Kutatásaink legfontosabb eredménye, hogy sikerült a transient receptor potential 1 (TRPV1) csatorna funkcionális arteriális expresszióját kimutatnunk. További kísérleteinkben felismertük, hogy a vaszkuláris TRPV1 farmakológiai tulajdonságai eltérnek a neuronális, fájdalomérzetért felelős receptorétól. Eredményeink arra utalnak, hogy a vaszkuláris TRPV1 egy független gyógyszergyári célpont lehet az erek összahúzására irányuló erőfeszítések esetében, továbbá, hogy a TRPV1 fájdalomcsillapítás érdekében történő gátlása nem kívánt vaszkuláris hatásokhoz vezethet.

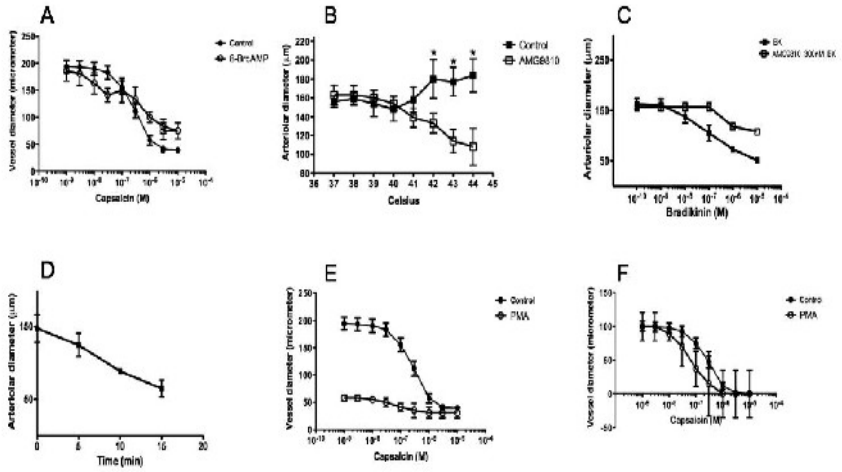
A részletes beszámolóban a publikációkban szereplő eredményeket rövidítve (a megjelent/elfogadott publikációk csatolása mellett), míg az azokban nem szereplő adatokat külön ábrákon mutatjuk be a pályázat eredeti munkaterve (vastag karakterekkel és kis betűvel idézve) alapján:

2007: A vanilloid receptor-1-et (TRPV1) expresszáló sejtek azonosítása vázizom, bőr, agy, szív és mezentériális erekben. A TRPV1 stimulációra bekövetkező vazoaktív hatások (konstrikció, dilatáció) jellemzése a fenti szövetekből izolált erekben. A TRPV1 fiziológiás értónushoz történő hozzájárulásának vizsgálata TRPV1 stimuláló patológiás körülmények esetén (alacsony pH, magas hőmérséklet és gyulladásos mediátorok jelenlétében (pl. bradikinin)).

A vanilloid receptor-1 (TRPV1) kimutatását immunhisztokémiai módszerekkel végeztük el. A hagyományos szenzoros neuronális jelenléten kívül, azzal összemérhető intenzitású vaszkuláris expressziót is kimutattunk. Behatóan két terület vizsgálatát végeztük el: vázizom és bőr arteriális TRPV1-et jellemeztünk morfológiai és funkcionális módszerekkel (Kark et al, Molecular Pharmacology 73, 1405-1412., 2008, a beszámolóhoz teljes terjedelemben csatolva). A cikkben szereplő legfontosabb, a munkaterv ezen pontjához releváns eredményeink:

1. A vázizomban futó artéria falában simaizomra jellemző TRPV1 festődést találtunk, míg a bőrben futó arteriola falában nem volt TRPV1 pozitivitás.
2. A vázizomban futó ér környezetében nem sikerült kimutatni TRPV1-et expresszáló szenzoros neuronok nyúlványainak jelenlétét, míg a bőrben futó artéria esetében ilyen képleteket nagy mennyiségben azonosítottunk.
3. A vázizomban futó artéria izolálását követően (kanülált ér, izobárikus körülmények) kapszaicin hatására vazokonstrikciót tapasztaltunk. A vazokonstrikció mértéke összemérhető volt a norepinefrinével (maximális konstrikció).
4. Ugyanezen érterületek in vivo kapszaicin válaszait tanulmányozva a bőrben vazodilatációt, a vázizomban vazokonstrikciót tapasztaltunk.

A TRPV1 fiziológiás értónushoz történő hozzájárulását pathológiás körülményeket modellező kísérletekben vizsgáltuk (1. ábra).



1. ábra Arteriális TRPV1 válaszkészséget befolyásoló tényezők

Eredményeink szerint a PKA útvonal aktiválása nem befolyásolta a TRPV1 stimuláció hatására bekövetkező vazokonstrikciót (1/A panel). A hőmérséklet emelkedése a vázizomból izolált artéria esetében vazokonstrikciót váltott ki, amely gátolható volt a TRPV1 antagonista AMG9810-vel (1/B panel). Ez arra utal, hogy a vaszkuláris TRPV1 képes lehet hőmérséklet-szenzorként viselkedni ebben a lokalizációban is, hozzájárulva a magas testhőmérséklet során megfigyelhető élettani jelenségekhez. A szenzoros neuronokban a TRPV1 egyik jól ismert aktivátora a bradikinin. Hatásait izolált vázizomereken tanulmányozva meglepetéssel tapasztaltuk, hogy az irodalomban elfogadott dilatáció helyett vazokonstrikció jelentkezett. Ezen vazokonstrikció gátolható volt a TRPV1 antagonista AMG9810-vel (1/C panel), ami arra utal, hogy a vázizomerekben bradikinin hatására bekövetkező konstrikciót a TRPV1 aktiválása magyarázhatja. Végül a PKC aktivátor PMA hatásait is vizsgáltuk a vaszkuláris TRPV1 érzékenységére. A PMA önmagában vazoaktívnak bizonyult, amennyiben lassan kifejlődő, de jelentős mértékű vazokonstrikciót váltott ki (1/D panel). A kapszaicin alkalmazása ilyen körülmények között kismértékben tovább csökkentette az érátmérőt (1/E panel), mely hatás dózis-függését a normalizált adatokból lehet jobban megítélni (1/F panel). A PMA által okozott feltehetően TRPV1 független konstrikció mellett nehéz pontos megállapításokat tenni, de a PKC aktivációja látszólagosan mintegy egy nagyságrenddel növelte a TRPV1 érzékenységet kapszaicinre. Az ábrán bemutatott eredményeink publikálására még nem került sor.

2008: TRPV1 agonista (capsaicin, SU-200, CHK-154, NADA, JYL-79, anandamide), antagonista (capsazepine, AMG 9810, JYL-1421, IBTU) és parciális agonista (JYL-1511, JYL-827) vegyületek struktúra-aktivitás viszonyainak jellemzése. A TRPV1 agonistákkal szembeni in vivo szenzitivitásának vizsgálata foszforilációt fokozó (8Br-cAMP, PMA, cyclosporine-A) kezelések mellett.

A tervben megfogalmazott kísérletek eredményeit közlésre elfogadták (Á Czikora; E Lizanecz; P Bakó; I Rutkai; F Ruzsnavszky; J Magyar; R Pórszász; T Kark; A Facskó; Z Papp; I Édes; A Tóth: Structure-activity relationships of vanilloid receptor agonists for arteriolar TRPV1, British Journal of Pharmacology, in press, az elfogadott kézirat a beszámolóhoz teljes terjedelemben csatolva). A közleményben bemutatott, a fenti tervhez közvetlenül kapcsolódó legfontosabb eredményeink:

1. A kapszaicin stimulációra bekövetkező arteriaális vazokonstrikció TRPV1 mediált, amennyiben specifikus antagonistával gátolható és TRPV1 knock out egérben nem figyelhető meg.
2. A vaszkuláris TRPV1 stimulációjára a simaizomsejt rétegben intracelluláris Ca2+ koncentráció emelkedés történik a kifejlődő vazokonstrikcióval párhuzamosan.
3. A TRPV1 stimuláció intracelluláris Ca2+ koncentráció fokozó hatását izolált simaizomsejtek esetében is meg lehet figyelni.

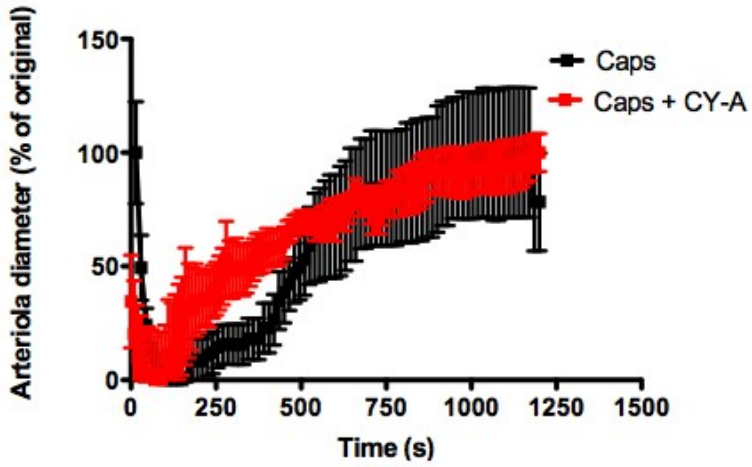
4. A vaszkuláris receptor farmakológiailag eltér a szenzoros neuronokban expresszálódó receptortól. A különböző TRPV1 agonistákkal történő stimulálás hatására a szenzoros irritáció nem mutat egyértelmű párhuzamot a vaszkuláris konstrikcióval.
5. Az agonisták hatására bekövetkező vaszkuláris TRPV1 stimuláció gyakran a receptor deszenzitizációjához vezet.
6. A deszenzitizáló hatáshoz a receptor 10% környéki mértékű stimulációja már elegendő.

A fenti célok közül a TRPV1-et potenciálisan foszforiláló kinázok aktiválásának hatásait már az 1-es ábrán bemutattuk. Ennek során a PKA stimulálásának nem találtunk hatását, míg a PKC stimuláció során a jelentős TRPV1 független konstrikció jelenléte tette a részletes analízist kevéssé kívánatossá (lásd 1. ábra). A TRPV1 foszforilációs szintjének emelkedését azonban a kináz aktivitások fokozódása mellett a foszfatáz aktivitások csökkenése is okozhatja. Korábbi eredmények alapján a TRPV1-et defoszforiláló foszfatáz a kalcineurin. A kalcineurin gátló ciklosporin hatásait a receptor farmakológiai tulajdonságaira egy kollaboráció keretében behatóan tanulmányoztuk (Pearce et al, Naunyn Schmiedebergs Arch Pharmacol. 377(2):149-57). A vaszkuláris receptorra ugyanakkor nem találtunk jelentős hatását a ciklosporinnak (2. ábra).

2009: A TRPV1 deszenzitizáció kinetikájának és mértékének vizsgálata különböző agonista és parciális agonista hatású molekulákkal. A deszenzitizáció foszforilációs szabályozásának vizsgálata. A TRPV1 stimuláció által kiváltott szenzoros-efferens hatások (lokális szenzoros neuronális hatások) karaketrizálása izolált ereken.

A 2008-ra kitűzött célok kapcsán bemutatott közleményünkben a vaszkuláris TRPV1 farmakológiai tulajdonságainak cizsgálata során valamennyi tervben megfogalmazott paramétert vizsgáltuk. A deszenzitizáció kinetikájának vizsgálata kapcsán eltéréseket találtunk. A kapszaicin válaszkészség 15 perc alatt teljesen eltűnt 1 mikromól kapszaicin jelenlétében. Ugyanakkor például az MSK-195 illetve a JYL-79 esetében egy hosszabb ideig fenntartott konstrikció volt megfigyelhető. Adataink arra utalnak, hogy a TRPV1 akut deszenzitizációja független az agonizmus mértékétől. Ennek a ténynek ékes példája a reziniferatoxin, amely akut vaszkuláris hatás nélkül volt képes a receptor teljes deszenzitizációját kiváltani.

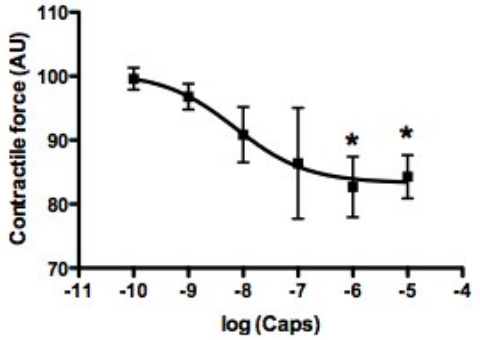
A foszforiláció TRPV1 érzékenységben betöltött szerepe, mint az már a fentebbi ábra és magyarázatok alapján is felszínre került, nehezen megítélhetőnek bizonyultak. A kalcineurin gátlás beható vizsgálatának ugyanakkor nem volt akadálya (2. ábra).



