endothelial cells via activation of purinergic receptors. As shown in Figure 2G, ATP induced a similar $Ca^{2+}$ signal in TRPV4$^{+/+}$ and TRPV4$^{-/-}$ MAECs, indicating that other aspects of $Ca^{2+}$ handling are not drastically changed in the knockout cells. Together, RT-PCR, Western blot analysis, and $[Ca^{2+}]_i$ measurements demonstrate that TRPV4 is functionally expressed in the plasma membrane of MAECs.

### CYP Epoxygenase Modulators Selectively Affect TRPV4 Responses to AA and Cell Swelling

The above results indicated that the increase in $[Ca^{2+}]_i$ in MAECs induced by 5,6-EET and 8,9-EET is fully dependent on TRPV4. These EETs, which are known to have vasorelaxant properties, are formed from AA through the action of CYP epoxygenases (Figure 3A). We therefore tested whether pharmacological modulation of CYP epoxygenase activity affected TRPV4 activity in MAECs.

To increase CYP2C expression, MAECs were preincubated with nifedipine. In line with previous work,[28] we found that this preincubation led to an increase in protein expression (Figure 3B). We investigated the effects of nifedipine pretreatment on the $[Ca^{2+}]_i$ response to the different TRPV4-activating stimuli in MAECs derived from TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice. The basal $[Ca^{2+}]_i$ was not affected by preincubation with nifedipine (data not shown), indicating that basal activity of TRPV4 is not affected by nifedipine. Direct application of nifedipine did not change $[Ca^{2+}]_i$ in MAECs ($\Delta[Ca^{2+}]_i = 2 \pm 1$ nmol/L; n=53). Importantly, pretreatment of TRPV4$^{+/+}$ MAECs with nifedipine significantly enhanced the response to AA (Figure 4B) and HTS (Figure 4C). This effect of nifedipine was not observed in TRPV4$^{-/-}$ MAECs (Figure 4B and 4C). Moreover, nifedipine pretreat-

