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## Hypotension Induced by TRPV4 Activation: Role of Ca<sup>2+</sup>-activated K<sup>+</sup> Channels and Sensory Nerves

Feng Gao\* and Donna H. Wang\*,†,‡

\*Department of Medicine, Michigan State University, Michigan

†Neuroscience Program, Michigan State University, Michigan

‡Cell and Molecular Biology Program, Michigan State University, Michigan

### Abstract

**Objective**—To examine the mechanisms involved in hypotension induced by transient receptor potential vanilloid 4 (TRPV4) activation.

**Methods**—Wistar rats were given 50 mg/kg capsaicin subcutaneously 1-2 days postnatally to cause degeneration of capsaicin-sensitive sensory nerves. Vehicle was given to the corresponding newborn rats as the control group. After being weaned, male rats were picked for further investigation. At the age of 8 weeks, mean arterial pressure (MAP) and its response to 4 $\alpha$ -phorbol 12,13-didecanoate (4 $\alpha$ -PDD, a selective TRPV4 activator, 2.5 mg/kg, i.v.) with/without CGRP<sub>8-37</sub> (1 mg/kg/min, i.v.), an antagonist of calcitonin gene-related peptide (CGRP, a potent vasodilator released from sensory nerves), in vehicle-/capsaicin-pretreated rats anesthetized with sodium pentobarbital (50 mg/kg, i.p.) were monitored to observe the contributions of neuropeptides released from sensory nerves to the 4 $\alpha$ -PDD-induced hypotension. To detect the roles of various vasodilating factors released by vascular endothelium in the hypotensive effect induced by TRPV4 activation, the corresponding inhibitors/blockers, including indomethacin (a cyclooxygenase inhibitor, 10 mg/kg, i.v.), N $\omega$ -Nitro-L-arginine (L-NA, a NO synthase inhibitor, 20 mg/kg, i.v.), apamin (a blocker of small-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (MaxiK) channels, 50  $\mu$ g/kg, i.v.) combined with charybdotoxin (a blocker of intermediate- and large-conductance MaxiK channels, 50  $\mu$ g/kg, i.v.), were used at various time before 4 $\alpha$ -PDD injection. Plasma CGRP and substance P levels of rats before/after administration were measured using the corresponding Radioimmunoassays. At last, immunohistochemistry stainings were performed to observe expression of TRPV4/CGRP/MaxiK in mesenteric resistant arteries and sensory neurons/nerve fibers.

**Results**—Intravenous administration of 4 $\alpha$ -PDD produced remarkable hypotension in vehicle-pretreated rats. The depressor effect was attenuated by degeneration of capsaicin-sensitive sensory nerves ( $P<0.05$ ) or administration of CGRP<sub>8-37</sub> ( $P<0.05$ ). In both vehicle- and capsaicin-pretreated rats, the combined administration of apamin and charybdotoxin markedly reduced the 4 $\alpha$ -PDD-induced hypotensive effect ( $P<0.05$ ), but i.v. administration of indomethacin and L-NA didn't produce the similar effect. Intravenous administration of 4 $\alpha$ -PDD increased plasma CGRP but not substance P levels in vehicle-pretreated rats only ( $P<0.05$ ), which was not affected by indomethacin, L-NA, or apamin plus charybdotoxin. Immunohistochemistry staining showed that TRPV4 colocalized with MaxiK channels in endothelium of mesenteric resistant arteries and with CGRP in sensory neurons/nerve fibers.

Correspondence to: Donna H. Wang, MD., FAHA Division of Nanomedicine and Molecular Intervention Department of Medicine, B316 Clinical Center, Michigan State University, East Lansing, MI, Fax number: 517-432-1326 Phone number: 517-432-0797  
Donna.Wang@ht.msu.edu.

**Disclosures** None.

**Conclusion**—Our data show that the hypotensive effect induced by TRPV4 activation attributes to, at least in part, activation of MaxiK channels and CGRP receptors upon CGRP release from sensory nerves.

### Keywords

blood pressure; calcitonin gene-related peptide; sensory nerves; Ca<sup>2+</sup>-activated K<sup>+</sup> channels

### Introduction

TRPV4, a member of the vanilloid subfamily of the transient receptor potential (TRP) channels, is a non-selective cation channel with a moderate Ca<sup>2+</sup> selectivity ( $P_{Ca^{2+}}/P_{Na^+}=6$ , in which P is permeability) [1]. Similarly as other TRP channels, TRPV4 has six transmembrane domains flanked by cytoplasmic N- and C-termini [2]. TRPV4 displays a broad expression in various cells and tissues and has been implicated in diverse physiological processes. TRPV4 can be activated by a variety of stimuli, including heat, cell swelling, exogenous phorbol ester compounds, or endogenous, physiologically active lipids such as 5',6'-epoxyeicosatrienoic acid (5',6'-EET) [3]. These TRPV4-activating stimuli promote the channel opening by two distinct pathways: the PLA<sub>2</sub>-cytochrome P<sub>450</sub> epoxygenase-dependent pathway or direct activation mediated by an aromatic residue at the N terminus of the third transmembrane domain [4].