2. ábra A kalcineurin gátlás (ciklosporin kezelés) hatásainak vizsgálata

Eredeményeink szerint a kapszaicin maximális vazokonstriktív hatása és a deszenzitizáció kinetikája sem különbözött ciklosporin-A jelenlétében.

Végül, komoly erőfeszítéseket tettünk annak érdekében, hogy sikerüljön az irodalomban leírt szenzoros neuronális hatásokat a vaszkulatúra tekintetében is jellemeznünk. Irodalmi adatok alapján a perivaszkuláris szenzoros neuronok stimulációjának vazodilatációhoz kell vezetnie. Ezt a jelenséget in vivo sikerült is kimutatnunk patkány bőr esetében (Kark et al, 2008).



3. ábra TRPV1 mediált dilatatív hatás patkány trachea esetében

Ugyanakkor a kísérleteink során a TRPV1 stimuláció hatására jellemzően vazokonstriktív hatásokat tapasztaltunk (vázizom és koronária erek), vagy a kapszaicin nem mutatott vazoaktív hatást (mezenteriális ér, baziláris artéria). Valójában az egyetlen kapszaicin kezelésre dilatációval válaszoló területnek a trachea bizonyult, jóllehet a dilatáció mértéke itt is csekély volt (3. ábra).

2010: A TRPV1 aktivátorok/inhibitorok hatásosságának ellenőrzése különböző in vivo körülmények között (egészséges, hipotenzív sokk, gyulladás, ischaemia, ischaemia-reperfúzió). A klinikai tesztekben hatékony molekulák vizsgálata az izolált ereken. A projekt financiális és adminisztratív lezárása.

A TRPV1 funkcionális jelentőségére ischaemia-reperfúzió során számos adat utal. A pályázat céljainak megvalósítása érdekében mindenekelőtt a TRPV1 szerepét kívántuk igazolni, ezért TRPV1 knockout egérrel végeztünk kísérleteket. Eredményeink szerint kapszaicin mind izolált koronária artériákon, mind working heart modellekben teljes koronária konstrikciót okoz, mely jelenség nem figyelhető meg a TRPV1 génhiányos egerekben. Jelenleg is folyó kísérleteinkben arra keressük a választ, hogy (1) jelent-e protekciót a TRPV1 jelenléte és aktiválása ischaemia-reperfúzió során; (2) ez a hatás mennyiben specifikus a TRPV1-re.

A klinikai tesztekben hatékonynak bizonyult TRPV1 antagonistákkal kapcsolatban sikerült azt kimutatnunk, hogy az SB-705498, BCTC, JYL-1421, AMG-517 egyaránt gátolta a vaszkuláris TRPV1-et is. Ezen eredményeink azt sugallják, hogy a fájdalomcsillapító hatásukért kifejlesztett TRPV1 antagonistáknak komoly kardiovaszkuláris hatásuk lehet, mely a vázimok mellett a koronária ereket is érintheti.

Végül a pályázati periódusban módunk nyílt a TRPV1 endogén ligandjának tekintett vegyület, az anandamid és a TRPV1 receptor kölcsönhatásainak bemutatására is egy összefoglaló közlemény erejéig. Ezen közleményben tárgyaltuk a vaszkuláris TRPV1 és anandamid vonatkozásában addig rendelkezézünkre álló adatokat is. Összességében azt a következtetést vontuk le, hogy az anandamid kevéssé képes a vaszkuláris TRPV1 aktivitását befolyásolni élettani körülmények között.

A pályázat megvalósítása során három, a pályázathoz közvetlenül kapcsolódó közleményt sikerült a laboratóriumunkból közölni, amely közlemények lefedik a pályázatban megfogalmazott célok túlnyomó részét. A közlemények impakt faktora 11, és a mai napig 27 hivatkozás született rájuk, jóllehet, az utolsó, és várhatólag legnagyobb hatású munkát az augusztus során fogadták el, és így még elektronikusan nem elérhető.

Ki kell ugyanakkor hangsúlyozni, hogy ezen, valóban csak ezen pályázathoz kötődő publikációkon

túl közvetetten a pályázat a laboratórium fenntartása és megalapozása révén hozzájárult számos további, ezen időszak során megjelent publikációnkhoz is. Végül, a kísérltek során felszínre került jelenségek kapcsán két további kéziratot is elkészítettünk: az egyiket major revision után szerkesztői döntésre vár a Life Sciences folyóiratban, amely munkánkban a TRPV1 endogén ligandjának vélt anandamid vaszkuláris hatásait karakterizáltuk részletesen és remélhetőleg napokon belül elfogadásra kerül, míg a másik munkában a tartós TRPV1 deszenzitizálás (születés utáni nagy dózisú kapszaicin kezelés) szenzoros és vaszkuláris hatásait tanulmányoztuk. Ezen munka közlésre történő előkészítése zajlik. Az eredményeit tekintve a legfontosabb az, hogy az amúgy magyar (Jancsó és Szolcsányi professzorok nevéhez kötődő) módszer hatékonyan eliminálja a TRPV1 mediált fájdalomhoz köthető folyamatokat (szenzoros irritáció, plazmaextravazáció), míg nem látszik hatékonynak a vaszkuláris TRPV1 mediált funkciók vonatkozásában.

Debrecen, 2011. szeptember 2.

Tóth Attila
témavezető

0026-895X/08/7305-1405–1412$20.00
MOLECULAR PHARMACOLOGY
Vol. 73, No. 5
43323/3328264

Mol Pharmacol 73:1405–1412, 2008
Printed in U.S.A.

Tissue-Specific Regulation of Microvascular Diameter: Opposite Functional Roles of Neuronal and Smooth Muscle Located Vanilloid Receptor-1

Tamás Kark, Zsolt Bagi, Erzsébet Lizanecz, Enikő T. Pásztor, Nóra Erdei, Ágnes Czikora, Zoltán Papp, István Édes, Róbert Pórszász, and Attila Tóth

Department of Pharmacology and Pharmacotherapy, Institute of Pharmacology (T.K., R.P.), and Division of Clinical Physiology, Institute of Cardiology (Z.B., E.L., E.T.P., N.E., Á.C., Z.P., I.É., A.T.), Medical and Health Science Center, University of Debrecen, Debrecen, Hungary

Received November 8, 2007; accepted February 5, 2008

ABSTRACT

The transient receptor potential type V1 channel (vanilloid receptor 1, TRPV1) is a Ca^{2+}-permeable nonspecific cation channel activated by various painful stimuli including ischemia. We hypothesized that TRPV1 is expressed in the arterioles and is involved in the regulation of microvascular tone. We found that TRPV1 stimulation by capsaicin (intra-arterial administration) of the isolated, perfused right hind limb of the rat increased vascular resistance (by 98 ± 21 mm Hg at 10 μg) in association with decreased skeletal muscle perfusion and elevation of skin perfusion (detected by dual-channel laser Doppler flowmetry). Denervation of the hind limb did not affect capsaicin-evoked changes in vascular resistance and tissue perfusion in the hind limb but reduced the elevation of perfusion in the skin. In isolated, pressurized skeletal (musculus gracilis) muscle arterioles (diameter, 147 ± 35 μm), capsaicin had biphasic effects: at lower concentrations, capsaicin (up to 10 nM) evoked dilations (maximum, 32 ± 13%), whereas higher concentrations (0.1–1 μM) elicited substantial constrictions (maximum, 66 ± 7%). Endothelium removal or inhibition of nitric-oxide synthase abolished capsaicin-induced dilations but did not affect arteriolar constriction. Expression of TRPV1 was detected by reverse transcriptase-polymerase chain reaction in the aorta and in cultured rat aortic vascular smooth muscle cells (A7r5). Immunohistochemistry revealed expression primarily in the smooth muscle layers of the gracilis arteriole. These data demonstrate the functional expression of TRPV1 in vascular smooth muscle cells mediating vasoconstriction of the resistance arteries. Because of the dual effects of TRPV1 stimulation on the arteriolar diameter (dilation in skin, constriction in skeletal muscle), we propose that TRPV1 ligands represent drug candidates for tissue-specific modulation of blood distribution.

The transient receptor potential type V1 channel (vanilloid receptor-1, TRPV1) is a nonselective cation channel, structurally belonging to the transient receptor potential family of ion channels. TRPV1 is found in sensory C and A-δ fibers (Caterina et al., 1997) and functions as a ligand-, proton-, and heat-activated molecular integrator of nociceptive stimuli in the periphery (Szallasi and Blumberg, 1999; Di Marzo et al., 2002a,b; Ross, 2003). Activation of TRPV1 leads to central (pain) as well as to local "sensory-efferent" effects (Szolcsányi, 1988).

It is well established that the sensory-efferent effects of TRPV1 stimulation include the release of neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P (SP) from the sensory nerve terminals. These peptides cause vasodilatation in different vascular beds such as mesenteric, hepatic, basilar, dural, and meningeal arterioles (Zygmunt et al., 1999; Ralevic et al., 2001; Harris et al., 2002; Dux et al., 2003; Akerman et al., 2004; O'Sullivan et al., 2004). It is interesting that TRPV1-induced release of SP from sensory neurons has been implicated recently in mediating pressure-induced myogenic constriction (Scotland et al., 2004). Likewise, previous studies have also proposed that in certain circumstances, TRPV1 activation may lead to vasoconstriction in mesenteric (Pórszász et al., 2002), coronary (Szolcsá-

The study was supported by the Hungarian Academy of Sciences OTKA (F048873 and K68077 to A.T., T046244 to R.P., and F-048837 to Z.B.) and by the Hungarian Ministry of Health (ETT 430/2006 to A.T., ETT 493/2006 to R.P., ETT-454-2006 to I.E.) grants. Z.B., Z.P., R.P., and A.T. were supported by Bolyai Fellowship of the Hungarian Academy of Sciences.

R.P. and A.T. served as cosenior authors.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.107.043323.

ABBREVIATIONS: TRPV1, transient receptor potential type V1 channel (vanilloid receptor 1); CGRP, calcitonin gene-related peptide; SP, substance P; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair(s); RTX, resiniferatoxin; AU, arbitrary units.

nyi et al., 2001), skeletal muscle (Lizanecz et al., 2006), and dural vessels (Dux et al., 2003), although the underlying mechanism remained obscure.

In the present study, the possible mechanisms of TRPV1-mediated vascular effects were investigated. It was found that TRPV1 stimulation results in opposite effects in different arterial beds from the same hind limb of the rat in vivo, namely vasodilation in the skin and vasoconstriction in the skeletal muscle. Moreover, investigation of the possible mechanisms of TRPV1-mediated responses confirmed TRPV1 expression in vascular smooth muscle cells and suggested cell type-specific differences in the capsaicin responsiveness.

Materials and Methods

Animals, Anesthesia, and General Preparation in the in Vivo Experiments. The experiments were performed on male Wistar rats weighing 250 to 450 g raised on a standard laboratory food and water ad libitum. Anesthesia was performed with 100 mg/kg i.p. thiobutobarbital (Inactin-Byk). The right common carotid artery and the left internal jugular vein were cannulated with polyethylene tubing for continuous measurement of arterial blood pressure and for administration of drugs, respectively. Respiratory movements were measured by means of a Statham transducer connected to one side of a Y-shaped cannula introduced into the trachea. The body core temperature was maintained at approximately 37°C with a temperature-controlled infrared heating lamp. Recordings were displayed on a polyphysiograph. All procedures used in this study are in agreement with the rules of the Ethics Committee on Animal Research.

Hind Limb Autoperfusion and Recording of Perfusion Pressure. Isolated hind limb autoperfusion and perfusion pressure recording were performed as described previously (Colquhoun et al., 1988). In brief, after administration of heparin sodium (1000 U/kg, i.v.), the right hind limb was perfused by means of a peristaltic pump (Masterflex; Cole-Parmer Instrument Co., Vernon Hills, IL) with blood taken from a catheter inserted in the left common carotid artery. The outlet side of the perfusion circuit (Masterflex Silicon tube platinum) was connected to the common iliac artery, which was approached through a midline abdominal incision. Perfusion pressure, measured from a side arm in the perfusion circuit between the pump and the iliac artery, was recorded using a Spectramed P23XL pressure transducer and displayed on a recorder. During the whole surgical procedure, the ischemic period of the leg never lasted longer than 3 to 5 min. The peristaltic pump was set to produce a constant flow rate (3 ml/min). This arrangement enabled us to record reproducible responses over 2 to 3 h without swelling of the paw. Capsaicin (8-methyl-*N*-vanillyl-*trans*-6-nonenamide), norepinephrine [(±)-4-(2-amino-1-hydroxyethyl)-1,2-benzenediol hydrochloride], and oxytocin (α-hypophamine) were administered into the perfusion cannula near to the iliac artery catheterization site. For administration, a Hamilton syringe was used, and the injected volumes varied between 10 and 100 μl. Acute cross-section of femoral, genitofemoral, and sciatic nerves were carried out in the right hind limb to prevent nociceptive reflexes from the perfused leg.