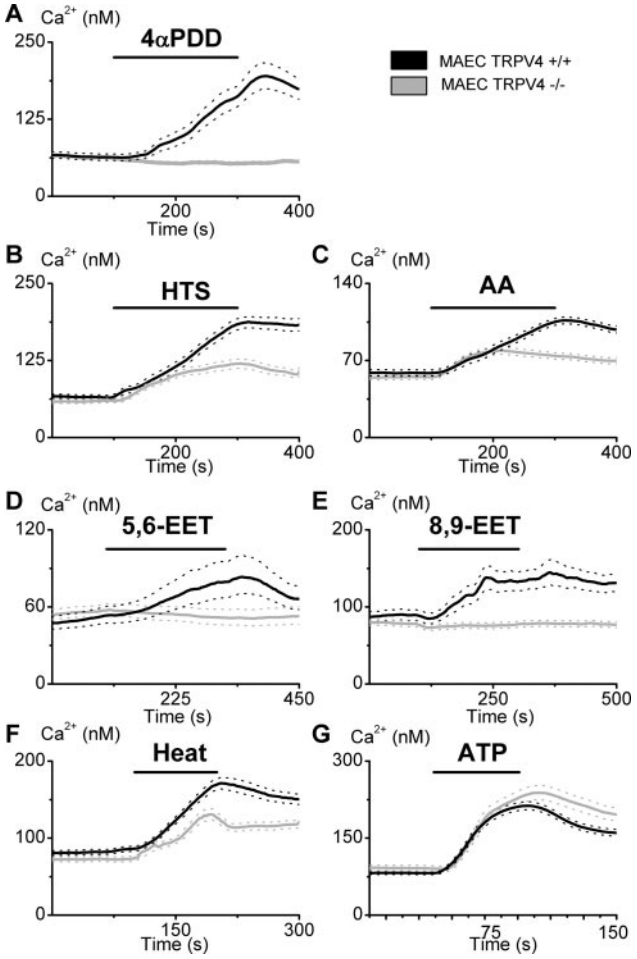


**Figure 2.** Different TRPV4 agonists to MAECs derived from wild-type and TRPV4-deficient mice. Effect of stimulation of MAECs with 4$\alpha$-PDD (1 $\mu$mol/L; A), HTS (B), AA (3 $\mu$mol/L; C) 5,6-EET (500 nmol/L; D), 8,9-EET (1 $\mu$mol/L; E), heat (42°C; F), and ATP (100 nmol/L; G) on $[Ca^{2+}]_i$. MAECs were derived from wild-type mice (black line, MAECs TRPV4$^{+/+}$), and MAECs were derived from TRPV4-deficient mice (gray line, MAECs TRPV4$^{-/-}$; dotted lines indicate SEs). In TRPV4$^{-/-}$ MAECs, responses to TRPV4-activating stimuli were significantly different from responses observed in TRPV4$^{+/+}$ MAECs (A through F), bar responses to the control stimuli ATP.

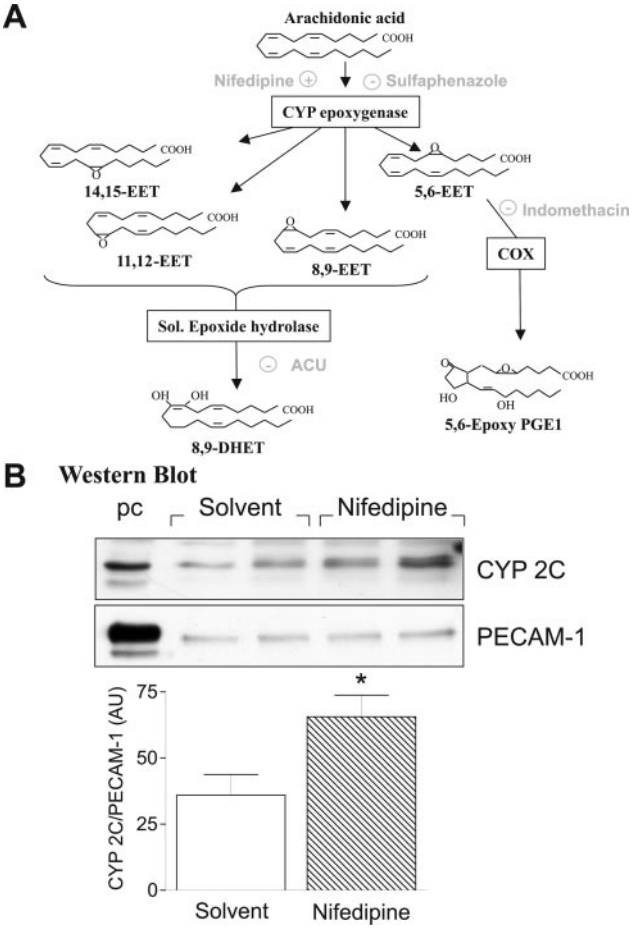


**Figure 3.** Nifedipine incubation increases the CYP 2C expression. A, Schematic overview of the cytochrome epoxygenase pathway. B, Mouse aortas were isolated, cleaned, and cut into rings that were incubated in culture medium containing either solvent or nifedipine (0.1 $\mu$mol/L) for 18 hours. Proteins were separated by SDS-PAGE and identified using specific antibodies to CYP 2C9 and PECAM-1. CYP 2C expression was normalized with respect to anti–PECAM-1 levels. Human endothelial cells overexpressing CYP 2C8 were used as a positive control (pc). The bar graph summarized the data obtained using tissue from 4 animals in each group. *Significant differences $P<0.05$; comparison with solvent; Student's *t* test).

ment did not affect the responses to 4$\alpha$-PDD (Figure 4A), exogenously applied 5,6-EET (Figure 4D), 8,9-EET (Figure 4E), or heat (Figure 4F) in MAECs from TRPV4$^{+/+}$ or TRPV4$^{-/-}$ mice. In addition, the TRPV4-independent response to ATP (100 nmol/L) was not affected by nifedipine pretreatment (data not shown).