TRPV4 is constitutively active at body temperature [5]. Recent findings reveal that TRPV4 plays an important role in systemic osmoregulation, mechanoregulation and thermoregulation [6-10]. Moreover, TRPV4 is also involved in the regulation of vascular tone, and activation of TRPV4 produces vasodilation [3]. We have previously shown that activation of TRPV4 by 4 $\alpha$ -phorbol 12, 13-didecanoate (4 $\alpha$ -PDD), a selective TRPV4 activator, leads to dose-dependent decreases in blood pressure in rats [11]. However, the mechanisms responsible for TRPV4-induced depressor effects are largely unknown.

Our previous studies also showed that release of calcitonin gene-related peptide (CGRP) played an important role in hypotension induced by activation of TRPV1, another member of the vanilloid subfamily. As well known, CGRP is one of the most potent vasodilatory neuropeptides and mainly released from sensory nerves expressing TRPV1 [12, 13]. Similar to TRPV1, TRPV4 has been reported to express in sensory nerves and colocalize with CGRP and substance P, which is also a vasodilatory neuropeptide [14, 15]. Based on the information stated above, we hypothesize that the depressor effect of 4 $\alpha$ -PDD is mediated, at least partly, through stimulus of sensory nerves and the subsequent release of vasodilatory neuropeptides. It is well established that capsaicin administered at 50 mg/kg subcutaneously 1-2 days postnatally when sensory nerves are not fully developed leads to permanent impairment and degeneration of capsaicin-sensitive sensory nerves [16, 17]. Using this model combined with the use of antagonists to block neuropeptide receptors as well as the measurement of plasma CGRP and substance P levels, we aimed at examining the role of neuropeptides released from sensory nerves in TRPV4-mediated depressor effects.

Vascular endothelium plays an important role in controlling the tone of the underlying vascular smooth muscle by releasing vasodilating factors including prostacyclin, nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF) [18, 19]. It has been shown that activation of TRPV4 in endothelial cells causes endothelium-dependent vascular relaxation in rat coronary artery [20]. Furthermore, the loss-of-function of TRPV4 results in a loss of shear stress-induced vasodilation, a response pattern critically dependent on endothelial TRPV4 expression [21]. These findings suggest that vasodilation induced by TRPV4 activation is, at least in part, endothelium-dependent. To identify which endothelial

pathway is involved in the TRPV4-mediated hypotensive effect, we used various inhibitors/blockers in combination with 4 $\alpha$ -PDD to achieve this goal. These inhibitors/blockers include indomethacin (a cyclooxygenase inhibitor), N $\omega$ -Nitro-L-arginine (L-NA, a NO synthase inhibitor), and apamin [an inhibitor of small-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (MaxiK) channels, SKCa] plus charybdotoxin [an inhibitor of intermediate- and large-conductance MaxiK channels, IKCa and BKCa].

Overall, the present study was designed to inquire the mechanisms involved in the hypotensive effects induced by TRPV4 activation. First, we determined the 4 $\alpha$ -PDD-induced hypotensive effect in vehicle- or capsaicin-pretreated rats to test if neuropeptides released from sensory nerves play a role in the depressor effect, and then, the specific endothelium-dependent pathway involved was identified by use of various inhibitors/blockers.

## Methods

### Animal preparation

All of the experiments were approved by the Institutional Animals Care and Use Committee. Pregnant Wistar rats (Charles River Laboratories, Wilmington, Massachusetts, USA) were housed in the animal facility for at least 1 week before parturition. Neonatal rats received capsaicin 50 mg/kg s.c. in a volume of 10 mL/kg 1-2 days postnatally as described [16, 17]. Control neonatal rats were treated with equal volumes of vehicle solution (5% ethanol, 5% Tween 80 in saline). After 3 weeks of weaning, male and female rats were separated, and only male rats were used at the age of 8 weeks in the present study.

### Surgical preparation

Under anesthesia with sodium pentobarbital at 50 mg/kg i.p., the left carotid artery was cannulated with a polyethylene tube containing isotonic heparin saline solution (5 U/ml) and connected with a Statham 231 D pressure transducer coupled to a Gould 2400s recorder for continuous monitoring of mean arterial pressure (MAP). The right jugular vein was cannulated for intravenous administration of drugs.

### Preparation of samples

When the depressor phase approached the lowest point in response to bolus administration of 4 $\alpha$ -PDD, i.e. 4-6 min after injection, the rats were killed by decapitation. Arterial and venous blood was collected and centrifuged for plasma CGRP and substance P assays. To determine colocalization of TRPV4 with MaxiK in mesenteric resistant arteries or with CGRP in dorsal root ganglia (DRG) neurons/perivascular sensory nerve fibers, vehicle-pretreated rats were killed by decapitation without subjecting to acute experiments, and mesenteric resistant arteries and DRG were collected for immunohistochemistry staining.