Measurement of Skin and Striated Muscle Blood Flow. Laser-Doppler recordings of microvascular blood flow changes were made in the middle of the paw covered by thin glabrous skin and from the flexor muscles of the thigh using a dual-channel laser Doppler flowmeter (MBF3D; Moor Instruments, Axminster, Devon, UK). The time constant was set to 1 s. During the experiment, the exposed skeletal muscle was kept moist by a wet chamber placed around the probe. Blood flow changes were recorded continuously throughout the experiment and were expressed as arbitrary units of flux (Escott and Brain, 1993; Pórszász and Szolcsányi, 1994). The zero level was verified at the end of the experiment. Disturbances caused by direct light were excluded by means of a piece of cotton wool placed onto the right hind limb. The peak of the changes was used to calculate the effect of drugs.

Long-Term Denervation of Hind Limb. In a group of animals (n = 15), the nervous supply of the right hind leg was denervated 7 to 20 days before the experiment under sodium pentobarbital [5-ethyl-5-(1-methylbutyl)-2,4,6-trioxohexahydropyrimidine] anesthesia (40 mg/kg, i.p.), as described previously (Sántha and Jancso, 2003). In brief, an incision was performed on the abdomen, and the right femoral, genitofemoral, and sciatic nerves were cross-sected transperitoneally. Then hemostasis was confirmed, and the wound was sutured. During recovery from the anesthesia, the animals were placed under an infrared heating lamp. Animals showing autotomy were not included in the experiments.

Isolation of Arterioles and Experimental Protocols. The isolation of the skeletal (gracilis) muscle arterioles of the rat and diameter measurement of the arterioles were performed as described earlier (Lizanecz et al., 2006). In brief, after spontaneous tone developed in response to intraluminal pressure of 80 mm Hg, arteriolar responses were obtained in maximal response to cumulative doses of the TRPV1 agonist, capsaicin (0.1 nmol/l to 1 μM). Capsaicin-induced responses were also observed after endothelium removal (Koller and Bagi, 2004) or after inhibition of NO synthase with N^{ω}-nitro-L-arginine methyl ester (Koller and Bagi, 2004). In separate experiments, intraluminal pressure was changed from 20 to 120 mm Hg, and changes in diameter were measured before and after capsaicin treatment (1 μM for 20 min followed by a 40-min regeneration period) (Scotland et al., 2004).

Immunohistochemistry. The immunohistochemical experiments were performed as described by Lizanecz et al. (2006) with minor modifications. In short, musculus gracilis, skin, and small mesenteric tissues were dissected from Wistar rats and were embedded in Tissue-Tek OCT compound (Electron Microscopy Sciences, Hatfield PA). Cryostat sections (thickness, 10 μm) were placed on adhesive slides and fixed in acetone for 10 min. The slices were blocked with normal goat sera (1.5% in phosphate-buffered saline; Sigma, St. Louis, MO) for 20 min and stained with anticapsaicin receptor antibodies [AB 5370P (rabbit) and AB 5566 (guinea pig); Millipore Bioscience Research Reagents, Temecula, CA; PC 547 (rabbit); Calbiochem, San Diego, CA; and RA 10110 (rabbit) and GP 14100 (guinea pig); Neuromics, Edina, MN] at a 1:500 dilution (for all TRPV1-specific antibodies), with smooth muscle actin antibody (NCL-SMA, dilution, 1:20; Novocastra Laboratories, New Castle, UK) or with a neurofilament-specific antibody (dilution, 1:100; Sigma) in the blocking buffer. Then the slices were incubated with anti-rabbit, mouse, and guinea pig antibodies conjugated with Texas red or Cy2. The pictures were captured by a Scion Corporation (Frederick, MA) digital camera attached to a Nikon Eclipse 80i fluorescent microscope (Nikon, Tokyo, Japan).

Detection of TRPV1 mRNA. Total RNA was isolated from rat aorta and A7r5 cells (obtained from American Type Culture Collection, Manassas, VA) (LGC Promochem, Wesel, Germany) and maintained in 10% fetal bovine serum containing Dulbecco's modified Eagle's medium, both were from Invitrogen (Carlsbad, CA) with RNeasy RNS isolation kit (Qiagen GmbH, Hilden, Germany) and cDNA was synthesized by a RevertAid H Minus kit (Fermentas UAB, Vilnius, Lithuania), according to the manufacturer's instruction. The RT-PCR was performed by a sense (5′-CTACCTGGAACACCAATGTGGG-3′) and an antisense primer (5′-GCTGGGTGGCATGTCTATCTCG-3′) designed to produce a 596-bp fragment from DNA and a 149-bp fragment from RNA. Glyceraldehyde-3-phosphate dehydrogenase was used as control. Polymerase chain reaction was performed in a volume of 25 μl consisting of 1 μl of cDNA, 1 μM primer, 200 μM dNTP, 6 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, and 2.5 U Long PCR Enzyme Mix (Fermentas UAB). The protocol was 94°C 5 min followed by 35 cycles of 20 s at 94°C, 20 s at 58°C, and 40 s at 72°C.

Materials and Solutions. Oxytocin (α-hypophamine), capsaicin (8-methyl-*N*-vanillyl-*trans*-6-nonenamide; Sigma), and resinifera-

toxin [RTX; 6,7-deepoxy-6,7-didehydro-5-deoxy-21-dephenyl-21-(phenylmethyl)daphnetoxin 20-(3-hydroxy-5-methoxybenzeneacetate] (from Sigma or from LC Laboratories, Woburn, MA) were dissolved in ethanol/Tween 80/physiological saline in the ratio 1:1:8 for a 10 mg/ml capsaicin and a 100 μg/ml RTX stock solution, and further dilutions were made with saline. Norepinephrine and oxytocin from Gedeon Richter (Budapest, Hungary) were dissolved in physiological saline.

Statistical Analysis. The peak increase or decrease in skin or muscle blood flow (arbitrary units of flux) was determined after drug administration for the assessment of the blood flow. The change of the evoked effect was related to the values measured before drug administration and expressed as percentages (mean ± S.E.M.). Changes in arteriolar diameter were expressed as the percentage change to baseline diameter (constrictions) or in the case of the arteriolar dilations, the percentage change of the diameter as was related to the maximal dilation, determined at 80 mm Hg intraluminal pressure in a Ca^{2+}-free medium. After the normality test, the statistical analysis was made by one-way analysis of variance, and pair-wise multiple comparisons were performed by Student-Newman-Keuls method. The diagrams were plotted using the computer program MicroCal Origin 5.0 (OriginLab Corp., Northampton, MA).

Results

It is well established that vanilloid receptor-1 (TRPV1)-mediated vasodilatation involves the activation of TRPV1 in sensory neuronal terminals and the consequent release of neurotransmitters evoking endothelial synthesis of NO (Zygmunt et al., 1999). The effects of TRPV1 stimulation by capsaicin on vascular resistance were tested in the isolated, perfused right hind limb of the rat in vivo. Changes in the systemic and perfusion pressure in the local blood flow in skeletal muscle and in skin of the same hind limb were measured simultaneously together with the respiration of the rat by pressure transducers and dual-channel laser-Doppler flowmetry (Fig. 1A). First, the responsiveness of the perfused rat hind limb preparations was tested by norepinephrine. Intra-arterial injection of norepinephrine (0.5 μg) resulted in an increase of both systemic and tissue blood pressure and a decrease in the blood flow in the perfused skeletal muscle, whereas no responses were detected in skin perfusion and respiration at this dose (Fig. 1). After a 15-min regeneration period, the effects of TRPV1 stimulations were

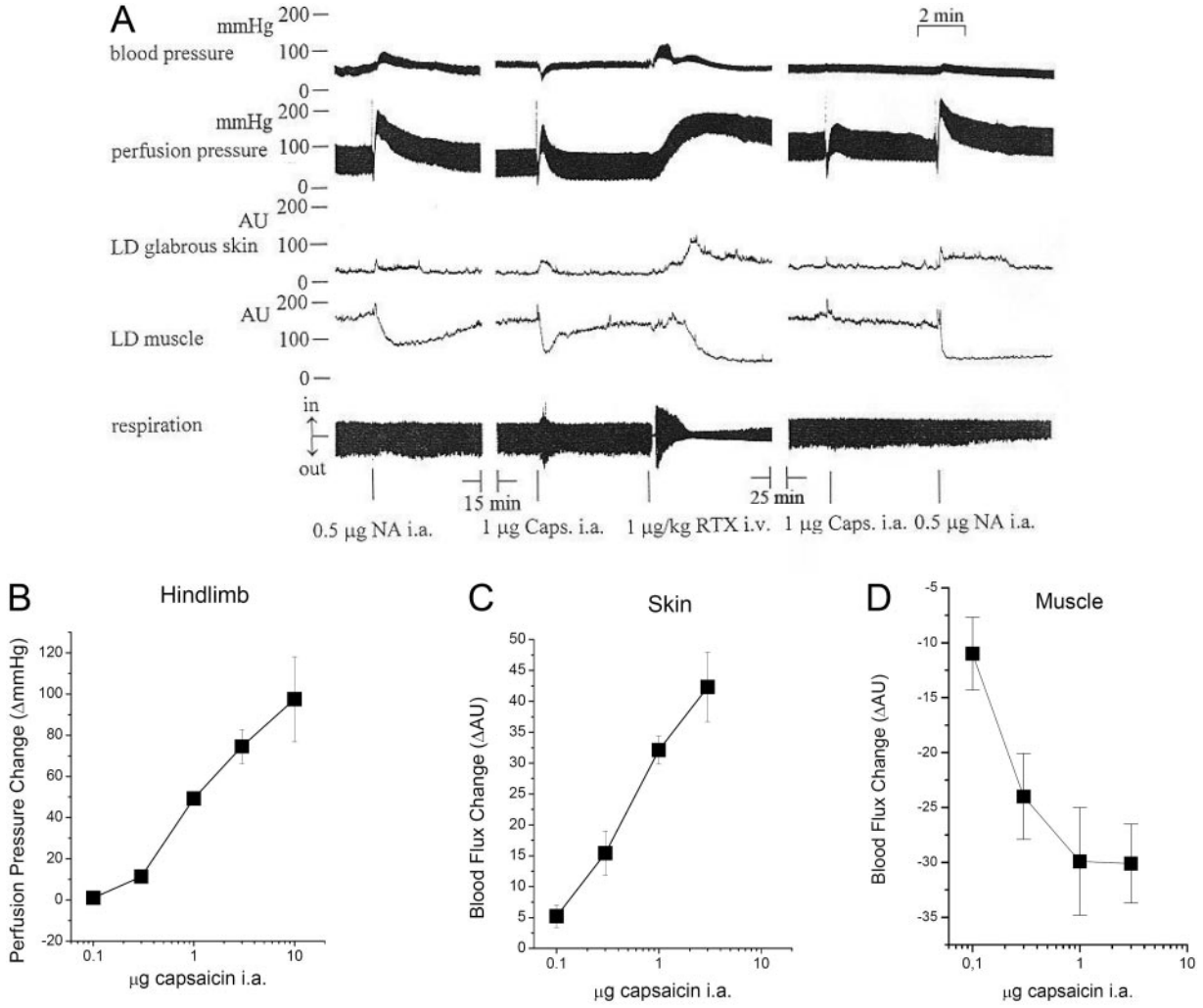


Fig. 1. Effects of TRPV1 stimulation in perfused hind limb of the rat. The left common carotid artery and the common iliac artery were cannulated for blood perfusion of the right hind limb of the rat (3 ml/min). Systemic (arterial blood pressure and respiration) and local (perfusion pressure, blood flow in the skin and skeletal muscle) effects of noradrenaline (NA, 0.5 μg i.a.), capsaicin (Caps, 1 μg i.a.), and RTX (1 μg/kg i.v.) were recorded on the same preparation. A representative of the in vivo experiments performed is shown in A. The dose-response of capsaicin is shown on the perfusion pressure (B), on the blood flow of the skin (C), and skeletal muscle (D) on the right hind limb. Values are average ± S.E.M.

tested. Injection of capsaicin (1 μg) into the arterial perfusion cannula of the blood-perfused right hind leg evoked a decrease in the systemic blood pressure, an increase in the perfusion pressure, a parallel increase in the cutaneous blood flow, and a decrease in blood perfusion of the muscle, whereas respiration was not affected by this dose. These responses were mimicked by the application of the ultrapotent TRPV1 agonist resiniferatoxin (1 μg/kg i.v.). In addition, the application of resiniferatoxin resulted in alterations in the respiration and desensitized vanilloid receptors, as shown by the unresponsiveness to repeated capsaicin stimulation after 25 min, whereas the noradrenaline responses were unaltered.

