

RESEARCH PAPER

Structure-activity relationships of vanilloid receptor agonists for arteriolar TRPV1

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Keywords

vanilloid receptor (TRPV1); resistance artery; vascular autoregulation

Received

21 January 2011

Revised

12 July 2011

Accepted

09 August 2011

BACKGROUND AND PURPOSE

The transient receptor potential vanilloid 1 (TRPV1) plays a role in the activation of sensory neurons by various painful stimuli and is a therapeutic target. However, functional TRPV1 that affect microvascular diameter are also expressed in peripheral arteries and we attempted to characterize this receptor.

EXPERIMENTAL APPROACH

Sensory TRPV1 activation was measured in rats by use of an eye wiping assay. Arteriolar TRPV1-mediated smooth muscle specific responses (arteriolar diameter, changes in intracellular Ca²⁺) were determined in isolated, pressurized skeletal muscle arterioles obtained from the rat and wild-type or TRPV1^{-/-} mice and in canine isolated smooth muscle cells. The vascular pharmacology of the TRPV1 agonists (potency, efficacy, kinetics of action and receptor desensitization) was determined in rat isolated skeletal muscle arteries.

KEY RESULTS

Capsaicin evoked a constrictor response in isolated arteries similar to that mediated by noradrenaline, this was absent in arteries from TRPV1 knockout mice and competitively inhibited by TRPV1 antagonist AMG9810. Capsaicin increased intracellular Ca²⁺ in the arteriolar wall and in isolated smooth muscle cells. The TRPV1 agonists evoked similar vascular constrictions (MSK-195 and JYL-79) or were without effect (resiniferatoxin and JYL-273), although all increased the number of responses (sensory activation) in the eye wiping assay. Maximal doses of all agonists induced complete desensitization (tachyphylaxis) of arteriolar TRPV1 (with the exception of capsaicin). Responses to the partial agonist JYL-1511 suggested 10% TRPV1 activation is sufficient to evoke vascular tachyphylaxis without sensory activation.

CONCLUSIONS AND IMPLICATIONS

Arteriolar TRPV1 have different pharmacological properties from those located on sensory neurons in the rat.

Abbreviations

AMG9810, (E)-3-(4-*t*-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylamide; CHO-TRPV1, CHO cells overexpressing rat TRPV1; DRG, dorsal root ganglion; JYL-1511, *N*-(4-*tert*-butylbenzyl)-*N'*-[3-methoxy-4-(methylsulphonylamino)benzyl]thiourea; JYL-273, 2-(4-*t*-butylbenzyl)-3-[[4-(4-hydroxy-3-methoxybenzyl)amino]carbothioyl]propyl pivalate; JYL-79, 2-(3,4-dimethylbenzyl)-3-[[4-(4-hydroxy-3-methoxybenzyl)amino]carbothioyl]propyl pivalate; MSK-195, *N*-[2-(3,4-dimethylbenzyl)-3-(pivaloyloxy)propyl]-2-[4-(2-aminoethoxy)-3 methoxyphenyl]acetamide; TRPV1, transient receptor potential vanilloid 1; TRPV1^{-/-}, B6.129X1-*Trpv1*^{tm1Jul/J} mice

Introduction

The transient receptor potential vanilloid 1 (TRPV1) is a non-selective cation channel, originally found in sensory C and

Aδ fibres (Caterina *et al.*, 1997). It functions as a ligand-, proton- and heat-activated molecular integrator of nociceptive stimuli (Szallasi and Blumberg, 1999; Di Marzo *et al.*, 2002; Ross, 2003) and hence represents a promising drug

target for analgesia (Szallasi *et al.*, 2007; Gunthorpe and Szallasi, 2008).

However, TRPV1 expression has recently been identified in many cells in addition to sensory neurons. In particular, TRPV1 expression was detected in various cell types in the brain (Toth *et al.*, 2005a), and in the periphery, including arteriolar receptors responsible for vasoconstriction (Kark *et al.*, 2008). Moreover, while functional expression of TRPV1 in the CNS remained elusive, activation of vascular TRPV1 has been shown to result in substantial vasoconstriction both *in vivo* and *in vitro* (Kark *et al.*, 2008). TRPV1 antagonists are in clinical trials for various conditions including dental pain, osteoarthritis, neuropathic pain, overactive bladder, chronic cough, rectal hypersensitivity, migraine, lower back pain and interstitial cystitis (Khairatkar-Joshi and Szallasi, 2009). Although some results of these trials are promising, they also revealed that TRPV1 antagonists can evoke serious hyperthermia (Gavva *et al.*, 2008). This hyperthermia is probably related to the involvement of TRPV1 in temperature regulation *in vivo* (Gavva *et al.*, 2007). However, the mechanism of this effect is not clear. Although some antagonists cause hyperthermia (Gavva *et al.*, 2008), others are without thermoregulatory effects in humans (Khairatkar-Joshi and Szallasi, 2009). This suggests that the TRPV1 responsible for analgesia is pharmacologically different from that involved in thermoregulation. The nature and identity of these TRPV1-dependent responses have not been identified yet, but it is plausible that a separate pool of receptors exists (Steiner *et al.*, 2007).

Capsaicin evokes vasoconstriction in skeletal muscle arteries presumably by activating TRPV1 located in smooth muscle (Kark *et al.*, 2008). Here we attempted to characterize this receptor pharmacologically. To achieve this, we chose a series of commercially available TRPV1 agonists and tested them in assays that measured not only their potency and efficacy, but also their kinetics of action and ability to induce desensitization (Toth *et al.*, 2005b). Our experiments revealed different pharmacological profiles for vascular TRPV1 when compared with that of TRPV1 responsible for sensory activation. These findings indicate that sensory neuronal and arterial receptor populations of TRPV1 can be selectively targeted.

Methods

The applied drug/molecular target nomenclature (e.g. receptors, ion channels) conforms to the *British Journal of Pharmacology's* Guide to Receptors and Channels (Alexander *et al.*, 2011).

Animals, anaesthesia and general preparation in the in vivo experiments

The experiments were performed on male Wistar rats ($n = 119$ rats) weighing 250–450 g and on male mice (six control C57BL/6J and five TRPV1^{-/-} knockout mice). Rats (WKY/NCrl) were obtained from Charles River (Isaszeg, Hungary), while mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and maintained on a standard laboratory food (CRLT/N chow from Szinbad Kft, Godollo, Hungary) and water *ad libitum*. Anaesthesia was induced by administration

of pentobarbital sodium (100 mg·kg⁻¹ i.p.). All animal care and experimental procedures complied with NIH guidelines and were approved by the Ethical and Experimental Animal Research Committee of the University of Debrecen.