### Experimental protocols Mean arterial pressure and drug administration

The effectiveness of degeneration of capsaicin-sensitive sensory nerves was verified by monitoring MAP responses to bolus administration of capsaicin at 30  $\mu$ g/kg i.v. to vehicle- or capsaicin-pretreated rats. Vehicle- and capsaicin-pretreated rats were further divided into five groups for i.v. administration of vehicle or 4 $\alpha$ -PDD in the presence or absence of indomethacin, L-NA, or a combined administration of apamin and charybdotoxin. Indomethacin at 10 mg/kg, L-NA at 20 mg/kg, or combination of apamin at 50  $\mu$ g/kg and charybdotoxin at 50  $\mu$ g/kg were given i.v. 15 min, 30 min or 20 min, respectively, before i.v. administration of 4 $\alpha$ -PDD at 2.5 mg/kg. The doses of indomethacin, L-NA, apamin and charybdotoxin were chosen based on previous studies [22-27]. The i.v. injection volume for

each drug was 400  $\mu$ l. At the above specified doses, these drugs nearly completely inhibited/ blocked activities of the corresponding endothelial pathways.

To examine the effect of CGRP<sub>8-37</sub>, an antagonist of CGRP receptor, on 4 $\alpha$ -PDD-induced hypotension, CGRP<sub>8-37</sub> was intravenously administered at the rate of 1 mg/kg per min for 2 min, followed by administration at the rate of 0.4 mg/kg per min for 15 min in vehicle-treated rats. 4 $\alpha$ -PDD at 2.5 mg/kg was given i.v. bolus 1 min after CGRP<sub>8-37</sub> administration. The dose and time frame for CGRP<sub>8-37</sub> were chosen based on the previous study [28].

### Radioimmunoassay

To determine immunoactive CGRP and substance P in plasma, commercially available rabbit anti-rat CGRP radioimmunoassay kit and rabbit anti-rat substance P radioimmunoassay kit (Bachem Americas Inc., Torrance, CA) were used. The assays were performed as recommended by the manufacturer.

### Immunohistochemistry

#### **Colocalization of TRPV4 and CGRP in perivascular sensory nerves innervating the mesenteric resistance arteries**

—Mesenteric resistance arteries were fixed with Zamboni's fixative solution containing 2% formaldehyde, 15% picric acid and 0.1 M phosphate buffer on ice for 2 hours, washed with dimethyl sulfoxide (1 $\times$ 10 min) and phosphate-buffered saline (PBS) (3 $\times$ 10 min) at room temperature, and incubated in 0.4% Triton-X 100 for permeabilization and 5% fetal bovine serum in PBS for 30 minutes at room temperature to block nonspecific binding. The arteries were subsequently incubated with goat anti-rat CGRP (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) and/or rabbit anti-rat TRPV4 (1:300, Alomone labs, Jerusalem, Israel), followed by a secondary antibody cocktail of donkey anti-goat CY3 (1:500, Jackson ImmunoResearch, West Grove, PA) and donkey anti-rabbit FITC (1:300, Jackson ImmunoResearch, West Grove, PA) for 30 minutes. After washing with PBS, the vessels were mounted to slides and optical sections of the vessels were viewed under Zeiss Pascal confocal laser scanning microscope using 488-nm and 543-nm laser. Negative control was performed by omission of primary antibodies, which showed no immunoreactivity.

**Colocalization of TRPV4 and CGRP in DRG neurons**—DRG was embedded in OCT compound and placed in liquid nitrogen. Samples of freshly frozen DRG were sectioned at 5  $\mu$ m on a Cryotome FSE (Thermo Electron, Pittsburgh, PA), placed on 3-aminoalkoxyethoxysilane coated slides, fixed in 75% Acetone/Ethanol (v/v) for 5 minutes and incubated in normal donkey serum (1:28, Jackson ImmunoResearch labs, West Grove, PA) for 30 minutes. DRG sections were examined for CGRP and TRPV4 expression as described above with the use of goat anti-rat CGRP (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-rat TRPV4 (1:50, Alomone labs, Jerusalem, Israel), followed by a secondary antibody cocktail of donkey anti-goat CY3 (1:136, Jackson ImmunoResearch labs, West Grove, PA) and donkey anti-rabbit FITC (1:136, Jackson ImmunoResearch labs, West Grove, PA) for 30 minutes. The slides were viewed under Zeiss Pascal confocal laser scanning microscope using 488-nm and 543-nm laser. Negative control was performed by omission of primary antibodies, which showed no immunoreactivity.