Next, the capsaicin-mediated responses were investigated in detail. Intra-arterial application of capsaicin (0.1–10 μg) resulted in a dose-dependent increase of perfusion pressure (98 $\pm$ 21 mm Hg increase at 10 μg, Fig. 1B), an increase in the blood flow in the skin (42 $\pm$ 5 AU increase at 3 μg; Fig. 1C) and a decrease in the blood flow in the skeletal muscle (30 $\pm$ 4 AU decrease at 3 μg; Fig. 1D).

To determine the role of neural elements in the capsaicin-induced responses, long-term denervation was performed. In a group of animals (n = 15), the genitofemoral, femoral, and sciatic nerves were cross-sected 7 to 20 days before the commencement of the experiment (see *Materials and Methods*). Denervation was not able to abolish capsaicin (1 μg, intra-arterial application)-evoked increase in the perfusion pressure (Fig. 2, increase in the perfusion pressure is 49 $\pm$ 3 mm Hg in control and 32 $\pm$ 3 mm Hg in denervated hind limbs), nor was it able to affect oxytocin (0.5 I.U.)-mediated elevations (49 $\pm$ 7 mm Hg increase in control and 56 $\pm$ 8 mm Hg in denervated hind limbs).

The effect of denervation was also tested on the local blood flow in the skin and in the skeletal muscle (Fig. 3). Capsaicin-evoked increases in the blood flow were decreased in the skin (28 $\pm$ 3 AU increase in the case of control versus 18 $\pm$ 2 AU in the case of denervated right hind limb; $P < 0.01$, n = 7), but the decrease in the blood flow in the skeletal muscle was not affected (9 $\pm$ 1.5 AU decrease in the case of control, versus 12 $\pm$ 1.5 AU in the case of denervated; P = 0.25, n = 7).

The presence of neurogenic innervation of the arteries in the skin and in the skeletal muscle was tested by immunohistochemistry (Fig. 4). Dense innervation of arteries was found in the skin (filled arrows in Fig. 4), but neurofilament-positive nerve terminals were not detected in the skeletal muscle arteries. In contrast, thicker nerves farther away from the vessels were detected in both skin and skeletal muscle tissue samples (labeled by open arrows in Fig. 4).

These results suggested non-neuronal vasoconstriction (decrease in the blood flow) upon TRPV1 stimulation in the skeletal muscle arterioles. To test this hypothesis, skeletal muscle (musculus gracilis) arteries were isolated and cannulated to directly measure the vasoactive effects of capsaicin. Effects of TRPV1 stimulations were tested after the spontaneous development of the myogenic tone in response to 80 mm Hg intraluminal pressure (spontaneous tone, 31 $\pm$ 4% of the maximal diameter). TRPV1 activation with capsaicin resulted in a biphasic effect on these arterioles. Low nanomolar concentrations (0.1–10 nM) of capsaicin resulted in substantial arteriolar dilation (maximum at 10 nM, 32 $\pm$ 13%, n = 12), which was abolished by the removal of endothelium (Fig. 5A) or by NO synthase inhibition with N^{ω}-nitro-L-arginine methyl ester (0.2 mM) (7 $\pm$ 5% dilation, n = 8, or 1 $\pm$ 2% constriction, n = 4, respectively). In contrast, higher concentrations of capsaicin (0.1–1 μM) elicited a significant vasoconstriction (Fig. 5A; apparent maximum at 1 μM, 66 $\pm$ 7%, n = 12), which was not affected by endothelium removal (Fig. 5A; apparent maximum at 1 μM, 68 $\pm$ 4%, n = 8 after endothelium removal). We have found that capsaicin-induced arteriolar constriction was transient (Fig. 5B), reaching its maximum at approximately 90 s (maximal constriction: 59 $\pm$ 10%, n = 5) and returning to the baseline diameter at the end of the 20-min treatment (3 $\pm$ 3% dilation, n = 5).

The role of the endogenous activation of TRPV1 on the determination of arteriolar diameter was also tested. Application of the TRPV1 antagonist capsazepine (10 μM) resulted in a significant vasodilation (28 $\pm$ 7%, n = 5) in isolated skeletal muscle arteries.

Based on these functional and immunohistochemical observations, we hypothesized that TRPV1 expression is not restricted to sensory neurons in the vasculature. The vascular expression of TRPV1 in the musculus gracilis arterioles was evaluated by immunohistochemistry. In these arterioles,

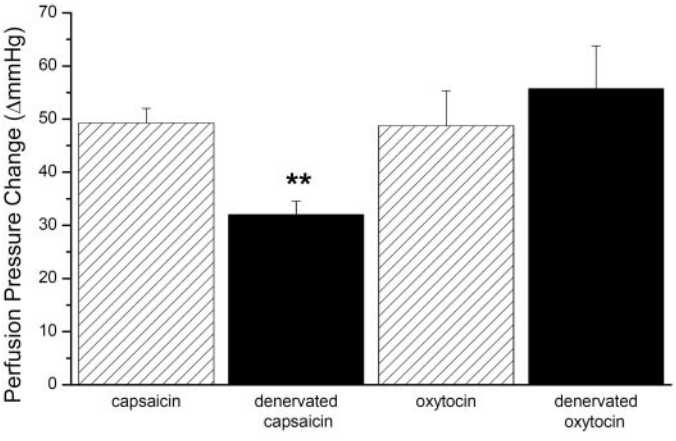


Fig. 2. Effects of denervation on the perfusion pressure changes of the rat right hind limb in vivo. In a group of animals (n = 15), the nerves innervating the right hind leg were transected 7 to 20 days before the measurement. The effects of capsaicin (1 μg i.a.) and oxytocin (0.5 I.U.) were tested on the perfusion pressure in control and in denervated hind limbs. Values are average $\pm$ S.E.M.

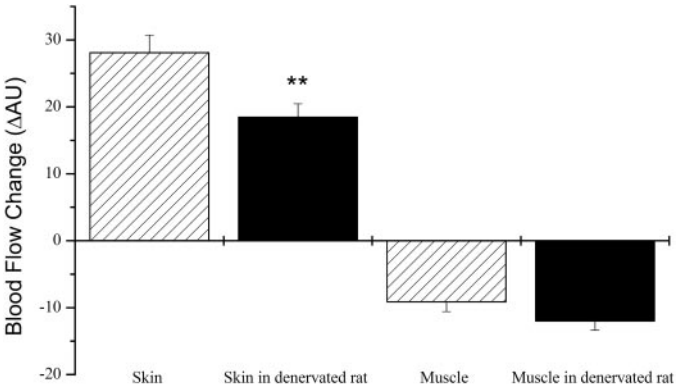


Fig. 3. Effects of denervation on the blood flow changes of the rat right hind limb in vivo. Denervation was performed as mentioned earlier. The effects of capsaicin (1 μg i.a.) on the blood flow of skin and skeletal muscle were tested in control and denervated hind limbs. Values are average $\pm$ S.E.M., n = 7.

TRPV1-expressing cells were costained with smooth muscle α-actin (Fig. 6B), demonstrating the expression of TRPV1 in the smooth muscle cells. To further confirm the specificity of the TRPV1 staining, arterioles were costained with different TRPV1 antibodies developed against different TRPV1 epitopes in different species, which resulted in overlapping staining patterns (data not shown). the presence of TRPV1 mRNA in aorta and in cultured A7r5 vascular smooth muscle cells was also confirmed by RT-PCR (Fig. 6A).

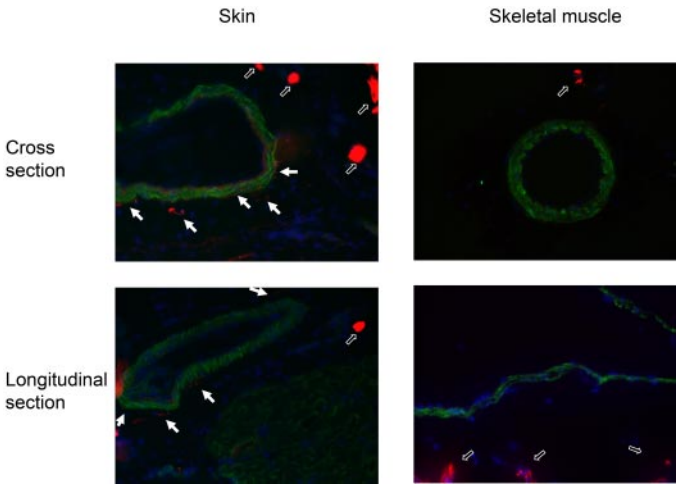


Fig. 4. Innervation of arteries in the skin and skeletal muscle of the rat hind limb. The skin (paw) and skeletal muscle (gracilis) of the hind limb were sectioned in a cryostate (thickness, 10 μm) and fixed in acetone. The presence of neuronal elements (innervation) was tested by a neurofilament specific antibody (dilution: 1:100; Sigma) and visualized by a secondary antibody conjugated with Texas red (red on the figures). For the staining of the arteries, a smooth muscle-specific antibody (dilution: 1:20; Novocastra) and a secondary antibody conjugated with Cy-2 (green on the figures) were used. Localization of these elements were visualized in cross and longitudinal sections. Thick nerves in the tissue sections are represented by open arrows, whereas thin neurits innervating the arteries are shown by closed arrows.

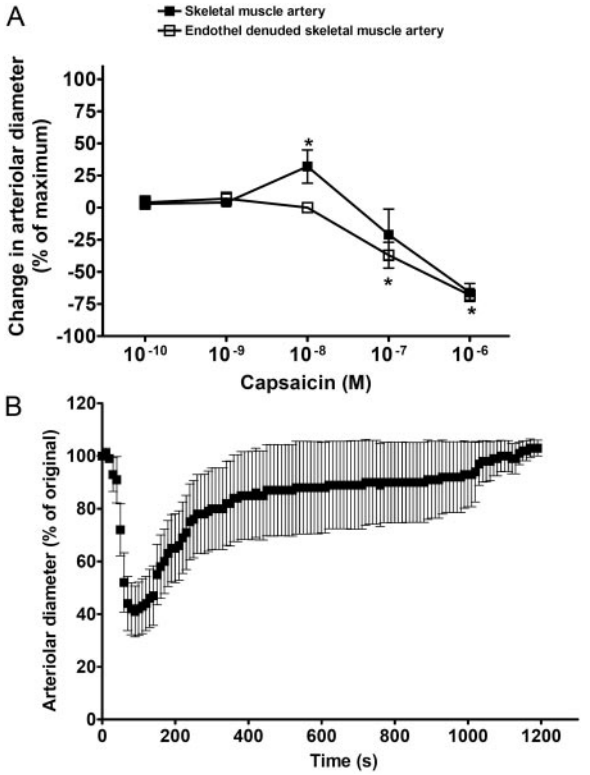


Fig. 5. Effects of TRPV1 stimulation in isolated arterioles. A, dose-response of capsaicin on isolated pressurized skeletal muscle arterioles (control, $n = 12$, and endothelium-denuded, $n = 8$). B, the effect of 20-min continuous application of 1 μM capsaicin. Values are average ± S.E.M.

Discussion

The main findings of the present study are that 1) in the hind limb, TRPV1 stimulation resulted in an increase of vascular resistance and reduction of tissue perfusion (Fig. 1), independently of the innervation (Fig. 2 and 3); 2) in the isolated skeletal muscle arterioles, capsaicin elicited vasoconstriction, which was not affected by endothelium removal (Fig. 5); 3) TRPV1 mRNA is detectable in both aorta and cultured smooth muscle cells (Fig. 6); and 4) TRPV1 is expressed in vascular smooth muscle cells of the skeletal muscle arterioles (Fig. 6). These data suggest functional expression of TRPV1 in the vascular smooth muscle cells of the skeletal muscle arterioles.