Next, we investigated the effect of sulfaphenazole,[29–31] an inhibitor of CYP2C enzymes, on the $[Ca^{2+}]_i$ response to the different TRPV4-activating stimuli in MAECs derived from TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice. Sulfaphenazole did not affect the basal $[Ca^{2+}]_i$ level in MAECs from either TRPV4 $^{+/+}$ or TRPV4$^{-/-}$ mice (data not shown), indicating that the compound does not directly affect TRPV4 activity. However, sulfaphenazole strongly reduced the response of TRPV4$^{+/+}$ MAECs to AA (Figure 4H) or HTS (Figure 4I). This inhibitory effect of sulfaphenazole was not observed in TRPV4$^{-/-}$ MAECs. Sulfaphenazole had no effect on the responses to 4$\alpha$-PDD (Figure 4G), exogenously applied 5,6-EET (Figure 4J), 8,9-EET, heating (Figure 4L), or ATP (data not shown) in cells from TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice.

Together, the results obtained with nifedipine and sulfaphenazole indicate that modulation of CYP2C activity selectively affects the TRPV4-dependent responses to AA and HTS. These data demonstrate that activation of TRPV4 in MAECs by HTS and AA requires the CYP2C-dependent formation of EETs, whereas 4$\alpha$-PDD and heat activate the channel via a different, CYP-independent pathway.

## Inhibition of EET Degradation Increases the TRPV4-Dependent $Ca^{2+}$ Responses to AA, Cell Swelling, and EETs

Most EETs are metabolized by soluble epoxide hydrolase (sEH)–dependent epoxide hydration into the biologically less active dihydroxyeicosatrienoic acids (DHETs)[32] (Figure 3A). We used a selective inhibitor of sEH, ACU,[27,33] to investigate the effect of reduced EET breakdown on the TRPV4 activity