Isolation of arterioles and measurement of vascular diameter

The isolation of skeletal muscle (m. gracilis) arterioles of the rat and measurement of the diameter of arterioles were performed as described previously (Lizanecz *et al.*, 2006). Briefly, arterioles were kept in a physiological saline solution (PSS; composition in mM: 110 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.0 MgSO₄, 1.0 KH₂PO₄, 5.0 glucose and 24.0 NaHCO₃ equilibrated with a gas mixture of 10% O₂, 5% CO₂ and 85% N₂, at pH 7.4.) at an intraluminal pressure of 80 mmHg until the development of spontaneous myogenic response (constriction to intraluminal pressure). Changes in intraluminal arteriolar diameter were measured after the various treatments. First ACh was used to determine dilator capacity and endothelium function, and then noradrenaline (NA) was applied to measure maximal constrictor response and smooth muscle function. Changes in diameter to TRPV1 agonists were tested next with cumulative doses of the agonists (capsaicin, 0.1 nM–1 µM; resiniferatoxin, 1 pM–10 nM; JYL-273, 0.1 nM–1 µM; MSK-195, 0.1 nM–3 µM; JYL-79, 3 pM–10 µM; JYL-1511, 1 nM–1 µM). The specificity of the capsaicin responses was tested by the application of the TRPV1 antagonist AMG9810. Cumulative dose-response curves for capsaicin were obtained in the absence and presence of 100, 300 and 1000 nM AMG9810 (obtained from Tocris Bioscience, Ellisville, MO, USA). Desensitization of arteriolar TRPV1 was tested in separate experiments. Acute desensitization (decrease in response in the continuous presence of agonist) was determined by measurement of arteriolar diameter during 20 min incubations with a high concentration of the drugs. This was followed by 40 min regeneration (in PSS solution) and tachyphylaxis (decrease of response upon re-administration of the agonist) was assessed by measuring the response to 1 µM capsaicin. Arterioles were isolated from wild-type and TRPV1 knockout mice as detailed for the rat. Experiments were also performed similarly; ACh was used to determine endothelial function, and NA was applied to estimate smooth muscle function. Changes in diameter to TRPV1 agonists were tested by measuring responses to cumulative doses of capsaicin (0.1 nM–30 µM).

Determination of antagonist equilibrium dissociation constant

A conventional Schild plot (Arunlakshana and Schild, 1959) was constructed based on the measured values. EC₅₀ of capsaicin was calculated in the absence (designated as A) or in the presence of AMG9810 (designated as A'), then log((A/A')-1) values were plotted as a function of the logarithm of AMG9810 concentration (Figure 2B). Data were fitted by linear regression, and the antagonist equilibrium dissociation constant was obtained from the x-intercept.

Parallel measurement of vascular diameter and intracellular Ca²⁺ concentrations

Skeletal muscle arterioles were isolated and cannulated from the gracilis muscle of the rat, as mentioned above. After the

arteries had been mounted in the tissue chamber, the physiological buffer was supplemented with 1% BSA and 5 μM Fura-2AM fluorescent Ca^{2+} indicator dye for 60–120 min until a spontaneous myogenic tone developed. Then, the tissue chamber was placed on the stage of a Nikon TS100 (Tokyo, Japan) inverted microscope to measure intracellular Ca^{2+} concentrations by an Incytm2 instrument (Intracellular Imaging Inc, Cincinnati, OH, USA) by recording images (cut-off >510 nm) excited alternatively by 340 and 380 nm light. Images were recorded every 2–5 s and evaluated offline. Outer diameter of the arteries was determined on each recorded image and arteriolar Ca^{2+} concentrations were detected by calculating ratios between averaged signal intensity at 340 and 380 nm excitation in the whole arteriolar segment (representing a minimum of 200 pixels). A movie representative of the full experiment has been uploaded as a supplementary video file, and additional movies can also be seen at our website (<http://www.debkard.hu/upload/file/klinfiz/kkk/Vascularsystem/Vascularsystem.html>).

Isolation of smooth muscle cells from canine coronary arteries

Adult beagle dogs (10–14 kg) were anaesthetized with an i.v. injection containing 10 $\text{mg}\cdot\text{kg}^{-1}$ ketamine hydrochloride (Calypsol, Richter Gedeon, Hungary) and 1 $\text{mg}\cdot\text{kg}^{-1}$ xylazine hydrochloride (Sedaxylan, Eurovet Animal Health BV, Bladel, the Netherlands). After the chest had been opened, the heart was rapidly removed and the right coronary artery was perfused with Ca^{2+} -free minimum essential Eagle's medium, Joklik modification solution, supplemented with taurine (2.5 $\text{g}\cdot\text{L}^{-1}$), pyruvic acid (175 $\text{mg}\cdot\text{L}^{-1}$), ribose (750 $\text{mg}\cdot\text{L}^{-1}$), allopurinol (13.5 $\text{mg}\cdot\text{L}^{-1}$) and NaH_2PO_4 (200 $\text{mg}\cdot\text{L}^{-1}$) equilibrated with a mixture of 95% O_2 and 5% CO_2 (similar to all further solutions) for 5 min to remove the blood. Then the solution was changed to Dulbecco's modified Eagle's medium (DMEM), and an approximately 2.5 cm long right coronary artery segment was isolated and cannulated at both ends. The cannulae were connected to a peristaltic pump, and the solution was pumped from the tissue chamber into the arteriolar lumen (from which it leaked back to the tissue chamber). Then DMEM was supplemented with 3 $\text{mg}\cdot\text{mL}^{-1}$ collagenase type I (Worthington, Lakewood, NJ, USA) for 30 min and with 1 $\text{mg}\cdot\text{mL}^{-1}$ elastase (Worthington) at 60 min. The vessel fell apart after about 90 min under these conditions; the cell-rich solution was then transferred into 24-well plates. After the adherence of the cells to the glass coverslips placed in the wells (about 10 min), the solution was replaced with DMEM to remove the digesting enzymes, and the cells were incubated for 60 min in a CO_2 thermostat. Then, the media was changed to DMEM containing 1 $\text{mg}\cdot\text{mL}^{-1}$ BSA and 5 μM fura2-acetoxymethyl ester (Molecular Probes, Eugene, OR, USA) for 2 h at room temperature. The cover slips were then placed in a suitable chamber for intracellular Ca^{2+} concentration measurements. These measurements were started by washing the cells with Dulbecco's PBS (DPBS) three times, and the measurements were performed in DPBS. The fluorescence of individual cells was measured with an Incytm2 fluorescence imaging system (Intracellular Imaging Inc., Cincinnati, OH, USA). The cells within a field were illuminated alternately at 340 and 380 nm. Emitted light at >510 nm was measured. The cells were treated with 1 μM capsaicin and

then with 100 mM KCl. Data were analysed with the InCyt 4.5 software and further processed with Excel (Microsoft Corp, Redmond, WA, USA) and Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA) software.

Measurement of eye wiping

The eye wiping assay was performed as described previously (Jakab *et al.*, 2005). In short, one drop (10 μL) of agonists (capsaicin, 1 μM ; resiniferatoxin, 10 nM; JYL-273, 1 μM ; MSK-195, 1 μM ; JYL-79, 1 μM ; JYL-1511, 1 μM) was put into the right or left conjunctiva of the rat (single treatment for each rat). The number of eye wipes was counted for 60 s. In the control group, the same volume of solvent was administered in a similar manner.

Materials and solutions

Chemicals were from Sigma-Aldrich (St. Louis, MO, USA) if not stated otherwise. Resiniferatoxin, JYL-273, MSK-195, JYL-79 and JYL-1511 were from Alexis (Enzo Life Sciences AG, Lausen, Switzerland). TRPV1 agonists were dissolved in ethanol.

Statistical analysis

Arteriolar diameter was measured in μm , determined at 80 mmHg intraluminal pressure. Results are shown as mean $\pm$ SEM. Statistical differences were evaluated by Student's *t*-test by comparing values before and after treatments (paired) or comparing eye wipes of vehicle-treated rats with those of TRPV1 agonist-treated rats (unpaired).

Results

Application of the TRPV1-specific agonist capsaicin (1 μM) resulted in a substantial constriction (decrease of arteriolar diameter from 210 ± 11 μm to 91 ± 17 μm , $n = 7$, $P < 0.01$) of skeletal muscle (m. gracilis) arterioles (Figure 1) similar to NA (10 μM , decrease of arteriolar diameter to 68 ± 9 μm , $n = 7$, Figure 1). In contrast, the endothelium-dependent vasodilator ACh evoked dilatation (increase in arteriolar diameter to 240 ± 20 μm , $n = 7$, $P = 0.028$, Figure 1).