Our present findings also indicate that TRPV1 stimulation results in diverse vascular effects in arterial beds of the rat hind limb. We propose that TRPV1 regulates arteriolar diameter primarily by two mechanisms (Fig. 7). In the case of skin, TRPV1 activation resulted in a neuronal vasodilation (Fig. 1 and 3), probably mediated by a sequence of events

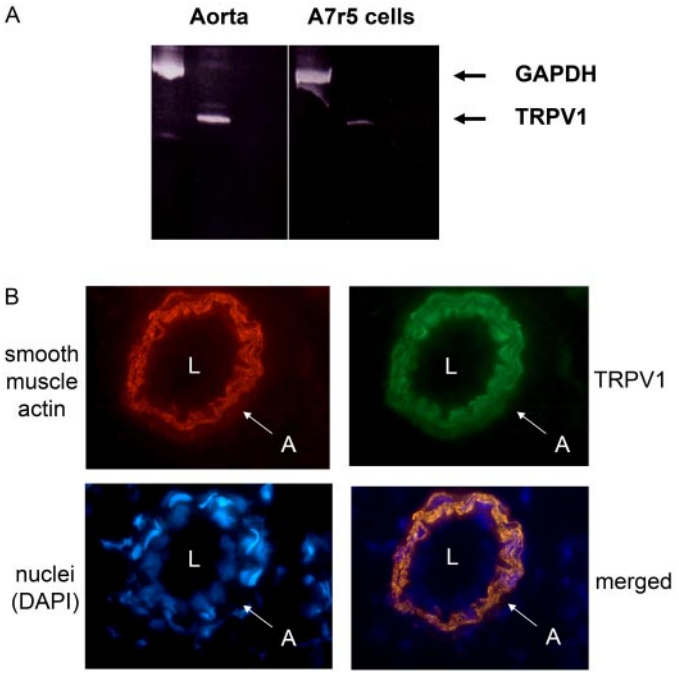


Fig. 6. Expression of TRPV1 in vascular smooth muscle cells. RT-PCR analysis was performed using RNA isolated from aorta and cultured vascular smooth muscle cells (A). TRPV1-specific primers were designed to yield a 149-bp fragment from mRNA (shown by the arrow) and a 596-bp fragment from DNA (not detected). Glyceraldehyde-3-phosphate dehydrogenase was used as control. To investigate the localization of TRPV1, immunohistochemistry was performed in the skeletal muscle (gracilis) tissue sections of the rat (B). TRPV1- (rabbit, 1:500 dilution, green; Calbiochem) and smooth muscle actin (monoclonal, 1:20 dilution, red; Novocastra)-specific staining was visualized by a fluorescent microscope. Slides were also processed in mounting media containing 4,6-diamidino-2-phenylindole for staining of nuclei (blue). The artery used in the in vitro functional studies is identified (L, lumen; A, artery).

such as the activation of sensory neurons, the subsequent local release of sensory neurotransmitters (like CGRP or SP), stimulation of endothelial cells by these neurotransmitters, activation of endothelial NO synthesis, and NO-mediated relaxation of smooth muscle cells, as proposed by Zygmunt et al. (1999). In contrast, TRPV1 stimulation by capsaicin had biphasic effects in the isolated skeletal muscle resistance arterioles (vasodilation at lower concentrations and vasoconstriction at higher concentrations; Fig. 5), suggesting a dual regulation of vascular tone. The dilatative effects of capsaicin were endothelium-dependent (Fig. 5) and nitric oxide-mediated. In addition, inhibition of TRPV1 in isolated, pressurized skeletal muscle arteries resulted in a vasodilation, indicating a physiological role of TRPV1 in the regulation of vascular diameter. It should also be noted that capsaicin evoked opposite effects in different vascular beds under in vivo conditions (Fig. 1A). The increase of pressure of the isovolumetric (3 ml/min) perfusion in the hind limb suggests higher local resistance in the vasculature of the hind limb (at least partly as a result of skeletal muscle arteriolar constriction, Fig. 5), whereas the simultaneous decrease in systemic blood pressure indicates a somewhat higher overall efficiency of vasodilatative receptors (probably mediated by mesenteric, dural, skin, pulmonary, or coronary arteries) in the whole vasculature.

The simplest explanation of the findings is that TRPV1 is functionally expressed in vascular smooth muscle cells. Although findings based on immunohistochemical data in general should be interpreted with caution, this proposal is also supported by RT-PCR results and the vasoconstrictive effect of TRPV1 stimulation in intact or endothelial denuded isolated skeletal muscle arteries. According to these data, we hypothesize that activation of TRPV1 in skeletal muscle arteries occurs both in sensory neurons and in vascular smooth muscle cells, leading to Ca^{2+} influx into both cell types. The elevated intracellular Ca^{2+} concentration in the smooth muscle directly results in vasoconstriction, whereas in the sensory nerves, it triggers neurotransmitter release and concomitant endothelial-dependent vasodilation (Fig. 7).

Capsaicin-evoked in vivo vasoconstriction of various arterial beds was discovered decades ago (Molnár and Gyorgy, 1967; Toda et al., 1972; Donnerer and Lembeck, 1982; Duckles, 1986; Edvinsson et al., 1990). In these initial and follow-up studies, TRPV1-mediated vasoconstriction was found in dog mesenteric (Toda et al., 1972; Pórszász et al., 2002), renal, and carotid artery (Toda et al., 1972); in cat middle cerebral (Duckles, 1986; Edvinsson et al., 1990), pial (Edvinsson et al., 1990), and pulmonary (Molnár and Gyorgy, 1967) arteries; in rat heart (Szolcsányi et al., 2001), small mesenteric (Scotland et al., 2004), dural (Dux et al., 2003), and skeletal muscle arteries (Lizanecz et al., 2006); and in mouse knee joint (Keeble and Brain, 2006). Multiple mechanisms leading to TRPV1-mediated vasoconstriction were suggested, including endothelin-1 (Szolcsányi et al., 2001) or SP (Scotland et al., 2004) release from sensory neurons, and yet uncharacterized smooth muscle-mediated effects were observed (Pórszász et al., 2002; Dux et al., 2003; Keeble and Brain, 2006). In addition to these possibilities, our data provide evidence for TRPV1 expression in vascular smooth muscle cells, suggesting a direct link between TRPV1 activation and smooth muscle contraction. Nevertheless, these data indicate that vasoconstrictive effects of TRPV1 stimulation are not restricted to a specific blood vessel or to a single species.

It is interesting that in some of these cases, like in the case of rat mesenteric arteries, both vasoconstriction (Scotland et al., 2004) and vasodilation (Ralevic et al., 2000) were observed upon capsaicin stimulation. It suggests that there are two pools of TRPV1 in these systems, but one of the receptor types is down-regulated under specific circumstances, and

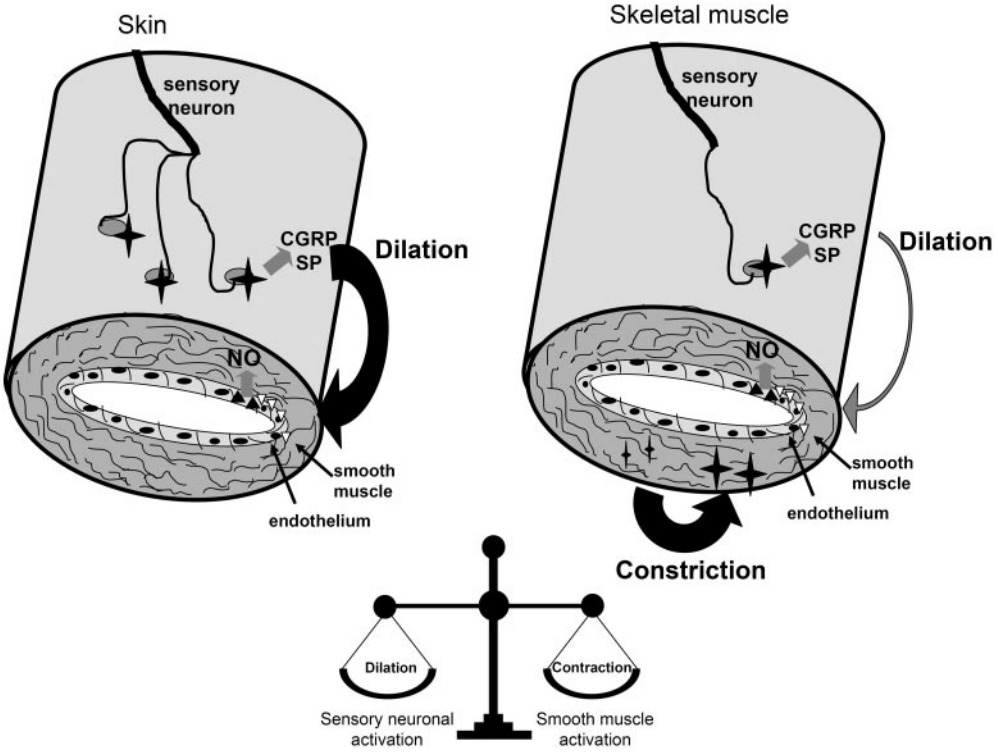


Fig. 7. Proposed mechanism of tissue-specific regulation of vascular diameter by TRPV1. Our data support the well known sensory neuronal dilation in the skin arteries involving the following events: 1) activation of sensory neuronal TRPV1; 2) elevation of intracellular Ca^{2+} in the neuronal terminals; 3) release of sensory neurotransmitters, including CGRP and SP; 4) activation of endothelial receptors of these neurotransmitters; 5) increase of endothelial NO synthesis; and 6) NO diffusion and smooth muscle relaxation. The same mechanism was found to be responsible for capsaicin-mediated dilation in skeletal muscle arteries. In contrast, functional expression of TRPV1 was also identified in vascular smooth muscle cells of gracilis artery. The activation of these smooth muscle-located receptors led to vasoconstriction. We propose that the balance of activities of sensory neuronal and smooth muscle-located TRPV1-mediated pathways determines the vasoactive effects of TRPV1 stimulation.

the physiological effect of capsaicin stimulation is dominated by the active receptor population. In accordance with this idea, dose-dependent biphasic effects were also noted in some studies: low dose capsaicin evoked dilation, whereas higher concentrations resulted in constriction (Edvinsson et al., 1990; Dux et al., 2003), similar to our findings.

Several mechanisms have been suggested to regulate TRPV1 activity, besides to the expressional regulation. These include protein kinase C (Bhave et al., 2003) or protein kinase A (Bhave et al., 2002)-mediated phosphorylation, calcineurin-mediated dephosphorylation (Docherty et al., 1996), interaction with calmodulin (Numazaki et al., 2003) or with phosphoinositides (Liu et al., 2005; Lukacs et al., 2007), besides others. As a matter of TRPV1 mediating skeletal muscle vasoconstriction, phosphorylation seems to be the most likely candidate (Lizanecz et al., 2006).

Some of the findings of this study suggest pharmacological differences in the TRPV1 pools mediating constriction and dilation, namely 1) higher sensitivity/effectivity of dilatative responses (dilation in the case of low capsaicin concentrations), with a profound constrictive responses at maximal stimulation; and 2) vasodilation evoked by short-term inhibition of TRPV1. In addition, earlier data suggest that the TRPV1 receptors mediating vasodilation can be easily desensitized by neonatal capsaicin treatments, whereas the capsaicin response of receptors mediating vasoconstriction remains intact or augmented (Donnerer and Lembeck, 1982). As a therapeutical consequence of these observations, it seems to be possible to design TRPV1 ligands preferably acting on receptors mediating constrictive or dilatative responses. Although there is no shortage of drug candidates regulating TRPV1 activity (Szallasi et al., 2007), their development was concentrated on their effects on sensory neuronal functions (mostly pain). One of the examples to emphasize the feasibility of such drug development is that it was possible to design an antagonist selective to the plasma membrane located TRPV1 over to the intracellular membrane-located receptors (Tóth et al., 2004). The drugs selective to receptors mediating dilation or constriction may be useful to regulate blood distribution in various pathophysiological conditions associated with ischemia. For example, sensory neuronal TRPV1 was found to be activated upon myocardial ischemia (Zahner et al., 2003; Pan and Chen, 2004) and beneficial in postischemic recovery (Wang and Wang, 2005), suggesting that selective activation of sensory neuronal TRPV1 may be beneficial in myocardial infarction.

Taken together, we report here that TRPV1 (a nonspecific Ca^{2+} channel) is expressed in smooth muscle cells, and its activation leads to vasoconstriction in skeletal muscle resistance arterioles. We propose that TRPV1 has a potential physiological/pharmacological role in the regulation of arteriolar tone in skeletal muscle (apparently in the range of 40% dilation to 60% constriction), which represents a promising new therapeutic strategy to control tissue-specific blood distribution.