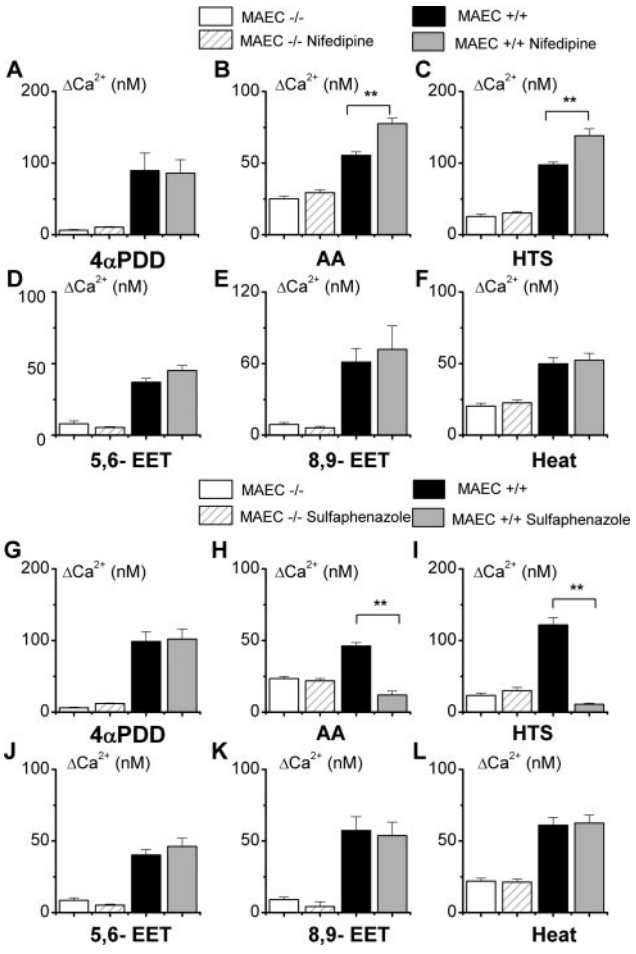


**Figure 4.** Effect of nifedipine and sulfaphenazole on TRPV4 $[Ca^{2+}]_i$ responses. Average increase in $[Ca^{2+}]_i$ in nontreated (black) and nifedipine- (A through F) or sulfaphenazole- (G through L) treated (gray) MAECs derived from wild-type TRPV4 (full box) and TRPV4-deficient mice (shaded box) in response to 3 $\mu$mol/L AA (A and G), 1 $\mu$mol/L 4$\alpha$-PDD (B and H), cells swelling (HTS; C and I), 500 nmol/L 5,6-EET (D and J), 1 $\mu$mol/L 8,9-EET (E and K) and heating to 42°C (F and L), respectively (n>18 from at least 3 independent experiments; **significant differences $P<0.001$; comparison with nontreated cells; Kolmogorov–Smirnoff test).

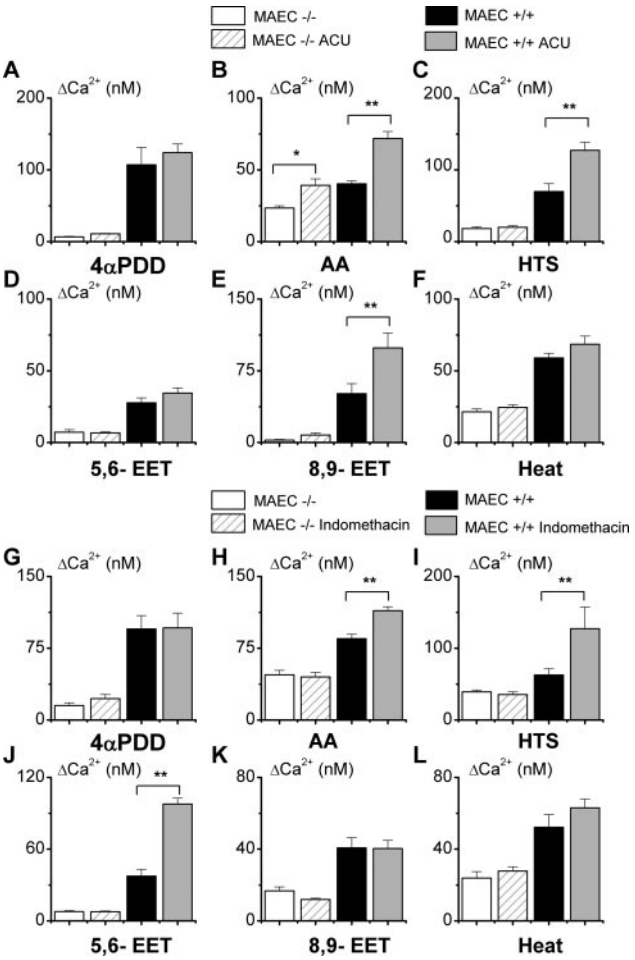


**Figure 5.** Effect of inhibiting EET degradation on the $[Ca^{2+}]_i$ responses after stimulation with specific TRPV4 agonists. Average increase in $[Ca^{2+}]_i$ in nontreated (black) and ACU- (2 $\mu$mol/L; A through F) or indomethacin- (G through L) treated (gray) MAECs derived from wild-type TRPV4 (full box) and TRPV4-deficient mice (shaded box) in response to 3 $\mu$mol/L AA (A and G), 1 $\mu$mol/L 4$\alpha$-PDD (B and H), cells swelling (HTS; C and I), 500 nmol/L 5,6-EET (D and J), 1 $\mu$mol/L 8,9-EET (E and K), and heating to 42°C (F and L), respectively (n>18 from at least 3 independent experiments; **significant differences $P<0.001$; comparison with nontreated cells; Kolmogorov–Smirnoff test).

in MAECs. In TRPV4$^{+/+}$ MAECs, ACU (2 $\mu$mol/L) significantly enhanced the responses to AA (Figure 5B), HTS (Figure 5C), and 8,9-EET (Figure 5E) but had no effect on the responses to 4$\alpha$-PDD (Figure 5A), 5,6-EET (Figure 5D), or heat (Figure 5F). In TRPV4-deficient cells, ACU only caused a modest enhancement of the response to AA (Figure 5B). ACU had no effect on the response to ATP stimulation in either TRPV4$^{-/-}$ or TRPV4$^{+/+}$ MAECs (data not shown).