The vast majority of published data suggest that vascular TRPV1 stimulation produces a dilatation. It was therefore necessary to test the TRPV1 specificity of these capsaicin-mediated contractile responses. First, a competitive antagonist of TRPV1 was applied. AMG9810 antagonized capsaicin-mediated contractions in a dose-dependent manner (Figure 2A). Moreover, the potency of AMG9810 determined in these assays (177 nM, Figure 2B) was in agreement with its potency determined in other TRPV1-specific systems (Gavva *et al.*, 2005). Nonetheless, the TRPV1 selectivity of these capsaicin-mediated contractile responses was also tested in TRPV1 knockout (TRPV1^{-/-}) mice. The potency of capsaicin (EC_{50}) was 137 nM (Figure 2C) and efficacy was 73% (decrease in diameter from 69 ± 8 μm to 24 ± 3 μm , $n = 6$, Figure 2C) in arteries from wild-type mice, while the same capsaicin treatments were without effect in TRPV1^{-/-} mice (Figure 2C, $n = 5$).

Next, the potential mechanism of TRPV1-mediated constrictions was evaluated. Activation of TRPV1 results in an

increase in intracellular Ca^{2+} concentrations in many TRPV1-expressing cell types and this contributes to the physiological effects. To detect capsaicin-mediated changes in intracellular Ca^{2+} concentrations, a Ca^{2+} imaging system was applied. Simultaneous measurement of intracellular Ca^{2+} concentration and vascular diameter (outer diameter in this case) of cannulated rat arterioles isolated from the gracilis muscle of the rat was performed (Figure 3). The capsaicin-evoked vasoconstriction was paralleled by an increase in intracellular Ca^{2+} concentration (supplementary video file and Figure 3A). Moreover, both vascular diameter and intracellular Ca^{2+} con-

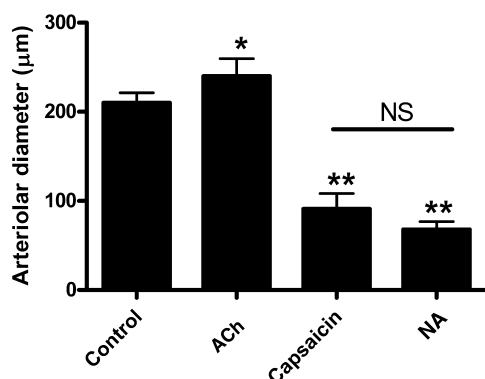


Figure 1

Functional effects of TRPV1 stimulation in skeletal muscle arteries. Internal diameter of cannulated gracilis arteries were measured at 80 mmHg intraluminal pressure before treatments (control). The existence of spontaneous myogenic tone and viability of endothelium was determined by ACh (10 μM)-evoked dilations. The constrictor response to TRPV1 agonist capsaicin (1 μM) was compared with the effect of NA (10 μM). Experiments were performed on the same ($n = 7$) arteries. Values are mean $\pm$ SEM. Significant differences are represented by asterisks (* $P < 0.05$ or ** $P < 0.01$).

centration increased in a dose-dependent manner, with potency in the nanomolar range (note maximal responses at 1 μM , Figure 3B). To identify the TRPV1-expressing cell type, arteriolar smooth muscle cells were isolated from canine coronary arteries (these arteries also responded to capsaicin treatment with a dose-dependent constriction; data not shown) and changes in intracellular Ca^{2+} concentrations to capsaicin (1 μM) and KCl (100 mM) treatments were tested (Figure 4). The capsaicin-mediated increase in intracellular Ca^{2+} concentrations in the cells responding to capsaicin (10 out of 28 cells, representative data in Figure 4A and B) was similar (increase in 340/380 ratio from 0.69 ± 0.10 to 0.93 ± 0.17 , Figure 4C) to the increase evoked by depolarization (100 mM KCl, 340/380 ratio was 1.04 ± 0.20 , Figure 4C).

Having established the TRPV1 specificity of capsaicin-evoked vasoconstriction, the pharmacological properties of these receptors on skeletal muscle arteries of the rat were characterized in detail. The potency of capsaicin on this receptor (EC_{50}) was 221 nM (Figure 5A), efficacy was $58 \pm 7\%$ constriction ($n = 7$), which was not significantly different from the efficacy of NA ($69 \pm 3\%$ constriction, $n = 6$, $P < 0.01$ vs. control, $P = 0.08$ vs. capsaicin). The kinetics of the vasoconstrictor response was determined by continuous application of capsaicin (1 μM) for 20 min. Maximal constriction (decrease of arteriolar diameter from $160 \pm 11 \mu\text{m}$ to $76 \pm 16 \mu\text{m}$, $n = 9$) was achieved at 90 s (Figure 5B). After that, an acute desensitization (decrease of response in the presence of agonist) was observed. Arteriolar diameter was similar to the control at the end of the 20 min treatment (gradual increase to $150 \pm 13 \mu\text{m}$, $n = 9$, Figure 5B). Finally, tachyphylaxis (decrease of response upon repeated application of the agonist) was measured by the re-application of capsaicin (1 μM) after a 40 min regeneration period. Arteriolar diameter decreased from $161 \pm 17 \mu\text{m}$ to $109 \pm 18 \mu\text{m}$ ($n = 6$), suggesting significant resensitization of the receptor (Figure 5C).

Resiniferatoxin was tested under the same conditions (Figure 6). Surprisingly no vascular effects were detected upon

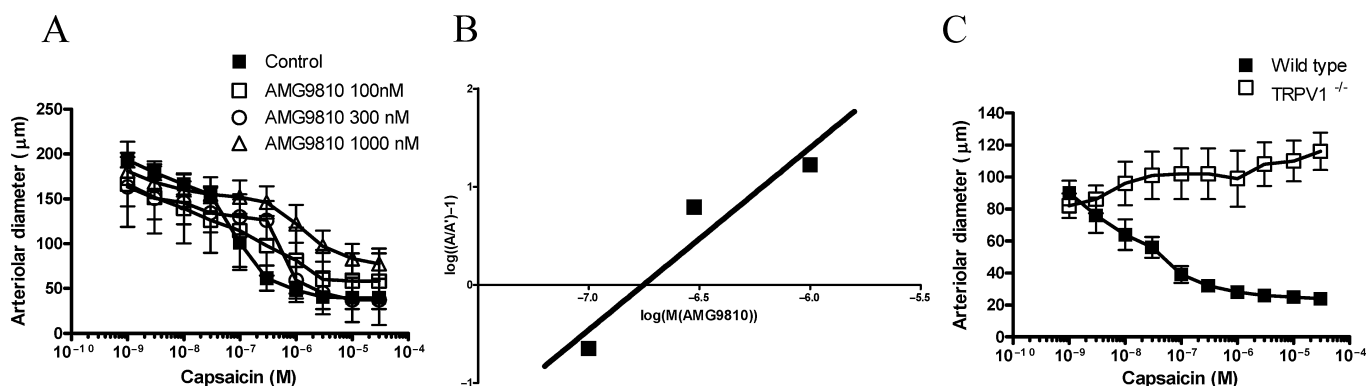


Figure 2

TRPV1 specificity of capsaicin-evoked vasoconstriction. Internal diameter of cannulated gracilis arteries was measured at 80 mmHg intraluminal pressure upon addition of different doses of capsaicin (cumulative dose-response curve) in the absence (control) and presence of the TRPV1 antagonist AMG9810 (100, 300 and 1000 nM, A). Symbols are means $\pm$ SEM of five to nine independent determinations. The equilibration dissociation constant of AMG9810 was determined by the conventional Schild plot (x-intercept, B). Finally, gracilis arterioles isolated from control (wild-type) and TRPV1 knockout (TRPV1 $^{-/-}$) mice were also tested for capsaicin-mediated vasoconstriction (C). Symbols are mean $\pm$ SEM of five to six independent determinations.