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Address correspondence to: Dr. Attila Tóth, Division of Clinical Physiology, Institute of Cardiology, Medical and Health Science Center, University of Debrecen, 22 Moricz Zs krt, 4032, Debrecen, Hungary. E-mail: atitoth@dote.hu

Structure-activity relationships of vanilloid receptor agonists for arteriolar TRPV1

Running title: Pharmacology of TRPV1 in peripheral arteries

Á. Czikora[1], E. Lizanecz[1], P. Bakó[1], I. Rutkai[1], F. Ruzsnavszky[2], J. Magyar[2], R. Pórszász[3], T. Kark[3], A. Facskó[4], Z. Papp[1], I. Édes[1], A. Tóth[1,*]

[1]Division of Clinical Physiology, Institute of Cardiology; [2]Department of Physiology, [3]Department of Pharmacology and Pharmacotherapy, Institute of Pharmacology; [4]Department of Ophthalmology, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary.

* E-mail: atitoth@dote.hu

Summary

Background and purpose: The vanilloid receptor 1 (TRPV1) plays a role in the activation of sensory neurons by various painful stimuli and became a therapeutic target. However, functional TRPV1 expression was also observed in the peripheral arteries affecting microvascular diameter.

Experimental approach: Sensory TRPV1 activation was measured by eye wiping tests. Arteriolar TRPV1 mediated smooth muscle specific responses (arteriolar diameter, changes in intracellular Ca^{2+}) were determined in isolated, pressurized skeletal muscle arterioles (from the rat and wild type or $TRPV1^{-/-}$ mice, n=130) or in isolated canine smooth muscle cells. Vascular pharmacology of TRPV1 agonists (potency, efficacy, kinetics of action and receptor desensitization) was determined in isolated skeletal muscle arteries of the rat.

Key results: Capsaicin evoked a similar constriction as norepinephrine, which was absent in TRPV knockout mice and was competitively inhibited by a TRPV1 antagonist AMG9810. Capsaicin activation resulted in an increase in intracellular Ca^{2+} in the arteriolar wall as well as in isolated smooth muscle cells. Other TRPV1 agonists evoked similar vascular constrictions (MSK-195, JYL-79) or were without effect (resiniferatoxin, JYL-273), although all resulted in a sensory activation (eye wiping). Maximal dose of agonists gave different kinetics of arteriolar response. A complete desensitization (tachyphylaxis) of arteriolar TRPV1 was observed (with the exception of capsaicin). Application of the partial agonist JYL-1511 suggested that about 10% TRPV1 activation is sufficient to evoke vascular tachyphylaxis without sensory activation.

Conclusions and implications: Our data suggests that arteriolar TRPV1 has different structure-activity relationship compared to sensory neuron located receptor in the rat.

Keywords: vanilloid receptor (TRPV1), resistance artery, vascular autoregulation

List of abbreviations:

TRPV1: Transient receptor potential vanilloid 1

AMG9810: (E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylamide

MSK-195: *N*-[2-(3,4-Dimethylbenzyl)-3-(pivalyloxy)propyl]-2-[4-(2-aminoethoxy)-3 methoxyphenyl]acetamide

JYL-79: 2-(3,4-Dimethylbenzyl)-3-{[(4-hydroxy-3-methoxy-benzyl)amino]carbothioyl}propyl pivalate

JYL-273: 2-(4-t-Butylbenzyl)-3-{[(4-hydroxy-3-methoxybenzyl)ami- no]carbothioyl}propyl pivalate

JYL-1511: *N*-(4-*tert*-Butylbenzyl)-*N*'-[3-methoxy-4-(methyl-sulfonylamino)benzyl]thiourea

BSA: Bovine serum albumin

TRPV1$^{-/-}$: B6.129X1-*Trpv1*^{tm1Jul}/J mice (Jackson Laboratories)

CHO-TRPV1: Chinese hamster ovary cells overexpressing rat TRPV1

PKC: Protein kinase C

PKA: Protein kinase A

DRG: dorsal root ganglion

i.p.: intraperitoneal injection

Introduction

The vanilloid receptor-1 (TRPV1) is a non-selective cation channel, originally found in sensory C and A-δ fibers (Caterina et al., 1997). It functions as a ligand-, proton- and heat-activated molecular integrator of nociceptive stimuli (Di Marzo et al., 2002; Ross, 2003; Szallasi et al., 1999) and hence represents a promising drug target for analgesia (Gunthorpe et al., 2008; Szallasi et al., 2007).

However, TRPV1 expression was identified in many cells, besides sensory neurons in the last years. 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 central nervous system remained elusive, activation of vascular TRPV1 resulted in a substantial vasoconstriction *in vivo* and *in vitro* (Kark et al., 2008). TRPV1 antagonists are in clinical trials for various indications like dental pain, osteoarthritis, neuropathic pain, overactive bladder, chronic cough, rectal hypersensitivity, migraine, lower back pain, interstitial cystitis (Khairatkar-Joshi et al., 2009). Although some results of these trials are promising, they also revealed that TRPV1 antagonists may evoke serious hyperthermia (Gavva et al., 2008). This hyperthermia is probably related to the contribution of TRPV1 to the temperature regulation, *in vivo* (Gavva et al., 2007). However, the mechanism of this effect is not clear. Although some antagonists are causing hyperthermia (Gavva et al., 2008), others are without thermoregulatory effects in human (Khairatkar-Joshi et al., 2009). It suggests that pharmacologically different TRPV1 is responsible for analgesia and for

thermoregulation. The nature and identity of these TRPV1 dependent responses have not been identified yet, but it seems to be plausible that a separate pool of receptors exists (Steiner et al., 2007).

Capsaicin evokes vasoconstriction in skeletal muscle arteries presumably by activating smooth muscle located TRPV1 (Kark et al., 2008). Here we made an effort to pharmacologically characterize this receptor. To achieve this goal we choose a series of commercially available TRPV1 agonists that were tested in assays measuring not only their potency and efficacy, but also their kinetics of action and desensitization (Toth et al., 2005b). Our experiments revealed different pharmacological profiles for vascular TRPV1 when compared to TRPV1 responsible for sensory activation. These findings implicate that TRPV1 may be selectively targeted (such as sensory neuronal and arterial receptor populations).

Methods

The applied drug/molecular target nomenclature (e.g. receptors, ion channels) conforms to BJP's Guide to Receptors and Channels (Alexander *et al.*, 2009).

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 (6 control C57BL/6J and 5 $TRPV1^{-/-}$ knockout mice). Rats (WKY/NCrl) were obtained from Charles River (Isaszeg, Hungary), while mice was obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and maintained on a standard laboratory food (CRLT/N chow from Szinbad Kft, Godollo, Hungary) and water ad libitum. Anaesthesia was performed with 100 mg/kg i.p. pentobarbital sodium.

Isolation of arterioles and measurement of vascular diameter

The isolation of skeletal muscle (m. gracilis) arterioles of the rat and diameter measurement of the arterioles were performed as described earlier (Lizanecz et al., 2006). Briefly, arterioles were kept in a physiological saline solution (PSS, composition in mM: 110 NaCl, 5.0 KCl, 2.5 $CaCl_2$, 1.0 $MgSO_4$, 1.0 KH_2PO_4, 5.0 glucose and 24.0 $NaHCO_3$ equilibrated with a gas mixture of 10% O_2 and 5% CO_2, 85% N_2, at pH 7.4.) at an intraluminal pressure of 80 mmHg until the development of spontaneous myogenic response (constriction to intraluminal pressure). Intraluminal arteriolar diameter was measured upon treatments. First, acetylcholine was used to determine dilative capacity and endothelium function, and then norepinephrine was applied to measure maximal

constrictive response and smooth muscle function. Changes in diameter to TRPV1 agonists were tested next with cumulative doses (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). Specificity of the capsaicin responses were tested by the application of the TRPV1 antagonist AMG9810. Cumulative dose responses for capsaicin were measured in the 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 KREBS) and tachyphylaxis (decrease of response upon re-administration of the agonist) was tested by 1 μM capsaicin. Arterioles were isolated from wild type and TRPV1 knockout mice as detailed for the rat. Experiments were also performed similarly: acetylcholine was used to determine endothelial function, and norepinephrine was applied to estimate smooth muscle function. Changes in diameter to TRPV1 agonists were tested with cumulative doses of capsaicin, (0.1 nM – 30 μM).

Determination of antagonist equlibrium dissociation constant

A conventional Schild plot (Arunlakshana et al., 1959) was constructed based on the measured values. EC_{50} 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 the function of the logarithm of AMG9810 concentration (Fig. 2B). Data were fitted by a

linear regression and the antagonist equilibrium dissociation constant was yielded as the x-intercept.

Parallel measurement of vascular diameter and intracellular Ca^{2+} concentrations

Skeletal muscle arterioles were isolated and cannulated from the gracilis muscle of the rat, as mentioned above. After mounting the arteries into 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 inverted microscope to measure intracellular Ca^{2+} concentrations by an IncyteIm2 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 at each 2-5 s and offline evaluated. 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 minimum 200 pixels). A movie showing the full representative 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/Vascular%20system/Vascular%20system.html).

Isolation of smooth muscle cells from canine coronary arteries

Adult beagle dogs (10–14 kg) were anaesthetised with an i.v. injection containing 10 mg/kg ketamine hydrochloride (Calypsol, Richter Gedeon, Hungary) and 1 mg/kg xylazine hydrochloride (Sedaxylan, Eurovet Animal Health BV, The Netherlands). After opening the chest, the heart was rapidly removed and the right coronary artery was perfused with Ca^{2+}-free JMM solution (Minimum Essential Medium Eagle, Joklik Modification), supplemented with taurine (2.5 g/l), pyruvic acid (175 mg/l), ribose (750 mg/l), allopurinol (13.5 mg/l), and NaH_2PO_4 (200 mg/l) 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 DMEM and an about 2.5 cm long right coronary artery segment was isolated and cannulated on 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 mg/ml collagenase type I (Worthington, Lakewood, NJ, USA) for 30 minutes and with 1 mg/ml elastase (Worthington, Lakewood, NJ, USA) at 60 min. The vessel fell apart in about 90 min under these conditions, when the cell-rich solution was transferred into 24-well plates. After the adherence of the cells to the glass coverslips placed into 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 thermostate. Then the media was changed to DMEM containing 1 mg/ml BSA and 5 M fura2-acetoxymethyl ester (Molecular Probes, Eugene, OR, USA) for 2 h at room temperature. Then the cover slips were placed in a suitable chamber for intracellular Ca^{2+} concentration measurements. These measurements were started by washing the cells with DPBS three times and then the measurements were performed in DPBS. The

fluorescence of individual cells was measured with an InCyt Im2 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 M capsaicin and then with 100 mM KCl. Data were analyzed 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

Eye wiping assay was performed as described previously (Jakab et al., 2005). In short, 1 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 of each rat). The number of eye wipes was counted during 60 s. In the control group the solvent was injected in the same volume and 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 ± S.E.M. Statistical differences were evaluated by Student's t-

test comparing values before and after treatments (paired) or comparing eye wipes of vehicle and 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 (Fig. 1) similarly to norepinephrine (10 µM, decrease of arteriolar diameter to 68±9 µm, n=7, Fig. 1). In contrast, the endothelium dependent vasodilator acetylcholine evoked a dilatation (increase in arteriolar diameter to 240±20 µm, n=7, $p=0.028$, Fig. 1).

A vast majority of published data suggested dilative effects upon vascular TRPV1 stimulation in the previous body of experimental evidence published in the literature. It was therefore necessary to test 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 (Fig. 2A). Moreover, the potency of AMG9810 determined in these assays (177 nM, Fig. 2B) was in agreement with its potency determined in other TRPV1 specific systems (Gavva et al., 2005). Nonetheless, TRPV1 selectivity of capsaicin mediated contractile responses were also tested in TRPV1 knockout ($TRPV1^{-/-}$) mice. The potency of capsaicin (EC_{50}) was 137 nM (Fig. 2C) and efficacy was 73% (decrease in diameter from 69±8 µm to 24±3 µm, n=6, Fig. 2C) on arteries from wild type mice, while the same capsaicin treatments were without effects on $TRPV1^{-/-}$ mice (Fig. 2C, n=5).