#### **Colocalization of TRPV4 and MaxiK in the endothelium of mesenteric resistant arteries**

—Mesenteric resistant arteries were embedded in OCT compound and placed in liquid nitrogen. Samples of freshly frozen mesenteric resistant arteries were sectioned at 5  $\mu$ m on a Cryotome FSE (Thermo Electron, Pittsburgh, PA), placed on 3-aminoalkoxyethoxysilane coated slides, fixed in 75% Acetone/Ethanol (v/v) for 5 minutes and incubated in normal donkey serum (1:28, Jackson ImmunoResearch labs, West Grove, PA)

for 30 minutes. Mesenteric resistant artery sections were incubated in avidin (Vector) and d-biotin (Sigma Chemical, St. Louis, MO) for 15 minutes. After washing with PBS, the arteries were incubated at 4 °C overnight in a primary antibody cocktail of goat anti-rat MaxiK $\beta$  (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-rat TRPV4 (1:100, Alomone labs, Jerusalem, Israel), followed by a secondary antibody cocktail of donkey anti-goat CY3 (1:136, Jackson ImmunoResearch labs, West Grove, PA) and donkey anti-rabbit FITC (1:136, Jackson ImmunoResearch labs, West Grove, PA) for 30 minutes. The slides were viewed under Zeiss Pascal confocal laser scanning microscope using 488-nm and 543-nm laser. Negative control was performed by omission of primary antibodies, which showed no immunoreactivity.

## Drugs

Capsaicin and 4 $\alpha$ -PDD (LC Laboratories, Woburn, MA) were dissolved in ethanol (5% v/v), Tween-80 (5% v/v) and saline to be used freshly. Indomethacin was dissolved in 0.24% Na<sub>2</sub>CO<sub>3</sub> solution. Apamin, charybdotoxin, L-NA, or CGRP8-37 was dissolved in saline.

## Statistical analysis

All values were expressed as means  $\pm$  SE. Differences between 2 groups were analyzed by the use of the unpaired Student *t* test. Differences among groups were analyzed using one-way ANOVA followed by a Bonferroni's adjustment for multiple comparisons. Differences were considered statistically significant at  $P < 0.05$ .

## Results

To examine the effectiveness of degeneration of capsaicin-sensitive sensory nerves, MAP responses to bolus i.v. injection of capsaicin, a selective TRPV1 channel activator, were examined in vehicle- or capsaicin-pretreated rats. Baseline MAP measured 1 hour after surgery was slightly but significantly lower in capsaicin pretreated compared to vehicle-pretreated rats ( $100 \pm 4$  mmHg vs  $116 \pm 3$  mmHg,  $P < 0.05$ ). Bolus i.v. injection of capsaicin caused a greater decrease in MAP in vehicle-pretreated rats ( $-20 \pm 1\%$ ) than in capsaicin-pretreated rats ( $-6 \pm 1\%$ ,  $P < 0.05$ ) (Fig. 1), indicating that capsaicin-sensitive sensory nerves were effectively denervated by capsaicin neonatal pretreatment [17, 28, 29].

Fig. 2 shows that i.v. administration of 4 $\alpha$ -PDD produced remarkable hypotension in vehicle-treated rats ( $-53 \pm 1\%$ ). MAP decreased promptly, reached the lowest point around 4-6 minutes and returned to baseline gradually over the course of 20 minute-recording. To identify which endothelial pathway is involved in the TRPV4-mediated hypotensive effect, various inhibitors/blockers, indomethacin, L-NA, and apamin plus charybdotoxin, were used in the present study. Intravenous administration of indomethacin or apamin plus charybdotoxin induced a very transient increase of MAP while L-NA caused a stable increase of MAP 20 min after injection (vehicle pretreatment,  $115 \pm 3$  mmHg vs  $143 \pm 4$  mmHg,  $P < 0.05$ ; capsaicin pretreatment,  $99 \pm 5$  vs  $124 \pm 4$  mmHg,  $P < 0.05$ ). 4 $\alpha$ -PDD was injected 10-15 minutes after MAP returned to baselines in the cases of indomethacin or apamin plus charybdotoxin administration and 30 minutes after L-NA administration. In vehicle-pretreated rats, the hypotensive effect of 4 $\alpha$ -PDD was attenuated by combined administration of apamin and charybdotoxin ( $-27 \pm 3\%$ ,  $P < 0.05$ ), but not by indomethacin ( $-52 \pm 3\%$ ) or L-NA ( $-51 \pm 2\%$ ), indicating that MaxiK channels play a key role in 4 $\alpha$ -PDD-induced hypotension.

Compared to vehicle-treated rats, denervation of capsaicin-sensitive sensory nerves significantly attenuated 4 $\alpha$ -PDD-induced hypotensive effects ( $-39 \pm 2\%$ ,  $P < 0.05$ ), implicating that sensory nerves and/or their neuropeptides regulate 4 $\alpha$ -PDD action.

Furthermore, the depressor effect of 4 $\alpha$ -PDD was further attenuated by combined administration of apamin and charybdotoxin ( $-15 \pm 1\%$ ,  $P < 0.05$ ), but not by indomethacin ( $-40 \pm 1\%$ ). Interestingly, administration of L-NA slightly enhanced the depressor effect of 4 $\alpha$ -PDD in capsaicin-pretreated rats ( $-49 \pm 2\%$ ,  $P < 0.05$ ). Taken together, these findings indicate involvement of MaxiK channels and sensory nerves in 4 $\alpha$ -PDD-induced hypotension and absence of contributions of NO and prostacyclin to the depressor effect.