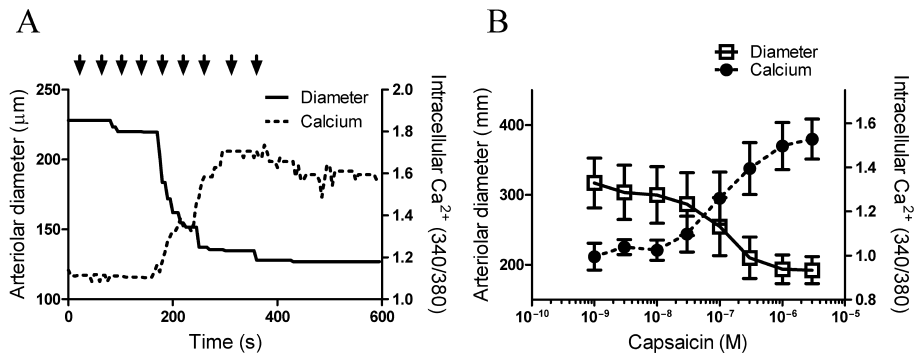


Figure 3

Mechanism of capsaicin-mediated vasoconstriction: skeletal muscle arteries. Capsaicin-evoked changes in arteriolar diameter were recorded in parallel with changes in intracellular Ca^{2+} concentrations of the vascular wall. An individual experiment is shown in (A) (the full recorded experiment also available in the supplementary movie). Solid line represents the arteriolar diameter (please note that in this specific case, the outer diameter is plotted), while dotted line shows intracellular Ca^{2+} concentrations expressed as 340/380 ratio. Capsaicin was administered in a cumulative fashion (indicated by the arrows, the applied capsaicin doses were: 3×10^{-10} , 10^{-9} , 3×10^{-9} , 10^{-8} , 3×10^{-8} , 10^{-7} , 3×10^{-7} , 10^{-6} , 3×10^{-6} M). (B) The mean responses $\pm$ SEM of $n = 5$ single determinations.

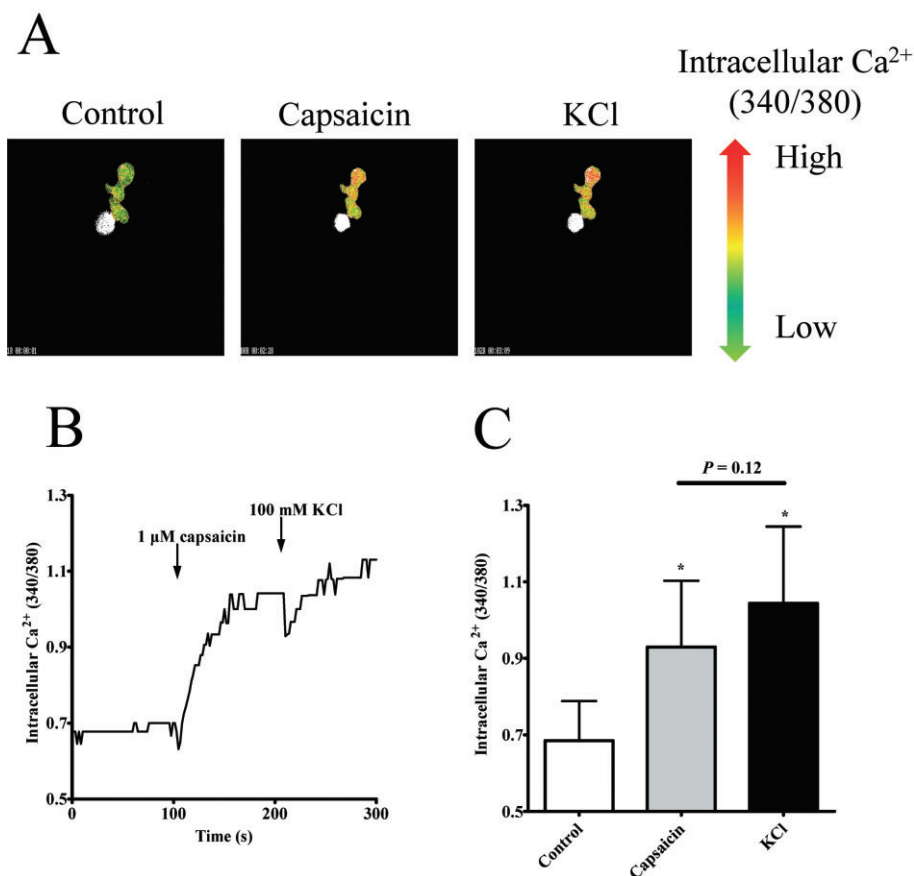


Figure 4

Mechanism of capsaicin-mediated vasoconstriction: isolated arteriolar smooth muscle cells. Canine, freshly isolated coronary arteriolar smooth muscle cells were loaded with fura-2 fluorescent Ca^{2+} -sensitive dye and treated with capsaicin (1 μM) and KCl (100 mM). Changes in intracellular Ca^{2+} concentrations were detected as changes in the 340/380 fluorescence ratio (a representative experiment is shown in A, where green pixels represent low values and red represent high values). Capsaicin evoked a fast increase in the intracellular Ca^{2+} concentrations in some cells, which was not increased further upon the addition of KCl (B). These observations were confirmed when responses of the capsaicin-sensitive cells (10 out of 28 viable cells) were evaluated (C). Columns represent mean $\pm$ SEM.

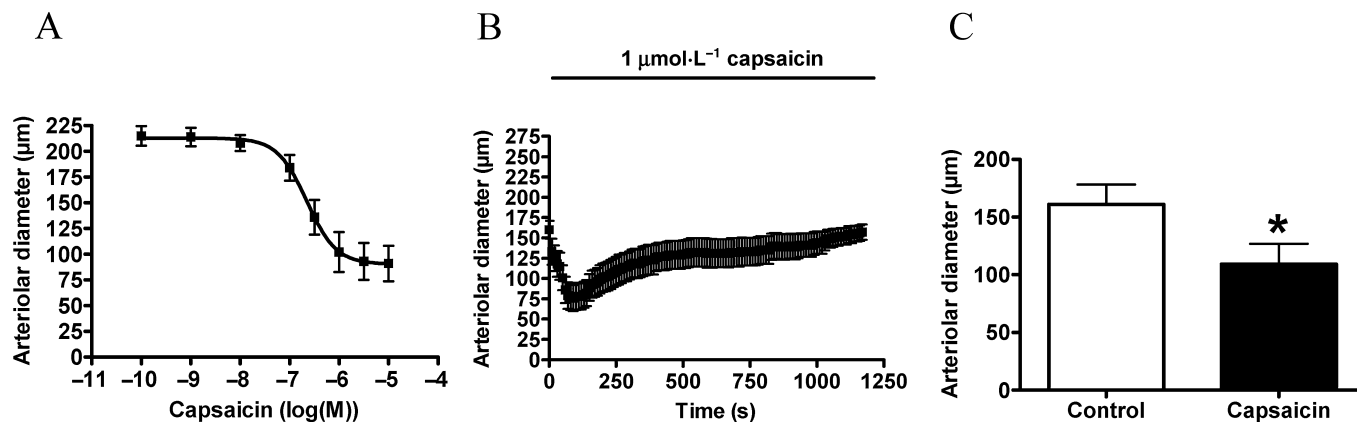


Figure 5

Pharmacological characterization of vascular responses to capsaicin. Experiments were performed on cannulated arteries as mentioned above. First, a cumulative dose-response curve was obtained (A, $n = 7$). Next on a separate set of arteries, the kinetics of response were measured by the application of $1 \mu\text{M}$ capsaicin for 20 min. Arteriolar diameter was measured at 10 s intervals (B, $n = 9$). After this 20 min treatment, the arteries were washed and were incubated in PSS solution for 40 min (regeneration). At the end of regeneration, vasoconstriction to the same dose of capsaicin ($1 \mu\text{M}$) was measured to determine tachyphylaxis (C, $n = 7$). Values are mean $\pm$ SEM, significant difference ($P < 0.05$) is represented by an asterisk.