Next the potential mechanism of TRPV1 mediated constrictions was evaluated. Activation of TRPV1 results in an increase of intracellular Ca^{2+} concentrations in many TRPV1 expressing cell types, contributing 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 specific case) of cannulated rat arterioles isolated from the gracilis muscle of the rat was performed (Fig. 3). Capsaicin evoked vasoconstriction was paralleled by an increase in intracellular Ca^{2+} concentration (supplementary video file and Fig. 3A). Moreover, both vascular diameter and intracellular Ca^{2+} concentration increased in a dose-dependent manner, with a potency in the nanomolar range (note maximal responses at 1 μM concentration, Fig. 3B). To identify the TRPV1 expressing cell type arteriolar smooth muscle cells were isolated from canine coronary arteries (these arteries have also responded to capsaicin treatment with a dose dependent constriction, data not shown) and changes in intracellular Ca^{2+} concentrations were tested upon capsaicin (1 μM) and KCl (100 mM) treatments (Fig. 4). The capsaicin mediated increase in intracellular Ca^{2+} concentrations in the cells responding to capsaicin (10 out of 28 cells, representative data in Fig. 4A and 4B) was similar (increase in 340/380 ratio from 0.69±0.10 to 0.93±0.17, Fig. 4C) to the increase evoked by depolarization (100 mM KCl, 340/380 ratio is 1.04±0.20, Fig. 4C).

Having established the TRPV1 specificity of capsaicin evoked vasoconstrictive effects, the pharmacological properties of these receptors were characterized in detail on skeletal muscle arteries of the rat. The potency of capsaicin on this receptor (EC_{50}) was 221 nM (Fig. 5A), efficacy was 58±7% constriction (n=7), which was not significantly different from the efficacy of norepinephrine (69±3% constriction, n=6, $p<0.01$ versus control, $p=0.08$ versus capsaicin). The kinetics of vasoconstrictive response was determined by continuous application of capsaicin (1 μM) for 20 min. Maximal

constriction (decrease of arteriolar diameter from 160±11 µm to 76±16 µm, n=9) was achieved at 90 s (Fig. 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±13 µm, n=9, Fig. 5B). Finally, tachyphylaxis (decrease of response upon repeated application of the agonist) was measured by the re-application of capsaicin (1 µM) after 40 min regeneration period. Arteriolar diameter decreased from 161±17 µm to 109±18 µm (n=6) suggesting significant re-sensitization of the receptor (Fig. 5C).

Resiniferatoxin was tested under the same conditions (Fig. 6). Surprisingly no vascular effects were detected upon application in a concentration range from 1 pM to 10 nM (Fig. 6A). Moreover, no effects were detected upon application of 10 nM for 20 min (Fig. 6B). However, capsaicin (1 µM) was without effects after 40 min regeneration (Fig. 6C), suggesting complete desensitization of arterial TRPV1 upon the otherwise ineffective resiniferatoxin treatments.

JYL-273 was ineffective in evoking arteriolar vasoconstriction in a concentration range between 0.1 nM and 1 µM (n=7, Fig. 7A), nor has it any effect at 1 µM applied for 20 min (n=5, Fig. 7B). However, this 20 min incubation resulted in complete desensitization of TRPV1 as evidenced by the missing response to capsaicin (n=4, Fig. 7C), similarly to resiniferatoxin.

MSK-195 had a potency of 120 nM and an efficacy of 71±11% (n=5, Fig. 8A). Application of 1 µM MSK-195 for 20 min resulted in a transient decrease in arterial diameter (decrease from 235±19 µm to 155±25 µm at 90 s, n=6, Fig. 8B). However, kinetics of acute desensitization was slower than that is for capsaicin, since original

arteriolar diameter was not restored during the 20 min incubation (arterial diameter after 20 min incubation was 193±25, p=0.03 versus before treatment, n=6, Fig. 8B). Similarly to all agonists mentioned above MSK195 also evoked a complete desensitization of capsaicin sensitive vascular TRPV1 (Fig. 8C).

JYL-79 was more potent on vascular TRPV1 (EC_{50}=3.9 nM, n=8, Fig. 9A) than capsaicin. Its efficacy was 36±8% (n=8, Fig. 9A). It also evoked a transient vasoconstriction when applied at a concentration of 1 µM (decrease of vascular diameter from 228±13 µm to 127±12 µm at 100 s, n=5, Fig. 9B). The desensitization of the receptor was not complete at the end of incubation (vascular diameter at 20 min was 204±13 µm, p=0.046 versus before treatment, n=5, Fig. 9B). Moreover, no response to capsaicin (1 µM) was observed after 40 min regeneration (n=5, Fig. 9C).

To estimate the threshold of TRPV1 stimulation which may cause complete desensitization of vascular TRPV1 a partial agonist (JYL-1511) was applied. Its efficacy as agonist was about 17% and its potency was 3 nM in a CHO-TRPV1 cell line (Wang *et al.*, 2003). JYL-1511 was without effects in a concentration range of 1 nM - 1 µM on the vascular diameter (n=6, Fig 10A). Application of 1 µM for 20 min was also without effects (Fig. 10B). A partial inhibition (tachyphylaxis) of capsaicin response (1 µM) was noted after 40 min regeneration (decrease of vascular diameter from 244±14 µm to 209±17 µm, p=0.02, n=6, Fig. 10C). The level of partial agonism/antagonism was also determined (Fig. 11). Application of JYL-1511 (1 µM) resulted in a decrease of arteriolar diameter (arterial diameter decreased to 94±3 %, n=6) and an immediate capsaicin treatment (1 µM) resulted in a decrease of diameter to 83±6 % (p=0.04, n=6). In contrast, the same capsaicin treatment alone evoked a decrease of diameter to 43±7 % (p<0.01,

n=7) in separate experiments. According to these data, the agonism of JYL-1511 is 10±5 % and antagonism is 70±11 % at the vascular TRPV1.

The goal of the study was to detect differences between TRPV1 populations responsible for sensory neuronal activation and vasoconstriction. A weakness of 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 eye wiping assays. JYL-1511 did not evoked significant effects, while all of the other agonists increased the number of eye wipes (Fig. 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 to evoke acute activation of vascular TRPV1 receptors (Fig. 6 and 7, respectively).

Discussion and conclusions

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

Vascular TRPV1 was characterized here by measuring the vasoconstriction upon TRPV1 stimulation. It was found earlier that TRPV1 is expressed in vascular smooth muscle cells, suggesting that activation of TRPV1 is directly linked to intracellular Ca^{2+} elevations in smooth muscle (Kark et al., 2008). Indeed, decrease in arteriolar diameter was paralleled by an increase in intracellular Ca^{2+} concentrations in the vascular wall in this present study (Fig. 3), moreover, direct intracellular Ca^{2+} concetration measurements revealed the presence of functional TRPV1 in isolated arteriolar smooth muscle cells for the first time (Fig. 4).

Vasoconstrictive response to TRPV1 stimulation was reported decades ago (Donnerer et al., 1982; Duckles, 1986; Edvinsson et al., 1990; Molnar et al., 1967; Toda et al., 1972) and this effect has been confirmed later (Dux et al., 2003; Keeble et al., 2006; Lizanecz et al., 2006; Scotland et al., 2004; Szolcsanyi et al., 2001). Nonetheless, these responses were not in the centre of interest. One of the reasons for this was that

pharmaceutical research has been 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 vasoconstrictive 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 (Dux et al., 2003; Edvinsson et al., 1990). This suggested the involvement of different receptors or different pharmacology for TRPV1 mediating vascular dilatations and constrictions.

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

We have recently shown that stimulation of TRPV1 in skeletal muscle arterioles resulted in a substantial vasoconstriction (Lizanecz et al., 2006). Moreover, intra-arterial injection of capsaicin into the hindlimb 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 a vasoconstriction) (Kark et al., 2008). These data suggested that vascular TRPV1 may have sensory neuron independent physiological effects.

TRPV1 specificity of capsaicin mediated arteriolar vasoconstriction was ultimately proven here. Most importantly, capsaicin mediated responses were absent in

TRPV1 knockout mice (Fig. 2C). Moreover, an effort was also made to investigate the potential mechanisms. Intracellular Ca^{2+} concentration measurements were performed which showed a capsaicin mediated increase in the arterial wall of skeletal muscle arteries (Fig. 3) as well as in isolated arteriolar smooth muscle cells (Fig. 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 were tested on this response. Application of a TRPV1 antagonist (AMG9810, previously tested on exogenous and sensory neuronal TRPV1) revealed inhibition of 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 (Kark et al., 2008; Lizanecz et al., 2006). 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). One of the observations was 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) was described for pulmonary chemoreflex (Szolcsanyi et al., 1990). A 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 where vasoconstriction occurs. Alternatively, tachyphylaxis may be the reason of irreversible activation of TRPV1 by resiniferatoxin (Jeffry et al., 2009) leading to a sustained Ca influx. To measure the level of sufficient activation 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 a significant tachyphylaxis, rather suggesting a role for desensitization than a role for sustained Ca^{2+} influx in this system. In addition, although the functional response to capsaicin was transient and the arteries completely desensitized to capsaicin, after 40 min regeneration period capsaicin was able to evoke vasoconstriction, suggesting re-sensitization of the arteries and only a partial tachyphylaxis. Taken together, vascular smooth muscle located receptor seems to have different ligand selectivity for desensitization than that is for TRPV1 expressed in CHO cells (Table 1) and also for TRPV1 responsible for eye irritation upon capsaicin treatment in vivo (Fig. 12).

It was observed that the kinetics of acute desensitization was different in the case of agonists evoking vascular constriction. In the case of capsaicin a complete acute desensitization was observed, while in other cases only a partial desensitization was found (JYL-79, MSK-195). 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 a matter of TRPV1 mediating skeletal muscle vasoconstriction phosphorylation seems to be the most likely candidate. It was found that anandamide (Lizanecz et al., 2006), similarly 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, we report here 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 a vasoconstriction. The pharmacological profile of the vascular TRPV1 differs from the TRPV1 population responsible for sensory irritation. Arteriolar TRPV1 was inhibited by a competitive TRPV1 antagonist developed as an analgesic agent suggesting that vascular TRPV1 activation may represent a side effect to analgesic application of TRPV1 antagonists, *in vivo*. Moreover, vascular TRPV1 may be a new therapeutic candidate 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); 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.

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Tables

Table 1 Pharmacological properties of TRPV1 agonists

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

Figures and Legends

Fig. 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 acetylcholine (10 μM) evoked dilatations. Next, the constrictive response to TRPV1 agonist capsaicin (1 μM) was compared to that the effect of norepinephrine (10 μM). Experiments were performed on the same (n=7) arteries. Values are mean±S.E.M. Significant differences are represented by asterisks ($p<0.05$ (*) or $p<0.01$ (**)).

Fig. 2 TRPV1 specificity of capsaicin evoked vasoconstriction

Internal diameter of cannulated gracilis arteries were measured at 80 mmHg intraluminal pressure upon capsaicin cumulative dose-response determinations in the absence (control) and presence of the TRPV1 antagonist AMG9810 (100, 300 and 1000 nM, Panel A). Symbols are mean±S.E.M. of 5-9 independent determinations. The equilibration dissociation constant of AMG9810 was determined by the conventional Schild plot (x-intercept, Panel B). Finally, gracilis arterioles isolated from control (Wild type) and TRPV1 knockout ($TRPV1^{-/-}$) mice were also tested for capsaicin mediated vasoconstriction (Panel C). Symbols are mean±S.E.M. of 5-6 independent determinations.

Fig. 3 Mechanism of capsaicin mediated vasoconstriction: skelatal 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 on Panel 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 Ca2+ concentrations expressed as 340/380 ratio. Capsaicin was administered in a cumulative fashion (indicated by the arrows, the applied capsaicin doses were: $3x10^{-10}$, 10^{-9}, $3x10^{-9}$, 10^{-8}, $3x10^{-8}$, 10^{-7}, $3x10^{-7}$, 10^{-6}, $3x10^{-6}$ M). Panel B shows the mean±S.E.M. of n=5 single determinations.

Fig. 4 Mechanism of capsaicin mediated vasoconstriction: isolated arteriolar smooth muscle cells

Freshly isolated canine coronary arteriolar smooth muscle cells were loaded with fura-2 fluorescent Ca^{2+} sensitive dye and treated with capsaicin (1 M) 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 panel A, where green pixels represent low and red 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 (panel B). These observations were confirmed when responses of the capsaicin sensitive cells (10 out of 28 viable cells) were evaluated (panel C). Bars are mean±S.E.M.