Plasma CGRP and substance P levels in each of the five groups are shown in Fig. 3. Intravenous administration of 4 $\alpha$ -PDD significantly increased plasma CGRP levels in vehicle-pretreated rats ( $35.8 \pm 1.0$  pg/ml vs  $42.3 \pm 1.1$  pg/ml,  $P < 0.05$ ), but not in capsaicin-pretreated group ( $32.3 \pm 2.1$  pg/ml vs  $36.3 \pm 1.5$  pg/ml). Indomethacin, L-NA, or combined administration of apamin and charybdotoxin had no effect on 4 $\alpha$ -PDD-induced increases in plasma CGRP levels in vehicle-pretreated rats, but in capsaicin-pretreated rats, administration of these inhibitors/blockers slightly elevated 4 $\alpha$ -PDD-induced release of CGRP though no significant changes were observed. In contrast, i.v. administration of 4 $\alpha$ -PDD with or without indomethacin, L-NA, or combined administration of apamin and charybdotoxin didn't affect plasma substance P levels in either vehicle-pretreated ( $7.9 \pm 0.6$  pg/ml vs  $9.1 \pm 0.4$  pg/ml) or capsaicin-pretreated rats ( $5.0 \pm 0.5$  pg/ml vs  $6.2 \pm 0.4$  pg/ml). These results indicate that activation of TRPV4 causes CGRP but not substance P release from sensory nerves only when sensory nerves are intact.

To assess the role of increased CGRP release in 4 $\alpha$ -PDD-induced hypotension in vehicle-pretreated rats, MAP responses to 4 $\alpha$ -PDD with or without CGRP<sub>8-37</sub> were examined. Blockade of CGRP receptors with CGRP<sub>8-37</sub> significantly attenuated the depressor effect of 4 $\alpha$ -PDD in vehicle-pretreated rats ( $P < 0.05$ ), indicating that activation of CGRP receptors upon CGRP release induced by 4 $\alpha$ -PDD contributes to TRPV4-mediated hypotension (Fig. 4). Given that the magnitude of MAP reduction in response to CGRP<sub>8-37</sub> plus 4 $\alpha$ -PDD treatment in vehicle-pretreated rats ( $-32 \pm 4\%$ ) was very similar to that induced by 4 $\alpha$ -PDD treatment in capsaicin-pretreated rats ( $-39 \pm 2\%$ ), these results indicate that inability of 4 $\alpha$ -PDD in inducing CGRP release in capsaicin-pretreated rats may account for attenuated hypotensive effects of 4 $\alpha$ -PDD in these rats bearing sensory nerve impairment.

Double staining revealed that TRPV4, expressed in sensory neurons in DRG and sensory nerve fibers innervating mesenteric resistant arteries, colocalized with CGRP (Fig. 5). Moreover, immunohistochemistry staining of mesenteric resistant artery sections showed the colocalization of TRPV4 and MaxiK channels in vascular endothelium (Fig. 5). Colocalization is indicated by blue arrows. These results provide anatomical support for the notion that TRPV4 activation leads to CGRP release or opening of MaxiK channels.

## Discussion

The present study aimed at defining the mechanisms underlying the hypotensive effects induced by TRPV4 activation. We found that 1) 4 $\alpha$ -PDD is less effective in decreasing blood pressure when sensory nerves are impaired by neonatal capsaicin pretreatment, indicating that sensory nerves and/or their neuropeptides contribute to 4 $\alpha$ -PDD-induced hypotension; 2) 4 $\alpha$ -PDD increases plasma CGRP but not substance P levels when sensory nerves are intact, and blockade of the CGRP receptors attenuates 4 $\alpha$ -PDD-induced hypotension. These results support the notion that CGRP receptor activation upon CGRP release triggered by TRPV4 activation contributes to 4 $\alpha$ -PDD-induced hypotension; 3) blockade of MaxiK channels but not inhibition of cyclooxygenase or NO synthase markedly attenuates 4 $\alpha$ -PDD-induced hypotension, indicating contribution of MaxiK channels in 4 $\alpha$ -PDD-induced hypotension and the lack of a significant role for NO and prostacyclin in the depressor effect induced by 4 $\alpha$ -PDD; and 4) TRPV4 colocalizes with MaxiK channels in

resistant vascular endothelium and with CGRP in sensory neurons/nerve fibers, providing anatomical evidence for interaction between TRPV4 and MaxiK channels and TRPV4-mediated CGRP release. Taken together, these data indicate that the hypotensive effect of TRPV4 attributes to opening of MaxiK channels and activation of CGRP receptors following CGRP release induced by activation of TRPV4.

A dense perivascular CGRP-containing sensory nerve network is found around blood vessels in virtually all vascular beds and counterbalances the prohypertensive effects of several neurohormonal systems to maintain normal blood pressure. In the present study, we were expecting to see an elevated baseline blood pressure in capsaicin-pretreated rats. However, we saw an opposite result in anesthetized rats. A possible explanation is that degeneration of capsaicin-sensitive sensory nerves leads to relatively increased sympathetic nerve activity and suppression of which by anesthesia lowers blood pressure.