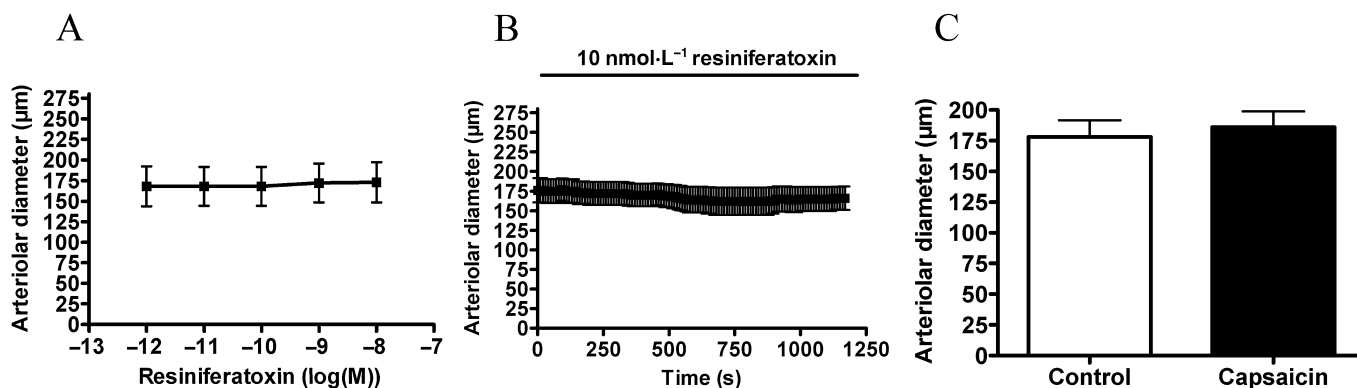


Figure 6

Arteriolar response to resiniferatoxin. Experiments were performed as mentioned in Figure 4 with resiniferatoxin. Responses to cumulative doses are shown in (A) ($n = 3$). No functional response was detected after 20 min of resiniferatoxin (B, 10 nM , $n = 5$). However, this treatment desensitized the receptors to capsaicin ($1 \mu\text{M}$) measured after regeneration (C, $n = 5$).

application in a concentration range from 1 pM to 10 nM (Figure 6A). Moreover, no effects were detected upon application of 10 nM for 20 min (Figure 6B). However, capsaicin ($1 \mu\text{M}$) was without effect after 40 min regeneration (Figure 6C), suggesting complete desensitization of arterial TRPV1 upon the otherwise ineffective resiniferatoxin treatments.

JYL-273 was ineffective at evoking arteriolar vasoconstriction in the concentration range 0.1 nM to $1 \mu\text{M}$ ($n = 7$, Figure 7A), nor did $1 \mu\text{M}$ JYL-273 applied for 20 min have any effect ($n = 5$, Figure 7B). However, similar to resiniferatoxin, this 20 min incubation resulted in complete desensitization of TRPV1 as evidenced by the lack of a response to capsaicin ($n = 4$, Figure 7C).

MSK-195 had a potency of 120 nM and an efficacy of $71 \pm 11\%$ ($n = 5$, Figure 8A). Application of $1 \mu\text{M}$ MSK-195 for 20 min resulted in a transient decrease in arterial diameter

(decrease from $235 \pm 19 \mu\text{m}$ to $155 \pm 25 \mu\text{m}$ at 90 s , $n = 6$, Figure 8B). However, the kinetics of this acute desensitization were slower than that for capsaicin, since the original arteriolar diameter was not restored during the 20 min incubation (arterial diameter after 20 min incubation was 193 ± 25 , $P = 0.03$ vs. before treatment, $n = 6$, Figure 8B). Similar to all the agonists mentioned above, MSK195 also evoked a complete desensitization of capsaicin-sensitive vascular TRPV1 (Figure 8C).

JYL-79 was found to be more potent on vascular TRPV1 ($\text{EC}_{50} = 3.9 \text{ nM}$, $n = 8$, Figure 9A) than capsaicin. Its efficacy was $36 \pm 8\%$ ($n = 8$, Figure 9A). It also evoked a transient vasoconstriction when applied at a concentration of $1 \mu\text{M}$ (decrease of vascular diameter from $228 \pm 13 \mu\text{m}$ to $127 \pm 12 \mu\text{m}$ at 100 s , $n = 5$, Figure 9B). The desensitization of the receptor was not complete at the end of the 20 min incuba-

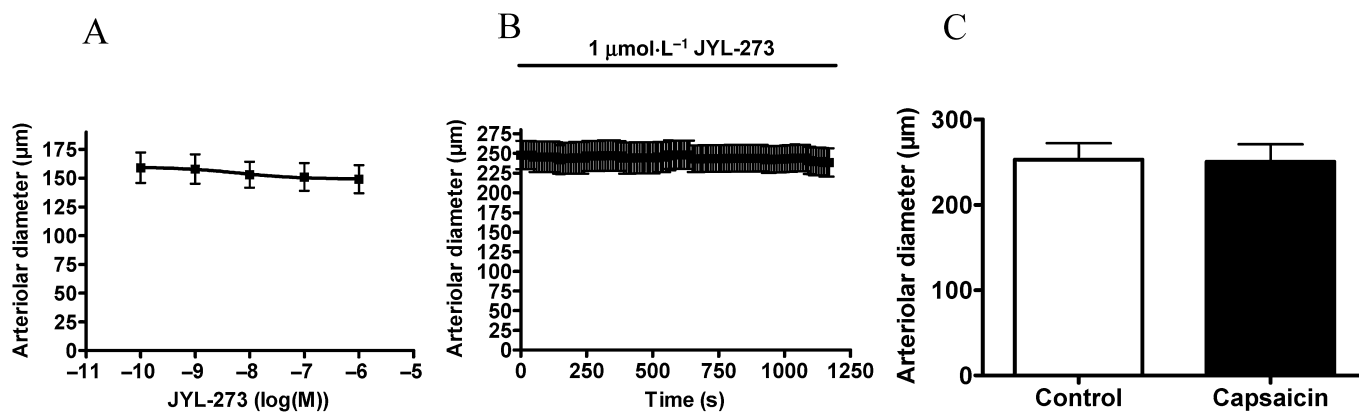


Figure 7

Arteriolar response to JYL-273. Experiments were performed as mentioned in Figure 4 with JYL-273. Responses to cumulative doses are shown in (A) ($n = 7$). No functional response was detected after 20 min of JYL-273 (B, $1 \mu\text{M}$, $n = 5$). However, this treatment desensitized the receptors to capsaicin ($1 \mu\text{M}$) measured after regeneration (C, $n = 4$).