The metabolism of 5,6-EET differs from that of the other EETs in that it is a much weaker substrate for sEH. In contrast, 5,6-EET is rapidly metabolized by cyclooxygenase (COX) enzymes to generate 5,6-epoxy-prostaglandin[34] (Figure 3A). To test the consequence of inhibition of 5,6-EET metabolism, we used the COX inhibitor indomethacin. Similar to the effects of ACU, the indomethacin (2 $\mu$mol/L) enhanced the $[Ca^{2+}]_i$ response to AA (Figure 5H) and HTS (Figure 5I) without affecting the responses to 4$\alpha$-PDD (Figure 5G) or heat (Figure 5I). Indomethacin potentiated the response to 5,6-EET (Figure 5J) but not that to 8,9-EET (Figure 5K), consistent with 5,6-EET but not 8,9-EET being a COX substrate. Again, effects of indomethacin were only observed in endothelial cells that expressed TRPV4 (Figure 5G through 5L), and indomethacin had no effects on basal $[Ca^{2+}]_i$ or on the response to ATP (data not shown).

Thus, inhibition of the metabolism of 5,6-EET and 8,9-EET increases the TRPV4 responses to AA and cell swelling. This indicates that the activity of sEH and COX enzymes reduces cellular EET levels in MAECs, leading to lowered activity of TRPV4.

## Effects of Modulating EET Generation and Degradation on Currents Through TRPV4 Channels

To investigate the consequences of modulating CYP activity on EET degradation on TRPV4 currents, we measured