Fig. 5 Pharmacological characterization of vascular responses to capsaicin

Experiments were performed on cannulated arteries as mentioned above. First, a cumulative dose-response was measured (panel A, n=7). Next on a separate set of arteries the kinetics of response was measured by the application of 1 μM capsaicin for 20 min. Arteriolar diameter was measured at 10 s intervals (panel B, n=9). After this 20 min treatment the arteries were washed and were incubated in KREBS solution for 40 min (regeneration). At the end of regeneration, vasoconstriction to the same dose of capsaicin (1 μM) was measured to determine tachyphylaxis (panel C, n=7). Values are mean±S.E.M., significant difference ($P<0.05$) is represented by asterisk.

Fig. 6 Arteriolar response to resiniferatoxin

Experiments were performed as mentioned in Fig. 4 with resiniferatoxin. Cumulative dose-response is shown on panel A (n=3). No functional response was detected in 20 min upon resiniferatoxin stimulation (panel B, 10 nM, n=5). However, this treatment desensitized the receptors to capsaicin (1 μM) measured after regeneration (panel C, n=5).

Fig. 7 Arteriolar response to JYL-273

Experiments were performed as mentioned in Fig. 4 with JYL-273. Cumulative dose-response is shown on panel A (n=7). No functional response was detected in 20 min upon JYL-273 stimulation (panel B, 1 μM, n=5). However, this treatment desensitized the receptors to capsaicin (1 μM) measured after regeneration (panel C, n=4).

Fig. 8 Arteriolar response to MSK-195

Experiments were performed as mentioned in Fig. 4 with MSK-195. Cumulative dose-response is shown on panel A (n=5). A transient vasoconstriction was observed upon MSK-195 stimulation for 20 min (panel B, 1 µM, n=6). In addition, this treatment desensitized the receptors to capsaicin (1 µM) measured after regeneration (panel C, n=6).

Fig. 9 Arteriolar response to JYL-79

Experiments were performed as mentioned in Fig. 4 with JYL-79. Cumulative dose-response is shown on panel A (n=8). A transient vasoconstriction was observed upon JYL-79 stimulation for 20 min (panel B, 1 µM, n=5). In addition, this treatment desensitized the receptors to capsaicin (1 µM) measured after regeneration (panel C, n=5).

Fig. 10 Arteriolar response to JYL-1511

Experiments were performed as mentioned in Fig. 4 with JYL-1511. Cumulative dose-response is shown on panel A (n=6). No functional response was detected in 20 min upon JYL-1511 stimulation (panel B, 1 µM, n=6). However, this treatment desensitized the receptors to capsaicin (1 µM) measured after regeneration (panel C, n=6).

Fig. 11 Partial agonism/antagonism of JYL-1511 on vascular smooth muscle located TRPV1

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 pretreatment) were also measured on a different set of arteries (capsaicin, n=7). Efficacy of JYL-1511 as partial agonist was expressed as the % of decrease in arteriolar diameter evoked by the application of capsaicin alone (100%, capsaicin). Efficacy as partial antagonist was expressed as the % of decrease in capsaicin constriction (100%, capsaicin) in the presence of JYL-1511 (1 µM, JYL-1511+capsaicin).

Fig. 12 Sensory neuronal irritation evoked by the applied 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 were counted for 60 s after application of 10 µl of the drugs onto the conjunctiva of the rats. Concentration of the drugs were chosen to represent the highest dose used in the vascular experiments (indicated in the figure). Bars are mean±S.E.M. (n=5), significant differences ($P<0.05$) from the control (wipes upon administration of the vehicle alone) are represented by asterisks.

Statement of conflicts of interest

The authors report no conflicts of interest.

Fig. 1

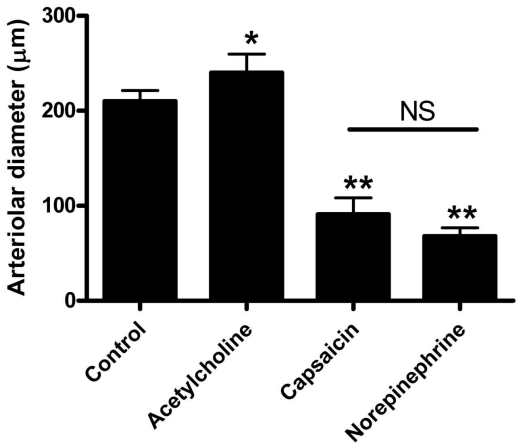

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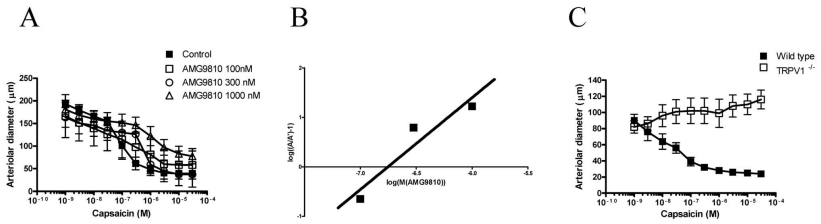
Fig. 2



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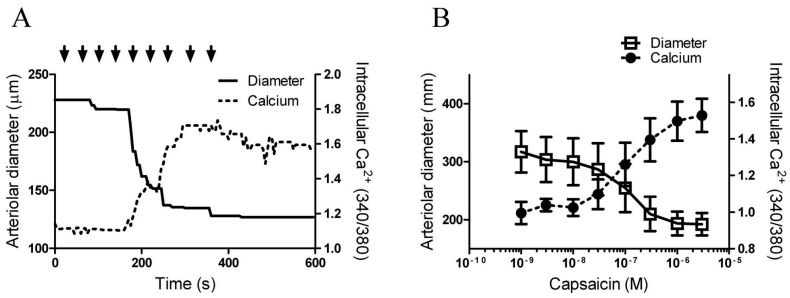

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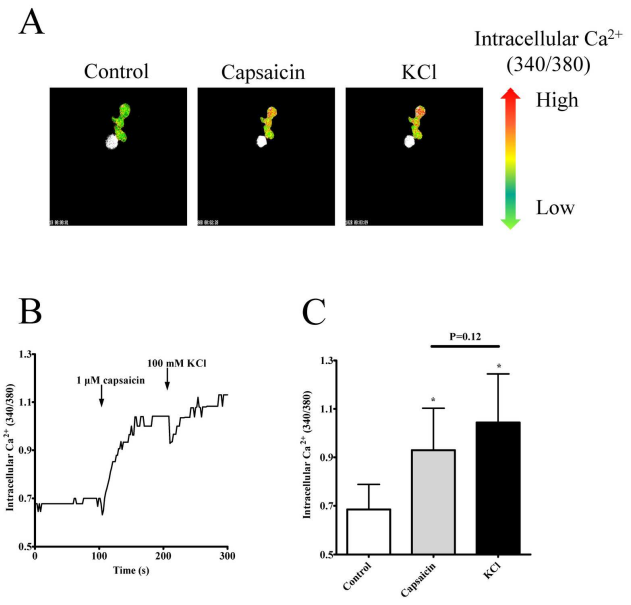

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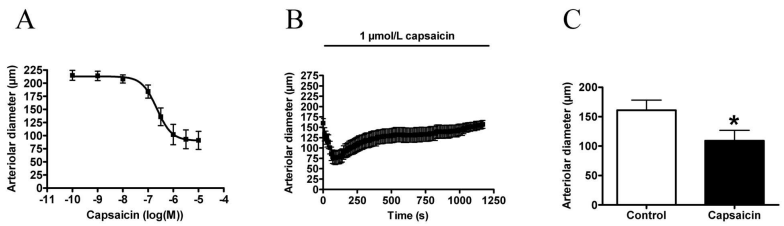
Fig. 5



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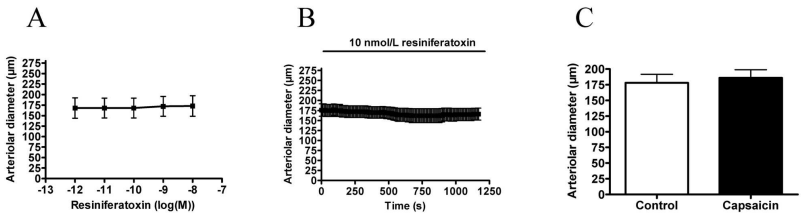

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Fig. 7

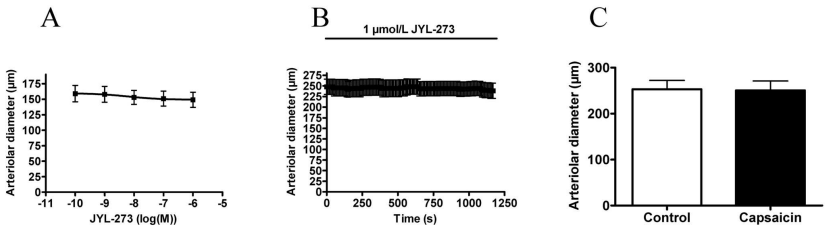

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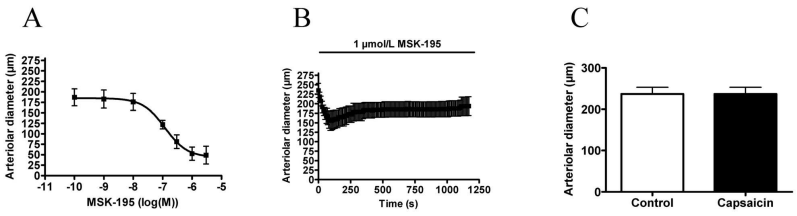
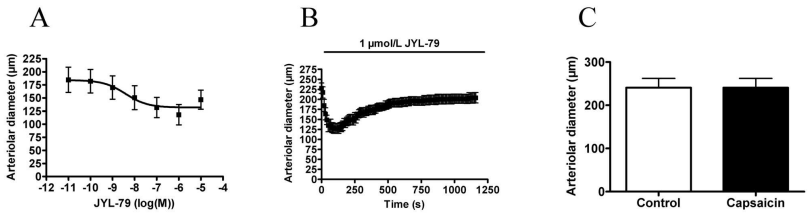
Fig. 8



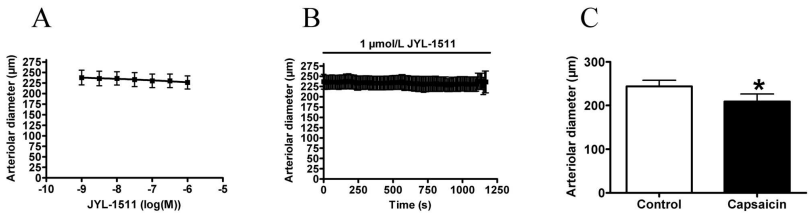
209x148mm (300 x 300 DPI)

Fig. 9



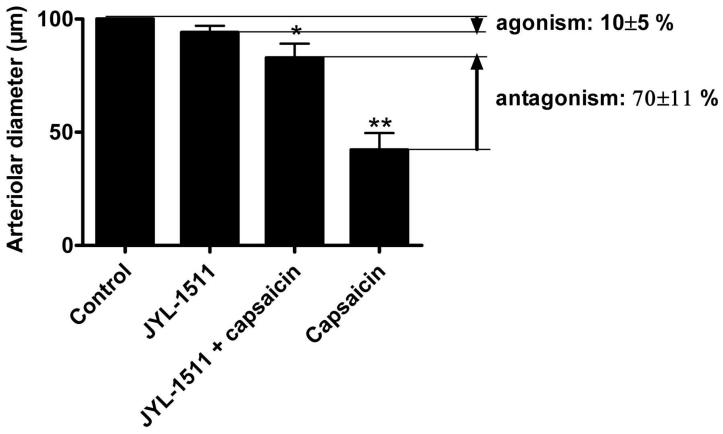
209x148mm (300 x 300 DPI)

Fig. 10



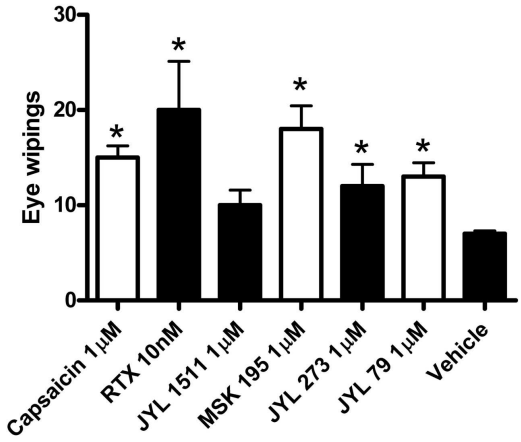
209x148mm (300 x 300 DPI)

Fig.11



209x148mm (300 x 300 DPI)

Fig. 12



209x148mm (300 x 300 DPI)