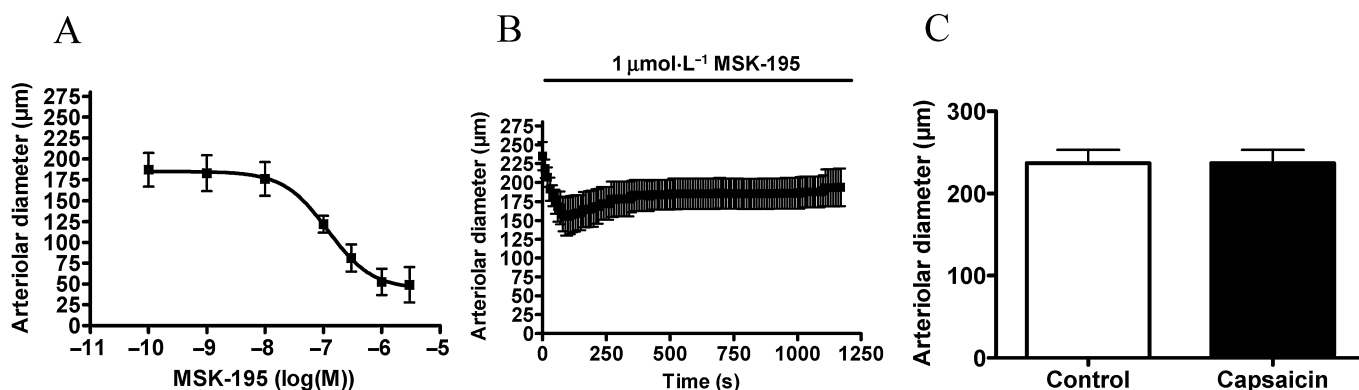


Figure 8

Arteriolar response to MSK-195. Experiments were performed as mentioned in Figure 4 with MSK-195. Responses to cumulative doses are shown in (A) ($n = 5$). A transient vasoconstriction was observed upon MSK-195 stimulation for 20 min (B, $1 \mu\text{M}$, $n = 6$). In addition, this treatment desensitized the receptors to capsaicin ($1 \mu\text{M}$) measured after regeneration (C, $n = 6$).

tion (vascular diameter at 20 min was $204 \pm 13 \mu\text{m}$, $P = 0.046$ vs. before treatment, $n = 5$, Figure 9B). Moreover, no response to capsaicin ($1 \mu\text{M}$) was observed after 40 min regeneration ($n = 5$, Figure 9C).

To estimate the threshold of TRPV1 stimulation, which causes complete desensitization of vascular TRPV1, a partial agonist (JYL-1511) was applied. Its efficacy as an agonist was about 17%, and its potency was 3 nM in a CHO cells overexpressing rat TRPV1 (CHO-TRPV1) cell line (Wang *et al.*, 2003). JYL-1511 was without effect on the vascular diameter in the concentration range 1 nM – $1 \mu\text{M}$ ($n = 6$, Figure 10A). Application of $1 \mu\text{M}$ for 20 min was also without effect (Figure 10B). A partial inhibition (tachyphylaxis) of the capsaicin response ($1 \mu\text{M}$) was noted after 40 min regeneration (decrease of vascular diameter from $244 \pm 14 \mu\text{m}$ to $209 \pm 17 \mu\text{m}$, $P = 0.02$, $n = 6$, Figure 10C). The level of partial agonism/antagonism was also determined (Figure 11). Application of JYL-1511 ($1 \mu\text{M}$) resulted in a decrease in the arteriolar diameter (arteriolar diameter decreased to $94 \pm 3\%$, $n = 6$) and immediate

capsaicin treatment ($1 \mu\text{M}$) resulted in a decrease in diameter to $83 \pm 6\%$ ($P = 0.04$, $n = 6$). In contrast, the same capsaicin treatment alone evoked a decrease in diameter to $43 \pm 7\%$ ($P < 0.01$, $n = 7$) in separate experiments. According to these data, the agonism of JYL-1511 is $10 \pm 5\%$ and antagonism is $70 \pm 11\%$ at the vascular TRPV1.

The aim of this study was to detect differences between TRPV1 populations responsible for sensory neuronal activation and vasoconstriction. A weakness in the previous characterization of the applied agonists is that their effects were tested only *in vitro*, many times only on TRPV1 receptors expressed exogenously (Table 1). Sensory neuronal activation was also tested here by use of the eye wiping assay. JYL-1511 did not evoke significant effects, while all of the other agonists increased the number of eye wipes (Figure 12). Note, that although these data were in complete agreement with previous *in vitro* results (Table 1), differences between sensory neuronal and vascular effects were also noted. In particular, resiniferatoxin and JYL-273 were both ineffective at evoking

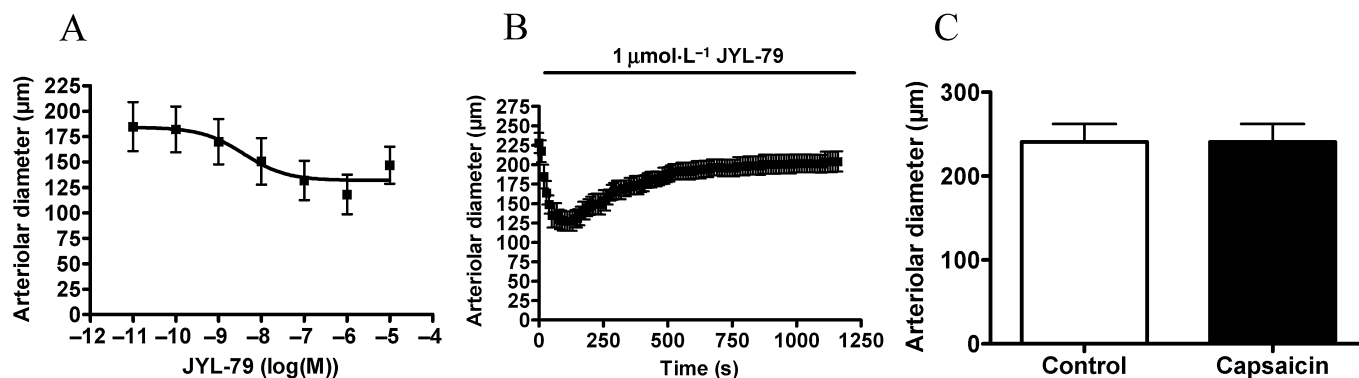


Figure 9

Arteriolar response to JYL-79. Experiments were performed as mentioned in Figure 4 with JYL-79. Responses to cumulative doses are shown in (A) ($n = 8$). A transient vasoconstriction was observed upon JYL-79 stimulation for 20 min (B, 1 μM , $n = 5$). In addition, this treatment desensitized the receptors to capsaicin (1 μM) measured after regeneration (C, $n = 5$).

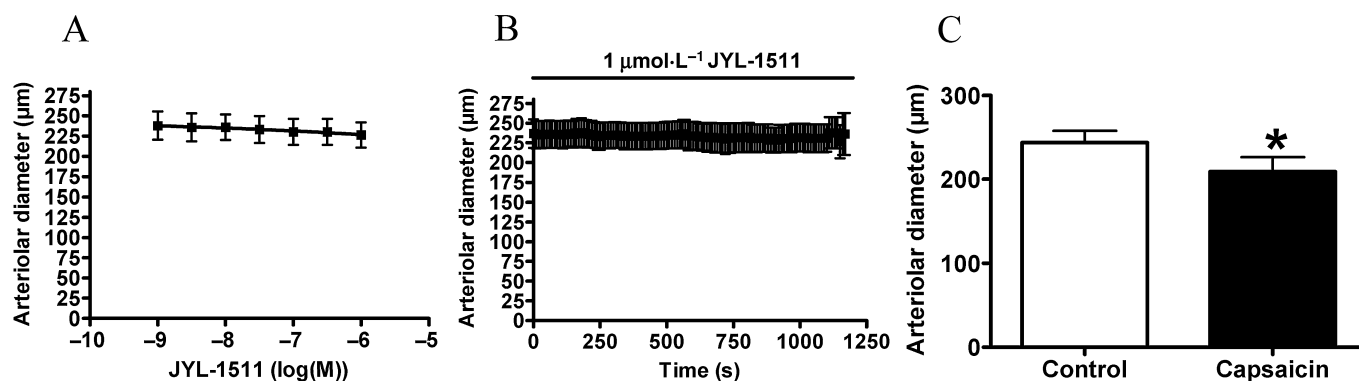


Figure 10

Arteriolar response to JYL-1511. Experiments were performed as mentioned in Figure 4 with JYL-1511. Responses to cumulative doses are shown in (A) ($n = 6$). No functional response was detected after 20 min of JYL-1511 (B, 1 μM , $n = 6$). However, this treatment desensitized the receptors to capsaicin (1 μM) measured after regeneration (C, $n = 6$).

acute activation of vascular TRPV1 receptors (Figures 6 and 7, respectively).