An endogenous cannabinoid lipid, anandamide, may activate TRPV1 leading to vasodilation and subsequent hypotension that depends on CGRP release from perivascular sensory nerves [30-32]. Similarly, TRPV4 is expressed in sensory neurons that contain CGRP [33], a finding consistent with the results obtained in the present study showing that TRPV4 colocalizes with CGRP in DRG and perivascular nerve fibers. Moreover, the data in the present study showed that TRPV4 activation increased plasma CGRP levels and that blockade of the CGRP receptors inhibited 4 $\alpha$ -PDD-induced depressor effects in a manner similarly as that of sensory nerve impairment. These findings indicate a key role for CGRP in mediating TRPV4-mediated hypotension.

Sensory nerve fibers may be classified as capsaicin-sensitive and capsaicin-insensitive populations. Capsaicin administered subcutaneously at a dose of 50 mg/kg to neonatal rats leads to an irreversible loss of 70-80% of small-diameter sensory neurons in DRG and depletion of neuronal stores of neuropeptides, whereas plasma levels of these neuropeptides may not be altered significantly due to the supplement from other tissues such as the thyroid gland [17, 28, 31, 32]. As shown in Fig. 3, plasma CGRP levels were at the similar level albeit tended to be lower in capsaicin-pretreated compared to vehicle-pretreated rats, a result consistent with previous findings.

CGRP may cause hypotension via its direct effect as a potent vasodilator or by inhibiting sympathetic nerve activity [13]. To date, CGRP is the most potent vasodilator discovered. It has been shown that plasma immunoreactive CGRP levels elevate in the animal models of acquired hypertension and that CGRP plays a compensatory role to buffer the increase of blood pressure [34]. In the present study, i.v. administration of indomethacin/apamin plus charybdotoxin induced a transient increase of blood pressure, and administration of L-NA led to a prolonged hypertensive effect. While these inhibitors/blockers had no effect on 4 $\alpha$ -PDD-induced CGRP release in vehicle-pretreated rats, these drugs tended to potentiate 4 $\alpha$ -PDD-induced CGRP release in capsaicin-pretreated rats albeit the enhancement was not statistically significant. Moreover, it has been shown that L-NAME (a derivative of L-NA)-induced hypertension increases sensitivity of vascular responses to CGRP [34]. Taken together, enhanced CGRP release as well as vascular responsiveness to CGRP may underlie the enhanced hypotension in capsaicin-pretreated rats given L-NA plus 4 $\alpha$ -PDD. Moreover, previous studies showed that cyclooxygenase-mediated increases in prostaglandin production participated in bradykinin-mediated CGRP synthesis and release [34, 35], which may explain the delayed hypotensive effects observed in rats treated with indomethacin plus 4 $\alpha$ -PDD.

Vascular endothelium controls the tone of the underlying vascular smooth muscle by releasing mainly three kinds of vasodilating factors including prostacyclin, NO, and EDHF

[18, 19]. It has been shown that 4 $\alpha$ -PDD activates TRPV4 expressed in endothelial cells leading to endothelium-dependent vascular relaxation in rat coronary and cerebral arteries [20]. Opening of TRPV4 channels, mimicking that of TRPV1 activation, leads to increases in Ca<sup>2+</sup> influx [36, 37] and possibly Ca<sup>2+</sup>-induced release of a vasodilator, NO [20]. An inhibitor of NO synthase was therefore used to examine the role of NO in TRPV4 action. We found however that inhibition of NO synthase had no effect on 4 $\alpha$ -PDD-induced decreases in blood pressure. Furthermore, inhibition of the cyclooxygenase pathway failed to attenuate 4 $\alpha$ -PDD-induced hypotension. These results indicate that neither NO nor prostacyclin may be a key player in TRPV4-induced hypotensive effects.

EDHF is synthesized and released by the endothelium, and the most likely candidates for EDHF include epoxyeicosatrienoic acids (EETs), endothelium-derived potassium ions (K<sup>+</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and C-type natriuretic peptide [38]. EDHF is as powerful as NO or prostacyclin (PGI<sub>2</sub>) in terms of regulating blood pressure, and impairment in the EDHF signaling contributes to several cardiovascular pathologies including hypertension, chronic renal failure, and diabetes [39]. EDHF may increase intracellular calcium concentrations that lead to the opening of MaxiK (SKCa, IKCa, and BKCa) channels and hyperpolarization of endothelial cells [40]. Accumulating evidence shows possible involvement of EDHF in arterial dilation induced by activation of TRPV4 [41-43]. The data in the present study showed that blockade of SKCa, IKCa, and BKCa by combined administration of apamin and charybdotoxin attenuated 4 $\alpha$ -PDD-induced hypotension possibly via preventing the opening of MaxiK channels, indicating that TRPV4-mediated depressor effects are, to a great extent, endothelial- and MaxiK channel-dependent.

In conclusion, our data demonstrate that TRPV4, co-expressed with MaxiK channels in resistant vessels and with CGRP in sensory neurons/nerve fibers, mediates profound hypotensive effects. The hypotensive effect of TRPV4 attributes to activation of MaxiK channels and CGRP receptors triggered by TRPV4-dependent CGRP release from sensory nerves. These findings may have important implications for future development of antihypertensive therapy.