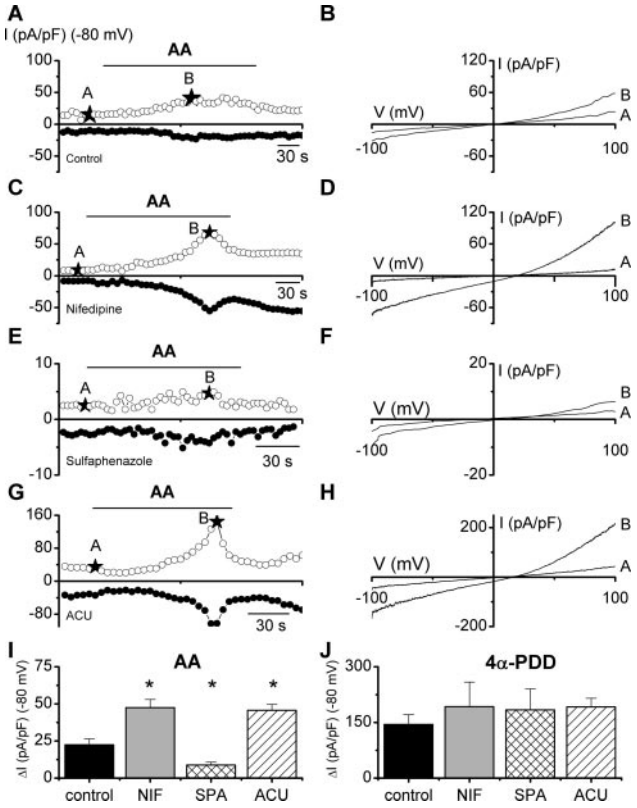


**Figure 6.** Effect of CYP modulators on the activation of TRPV4 by AA and 4$\alpha$-PDD. A through H, Time course of whole-cell currents at −80 and +80 mV (black and white circles, respectively; A, C, E, and G) and current–voltage relations obtained from time points indicated by black stars (B, D, F, and H) in TRPV4-transfected EA cells, showing the current activation in nontreated TRPV4-transfected EA cells (A and B), in nifedipine- (C and D), sulfaphenazole- (E and F) and ACU- (G and H) treated TRPV4-transfected EA cells on stimulation with 3 $\mu$mol/L AA. I, Maximal increases in inward-current density at −80 mV on stimulation with 3 $\mu$mol/L AA in nontreated cells (control; n=10), nifedipine- (NIF; n=8), sulfaphenazole- (SPA; n=11), and ACU- (n=11) treated TRPV4-transfected EA cells. J, Comparison of the average inward current activated by 1 $\mu$mol/L 4$\alpha$-PDD in nontreated cells (n=9), nifedipine- (n=6), sulfaphenazole- (n=7), and ACU- (n=8) treated TRPV4-transfected EA cells (*significant difference; $P<0.01$ compared with nontreated TRPV4-transfected EA cells; Students' $t$ test).

TRPV4 channel activation directly using the whole-cell patch-clamp technique. For this purpose, we used HUVEC-derived EA.Hyb926 cells (EA cells) transiently transfected with TRPV4, which yield more robust TRPV4 current responses than native MAECs. In $[Ca^{2+}]_i$ measurements on TRPV4-transfected EA cells (data not shown), we obtained qualitatively identical results as in TRPV4$^{+/+}$ MAECs. In whole patch-clamp experiments using TRPV4-transfected EA cells, AA (3 $\mu$mol/L) activated large currents showing the characteristic features of the TRPV4 channel: moderate outward rectification, a reversal potential around +10 mV, and S-shaped current–voltage curves.[2] In agreement with its effects in MAECs, AA-induced current amplitudes were larger in cells pretreated with nifedipine to increase CYP2C expression (Figure 6C, 6D, and 6I), smaller in cells treated with the CYP2C inhibitor sulfaphenazole (Figure 6E, 6F, and 6I) and larger in cells treated with the sEH inhibitor ACU (Figure 6G through 6I). In contrast, current responses to application of 4$\alpha$-PDD (1 $\mu$mol/L) were unaffected by nifedipine, sulfaphenazole, or ACU (Figure 6J).

## Discussion

In this study, we evaluated whether the $Ca^{2+}$ entry channel TRPV4 functions as a molecular target for EETs in MAECs and assessed the effects of pharmacological modulation of EET metabolism on TRPV4 activity. The results of the present investigation demonstrate that the response of TRPV4 to AA and cell swelling is modulated by formation and breakdown of EETs. Indeed, stimulation of the formation of EETs from AA, by increasing CYP2C expression, enhanced the TRPV4 response to AA or cell swelling, whereas inhibition of CYP2C expression blocked the response to these stimuli. Inhibition of COX and sEH enzymes to reduce EET breakdown enhanced the effect of AA, cell swelling, and EETs. By using MAECs from TRPV4$^{-/-}$ mice, we confirmed that these effects were mediated by TRPV4. Moreover, responses to 4$\alpha$-PDD and heat were independent of EET metabolism, confirming that they activate TRPV4 via separate pathways.

We initially tested the effects of different well-described TRPV4-activating stimuli on $Ca^{2+}$ responses in these cells and observed that stimulation with 4$\alpha$PDD, AA, cell swelling, heat, 5,6-EET, and 8,9-EET all resulted in robust responses in TRPV4$^{+/+}$ cells. In TRPV4$^{-/-}$ MAECs, we did not detect any response to 4$\alpha$PDD, 5,6-EET, and 8,9-EET, whereas clear but reduced responses were obtained with AA, cell swelling, and heat. A possible explanation for this residual effect in the knockout cells could be that AA or downstream metabolites of AA activate $Ca^{2+}$ permeable channels other than TRPV4 or a possible upregulation of other channel types. Likewise, other temperature-sensitive TRP channels such as TRPV3, which also demonstrate an activation threshold between 25°C and 42°C,[35] may account for the residual response to heat. The presence of other swelling-activated $Ca^{2+}$ channels like TRPV2 or TRPM3[36,37] could explain the $Ca^{2+}$ increase caused by cell swelling in TRPV4$^{-/-}$ cells.