Discussion and conclusions

Here we report an analysis of the pharmacological properties of a vasoconstrictor population of TRPV1. Changes in vascular diameter in response to various agonists and partial agonist/antagonists of the receptor were measured. Our data suggest that significant differences in the pharmacological properties of endogenous TRPV1 pools exist. There are at least two important consequences of this observation. Firstly, TRPV1 antagonists being developed as analgesic agents should be tested for side effects on the circulation. Secondly, selective modulation of vascular TRPV1 may also be a therapeutic target. Pharmacological exploitation of vascular TRPV1 seems to be a reasonable aim with the substantial chemical libraries constructed to develop successful TRPV1 antagonists.

Vascular TRPV1 was characterized here by measuring the vasoconstriction upon TRPV1 stimulation. Previously, TRPV1 was shown to be expressed in vascular smooth muscle cells and it was suggested that activation of TRPV1 is directly linked to intracellular Ca^{2+} elevations in smooth muscle (Kark *et al.*, 2008). Indeed, in the present study we found that a decrease in arteriolar diameter was paralleled by an increase in intracellular Ca^{2+} concentrations in the vascular wall (Figure 3); moreover, direct intracellular Ca^{2+} concentration measurements revealed, for the first time, the presence of functional TRPV1 in isolated arteriolar smooth muscle cells (Figure 4).

Vasoconstriction in response to TRPV1 stimulation was reported decades ago (Molnar and Gyorgy, 1967; Toda *et al.*, 1972; Donnerer and Lembeck, 1982; Duckles, 1986; Edvinsson *et al.*, 1990) and this effect was confirmed later (Szolcsanyi *et al.*, 2001; Dux *et al.*, 2003; Scotland *et al.*, 2004; Keeble and Brain, 2006; Lizanec *et al.*, 2006). Nonetheless, these responses were not thought to be of interest. One of the reasons for this was that pharmaceutical research had

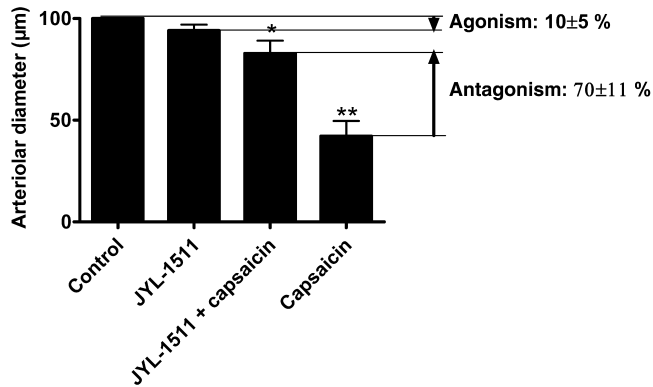


Figure 11

Partial agonism/antagonism of JYL-1511 on TRPV1 located in vascular smooth muscle. Changes in internal diameter of the arteries were measured before treatments (control, $n = 6$) and after addition of 1 μM JYL-1511 (JYL-1511, $n = 6$). Capsaicin responses were also determined in the presence of 1 μM JYL-1511 (JYL-1511 + capsaicin, $n = 6$). Finally, capsaicin responses alone (1 μM , without any pre-treatment) were also measured on a different set of arteries (capsaicin, $n = 7$). The efficacy of JYL-1511 as a partial agonist was expressed as the percentage of decrease in arteriolar diameter evoked by the application of capsaicin alone (100%, capsaicin). Its efficacy as a partial antagonist was expressed as the percentage of decrease in capsaicin constriction (100%, capsaicin) in the presence of JYL-1511 (1 μM , JYL-1511 + capsaicin).

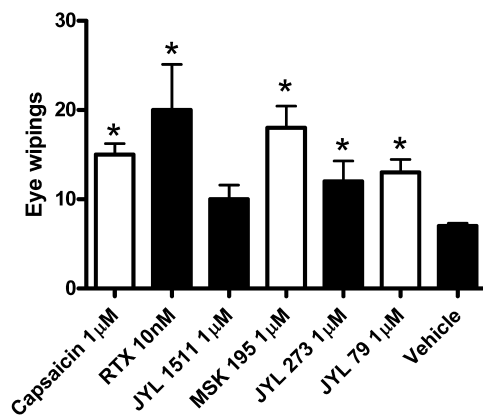


Figure 12

Sensory neuronal irritation evoked by application of the TRPV1 ligands. TRPV1 agonists or vehicle were applied in the eye of rats to determine their ability to evoke sensory neuronal irritation. Number of eye wipes was counted for 60 s after application of 10 μL of the drugs onto the conjunctiva of the rats. Concentrations of the drugs were chosen to represent the highest dose used in the vascular experiments (indicated in the figure). Columns represent mean $\pm$ SEM ($n = 5$), significant differences ($P < 0.05$) from the control (wipes upon administration of the vehicle alone) are represented by asterisks.

Table 1
Pharmacological properties of TRPV1 agonists

Agonist	Dorsal root ganglion/CHO-TRPV1		CHO-TRPV1		Arteriolar TRPV1 (vasoconstriction)		
	K_i (binding)	EC_{50} ($^{45}\text{Ca}^{2+}$ uptake)	EC_{50} (intracellular Ca^{2+})	Acute desensitization	Potency	Efficacy	Tachyphylaxis
Capsaicin	$1.8 \pm 0.3 \mu\text{M}$ (Wang <i>et al.</i> , 2003)	$95 \pm 8 \text{ nM}$ (Pearce <i>et al.</i> , 2008)	$35 \pm 11 \text{ nM}$ (Toth <i>et al.</i> , 2005b)	+	221 nM	$58 \pm 7\%$	-
Resiniferatoxin	23 pM (Lee <i>et al.</i> , 2001)	$1.5 \pm 0.3 \text{ nM}$ (Pearce <i>et al.</i> , 2008)	$81 \pm 20 \text{ pM}$ (Toth <i>et al.</i> , 2005b)	-	>10 nM	No effect at 10 nM	+
JYL-273	$11 \pm 4 \text{ nM}$ (Lee <i>et al.</i> , 2001)	$361 \pm 54 \text{ nM}$ (Lee <i>et al.</i> , 2002)	No data	No data	>3 μM	No effect at 1 μM	+
MSK-195	No data	$162 \pm 33 \text{ nM}$ (Lee <i>et al.</i> , 2002)	$52 \pm 12 \text{ nM}$ (Toth <i>et al.</i> , 2005b)	-	120 nM	$71 \pm 11\%$	+
JYL-79	$19 \pm 4 \text{ nM}$ (Lee <i>et al.</i> , 2001)	$58 \pm 8 \text{ nM}$ (Lee <i>et al.</i> , 2002)	$2.4 \pm 1.0 \text{ nM}$ (Toth <i>et al.</i> , 2005b)	-	3.9 nM	$36 \pm 8\%$	+
JYL-1511	$50 \pm 17 \text{ nM}$ (Wang <i>et al.</i> , 2003)	$3.4 \pm 0.5 \mu\text{M}$ (Wang <i>et al.</i> , 2003)	No data	+	N/A	No effect at 1 μM	+/-

concentrated on the exploitation of the obvious potential of sensory neuronal TRPV1 as an analgesic target. Another reason was that stimulation of sensory neuronal TRPV1 in the perivascular nerves evokes vasodilatation (Zygmunt *et al.*, 1999), probably obscuring the vasoconstrictor response in many cases. In accordance with this latter mechanism, earlier reports showed concentration-dependent biphasic effects of TRPV1 stimulation; low dose capsaicin evoked dilatation, while higher concentrations resulted in constriction (Edvinsson *et al.*, 1990; Dux *et al.*, 2003). This suggested the involvement of different receptors or different pharmacology for TRPV1-mediated vascular dilatation and constriction.