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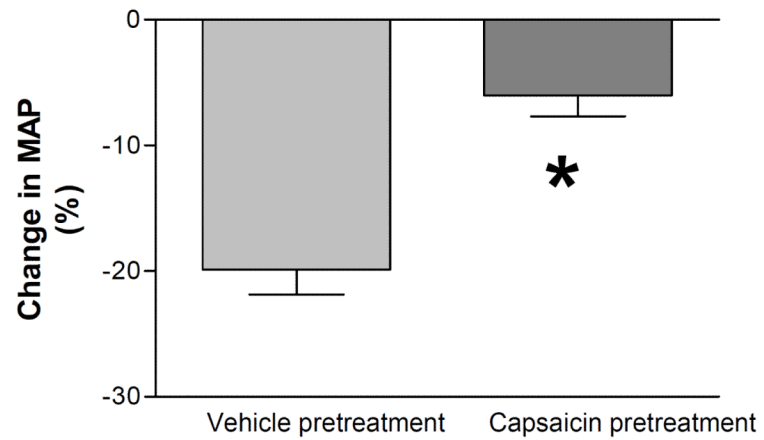
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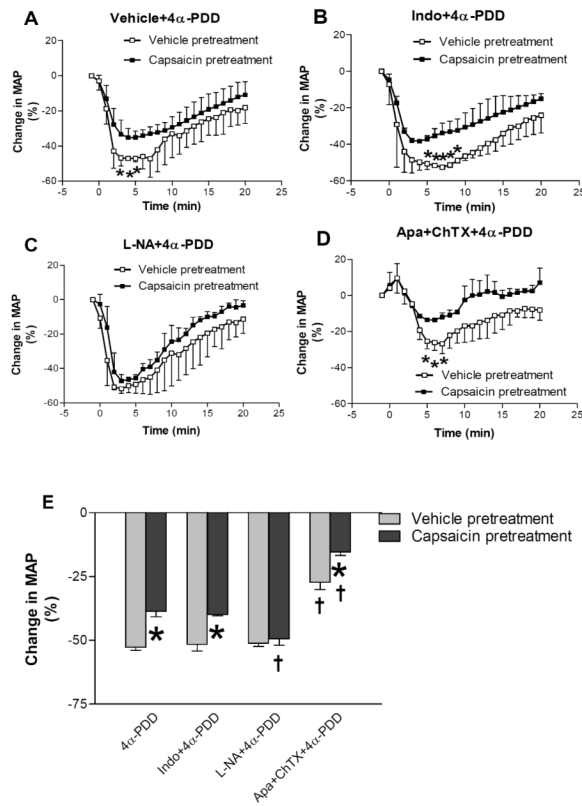


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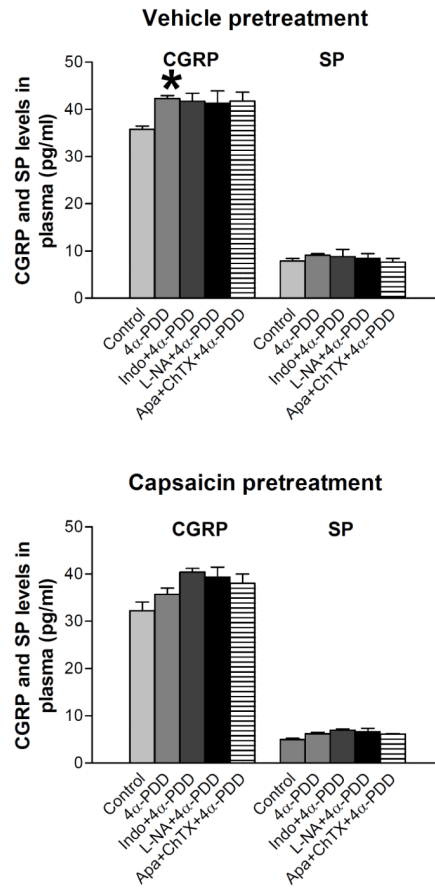


**Figure 1.** MAP responses to intravenous injection of capsaicin (CAP, 30  $\mu\text{g}/\text{kg}$ ) in anesthetized vehicle- or capsaicin-pretreated rats. Values are mean  $\pm$  SE (n=5 to 6). \*P<0.05 compared with the corresponding vehicle-pretreated group.