Our data clearly illustrate the existence of several ways in which TRPV4 channels can be activated. Activation by AA and HTS is highly sensitive to pharmacological agents that affect EET metabolism. AA is the substrate for CYP epoxygenases expressed in endothelial cells, which mediate the production of the TRPV4-activating EETs 5,6-EET and 8,9-EET. Activation of TRPV4 by cell swelling can most probably be attributed to the liberation of endogenous AA from phospholipids attributable to activation of phospholipase $A_2$.[2,8] The mode of TRPV4 activation by $\alpha$-phorbols and heat is clearly distinct from that by AA or swelling; CYP induction, CYP inhibition, and inhibition of sEH or COX failed to affect TRPV4 activation by these stimuli. Moreover, activation of TRPV4 by $\alpha$-phorbols and heat depends on a specific tyrosine residue, Tyr555, which likely forms part of the $\alpha$-phorbol binding site.[8]

The results of the present study indicate that a novel way of modulating TRPV4 activity is to interfere with the breakdown of the EETs into DHETs. We found that preventing the

breakdown of EETs using the selective sEH inhibitor ACU increased the TRPV4-dependent responses to AA, 8,9-EET, and cell swelling but not those induced by 4$\alpha$-PDD or heat. These data also imply that DHETs are no longer able to activate TRPV4, in line with a previous report.[17] The fact that ACU enhanced the AA-induced $Ca^{2+}$ response in TRPV4$^{-/-}$ MAECs could indicate that EETs also affect the activity/open probability of other MAEC channels. The response of endothelial cells to the exogenous application of 5,6-EET was not affected by ACU but was potentiated by the COX inhibitor indomethacin, which is consistent with the fact that 5,6-EET is the only regioisomer metabolized by COX.[34]

EETs are biologically important signal transduction molecules that have been implicated in numerous biological processes including communication between endothelial and vascular smooth muscle cells.[38] Although some of the effects attributed to EETs, such as enhanced cell–cell communication[39] and angiogenesis,[40] are membrane potential independent and most likely related to the activation of protein kinase A, it is tempting to speculate that the activation of TRPV4 channels underlies some of the anti-inflammatory and antihypertensive effects of the EETs. Our data not only highlight the potential role of TRPV4 in the regulation of vascular homeostasis but also underscore the role of sEH in the regulation of endothelial cell signaling. Indeed, our finding that sEH inhibitors increase endothelial cell $[Ca^{2+}]_i$ via TRPV4 indicate that these drugs have the potential to affect a number of $Ca^{2+}$-dependent processes in endothelial cells. Enhanced TRPV4 activation may account, at least partially, for the hypotensive effects of sEH inhibitors in vitro and in vivo.[32,41,42]

## Acknowledgments

This work was supported by the Human Frontiers Science Programme (HFSP research grant RGP 32/2004), the Belgian Federal Government, the Flemish Government, the Onderzoeksraad KU Leuven (GOA 2004/07, FWO G.0214.99, FWO G. 0136.00; FWO. G.0172.03, and the Interuniversity Poles of Attraction Program, Prime Ministers Office IUAP), Philip Morris Inc. (research grant to I.F.), and the Deutsche Forschungsgemeinschaft (FI 830/2-1 to B.F. and R.B.). We thank M. Caterina (Johns Hopkins School of Medicine, Baltimore, Md) and Professor F. Wuytack (Katholieke Universiteit Leuven, Belgium) for providing anti-TRPV4 and anti-SPCA1 antibodies, respectively. J.V. is a postdoctoral fellow of the FWO (Fonds voor Wetenschappelijk Onderzoek, Flanders).

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