Although the mechanism of the vasoconstrictor effects of TRPV1 agonists were generally not investigated in detail (Porszasz *et al.*, 2002; Dux *et al.*, 2003; Keeble and Brain, 2006), it was suggested that TRPV1-induced vasoconstriction is probably mediated by endothelin-1 (Szolcsanyi *et al.*, 2001) or substance P (Scotland *et al.*, 2004) release from sensory neurons.

We have recently shown that stimulation of TRPV1 in skeletal muscle arterioles results in a substantial vasoconstriction (Lizanecz *et al.*, 2006). Moreover, intra-arterial injection of capsaicin into the hind limb evoked a dose-dependent increase in blood flow in the skin (probably representing vasodilatation in this organ) and simultaneously, a decrease of blood flow in skeletal muscle (representing vasoconstriction) (Kark *et al.*, 2008). These data suggested that vascular TRPV1 have sensory neuron-independent physiological effects.

The TRPV1 specificity of capsaicin-mediated arteriolar vasoconstriction was ultimately proven here. Most importantly, capsaicin-mediated responses were absent in TRPV1 knockout mice (Figure 2C). Moreover, an effort was also made to investigate the potential mechanisms. The intracellular Ca^{2+} concentration measurements we obtained showed that capsaicin increased the diameter of the arterial wall of skeletal muscle arteries (Figure 3), as well as in isolated arteriolar smooth muscle cells (Figure 4). Although only 10 out of 28 isolated smooth muscle cells responded to capsaicin, these data strongly suggest that functional TRPV1 is expressed in arterial smooth muscle cells and that the activation of these receptors leads to an increase in smooth muscle intracellular Ca^{2+} concentrations and to vasoconstriction. Next, the effects of pharmacological TRPV1 inhibition on this response were tested. The TRPV1 antagonist AMG9810 (previously tested on exogenous and sensory neuronal TRPV1) inhibited this capsaicin-evoked arteriolar constriction in a competitive fashion. These findings suggest that TRPV1 antagonists developed as analgesic agents may also interfere with skeletal muscle blood perfusion by inhibiting vascular TRPV1.

Nonetheless, the major goal of this present work was to investigate the structure-activity relationship of TRPV1 agonists for the vascular TRPV1 in functional assays. Our results confirmed that TRPV1 stimulation by capsaicin evokes a substantial constriction in isolated cannulated skeletal muscle arteries (Lizanecz *et al.*, 2006; Kark *et al.*, 2008). Here, a series of commercially available agonists were also tested in addition to capsaicin. Significant differences in potency, efficacy and desensitization were found (Table 1). It was observed that some of the TRPV1 agonists (such as resiniferatoxin, JYL-273) were able to desensitize vascular TRPV1 without any apparent

vascular effects. This behaviour of resiniferatoxin in the vascular diameter assay is not unprecedented; a very similar action ('desensitization' to capsaicin without prior activation) has been demonstrated for pulmonary chemoreflex (Szolcsanyi *et al.*, 1990). One hypothesis for this desensitization is that low level activation of TRPV1 with certain structures may be sufficient to evoke complete tachyphylaxis, without increasing the intracellular Ca^{2+} concentrations to those levels needed to induce vasoconstriction. Alternatively, tachyphylaxis may be the reason for the irreversible activation of TRPV1 by resiniferatoxin (Jeffrey *et al.*, 2009) leading to a sustained Ca influx. To measure the level of activation needed to evoke tachyphylaxis a partial agonist (JYL-1511) was used. Its partial antagonism was confirmed on vascular receptors (about 10% agonism and 70% antagonism), and its application resulted in significant tachyphylaxis, suggesting a role for desensitization rather than sustained Ca^{2+} influx in this system. In addition, although the functional response to capsaicin was transient and the arteries were completely desensitized to capsaicin, after a 40 min regeneration period, capsaicin was able to evoke vasoconstriction, suggesting resensitization of the arteries and only a partial tachyphylaxis. Taken together, these results suggest the vascular smooth muscle-located receptor and also the TRPV1 responsible for eye irritation upon capsaicin treatment *in vivo* (Figure 12) seem to have different ligand selectivity for desensitization from that of the TRPV1 expressed in CHO cells (Table 1).

It was observed that the kinetics of acute desensitization were different in the case of agonists evoking vascular constriction. With capsaicin, complete acute desensitization was observed, while for other agonists, JYL-79, MSK-195, only a partial desensitization was observed. The fact that different agonists evoked responses with different durations suggests that TRPV1 agonists may be tailored to desired duration of vascular effects.

Several mechanisms have been suggested to regulate TRPV1 sensitivity and desensitization. These include PKC- (Bhave, 2003) or PKA- (Bhave *et al.*, 2002) mediated phosphorylation and calcineurin-mediated dephosphorylation (Docherty *et al.*, 1996). As regards TRPV1 mediating skeletal muscle vasoconstriction, phosphorylation seems to be the most likely candidate. It was found that anandamide (Lizanecz *et al.*, 2006), similar to resiniferatoxin and JYL-273 (shown in this report), evokes complete tachyphylaxis on vascular TRPV1 without functional effects. However, it was also shown, that the anandamide-mediated tachyphylaxis was antagonized by a protein phosphatase 2B (calcineurin) inhibitor (Lizanecz *et al.*, 2006). Moreover, in accordance with this hypothesis, TRPV1 responses to agonists were differently modulated by inhibition of calcineurin in a CHO-TRPV1 cell line (Pearce *et al.*, 2008), suggesting ligand selectivity for phosphorylation-dependent TRPV1 sensitization/desensitization/tachyphylaxis.

Taken together, our results indicate that TRPV1 (a non-specific Ca^{2+} channel) activation leads to an increase in intracellular Ca^{2+} concentrations in isolated coronary smooth muscle cells and in the wall of isolated skeletal muscle arteries, resulting in vasoconstriction. The pharmacological profile of the vascular TRPV1 differs from that of the TRPV1 population responsible for sensory irritation. Arteriolar

TRPV1 was inhibited by a competitive TRPV1 antagonist developed as an analgesic suggesting that vascular TRPV1 activation may represent a side effect of TRPV1 antagonists when used as analgesics *in vivo*. Moreover, vascular TRPV1 may be a new therapeutic target for the regulation of tissue blood distribution.

Acknowledgements

The study was supported by the Hungarian Academy of Sciences OTKA (K68077 and K84300 to AT and T046244 to RP) and by the Hungarian Ministry of Health (ETT 377/2009 to AT); the work is supported by the TÁMOP 4.2.1./B-09/1/KONV-2010-0007 project (to AT and ZP). The project is implemented through the New Hungary Development Plan, co-financed by the European Social Fund; and by the National Innovation Office of Hungary (Baross Gábor Project, ÉletMent). Finally, AT was supported by the Bolyai János Research Scholarship of the Hungarian Academy of Sciences.

Conflicts of interest

The authors report no conflicts of interest.

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