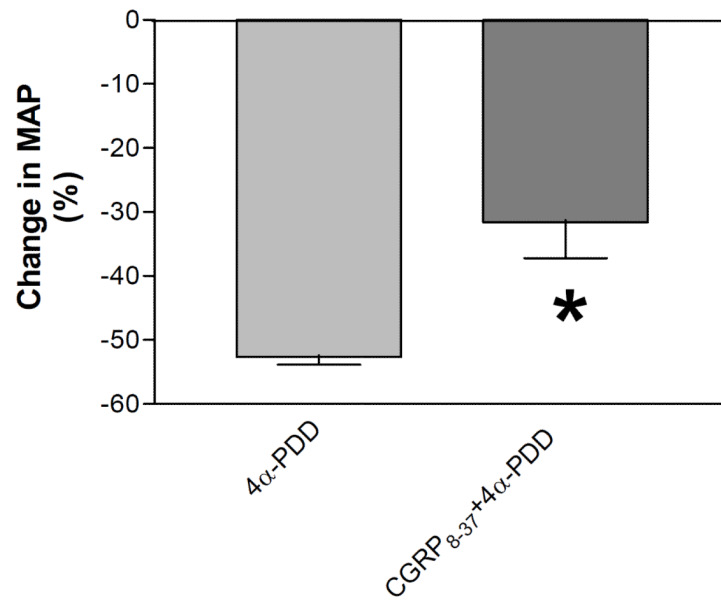


**Figure 2.**

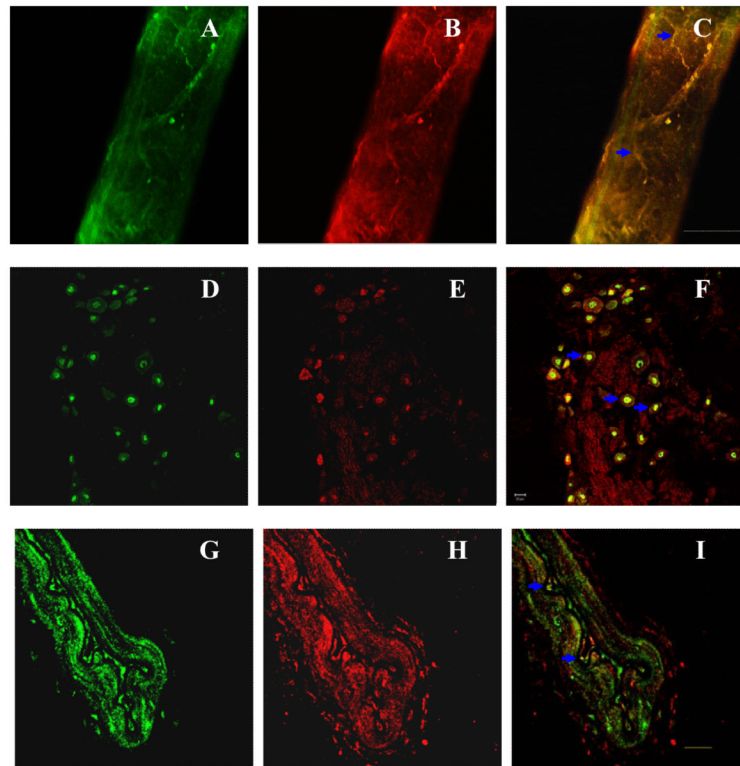
MAP responses to intravenous injection of 4 $\alpha$ -PDD (2.5 mg/kg) with or without indomethacin (10 mg/kg, i.v.), L-NA (20 mg/kg, i.v.) and apamin (50  $\mu$ g/kg, i.v.) plus charybdotoxin (50  $\mu$ g/kg, iv.) in anesthetized vehicle- or capsaicin-pretreated rats. A: Time-course responses of mean arterial pressure (MAP) to bolus injection of 4 $\alpha$ -PDD in rats. B-D: Time-course responses of mean arterial pressure (MAP) to bolus injection of 4 $\alpha$ -PDD with indomethacin, L-NA or apamin plus charybdotoxin, respectively. E: maximal changes of MAP in response to intravenous administration of drugs. Values are mean  $\pm$  SE (n=5 to 6). \*P<0.05 compared with the corresponding vehicle-pretreated group, †P<0.05 compared with the corresponding group administered 4 $\alpha$ -PDD.



**Figure 3.** Plasma CGRP and substance P (SP) levels in response to bolus injection 4α-PDD (2.5 mg/kg) with or without indomethacin (10 mg/kg, i.v.), L-NA (20 mg/kg) and apamin (50 μg/kg, i.v.) plus charybdotoxin (50 μg/kg, i.v.). Values are mean ± SE (n=4-7). \* $P < 0.05$  compared with the corresponding control group.



**Figure 4.** Mean arterial pressure responses to bolus injection of 4α-PDD (2.5 mg/kg) with or without CGRP8-37 in vehicle-pretreated rats. Values are mean ± SE (n=5 to 6). \*P<0.05 compared with the corresponding 4α-PDD-treated group.



**Figure 5.** Confocal microscope images of double-immunofluorescence staining of mesenteric resistant arteries (A, B, C, G, H, I) and DRG neurons (D, E, F). A, D and G: FITC-labeled TRPV4 receptor staining (green). B and E: Cy3-labeled CGRP staining (red). C and F: colocalization of TRPV4 and CGRP (yellow). H: Cy3-labeled MaxiK channels staining (red). I: colocalization of TRPV4 and MaxiK channels (yellow). Blue arrows: the colocalized areas. Negative control not shown. Scale bars, 20  $\mu\text{m}$ .