

**SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)**

# **Vascular effects of capsaicin receptor (TRPV1)**

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# 1. INTRODUCTION

## The TRPV1 receptor

The TRPV1 is a nonselective cation channel ( $P_{Ca}/P_{Na} \leq 10$ ). TRPV1 belongs to the TRPV subfamily of the large TRP (transient receptor potential) ion channel super family, whose prototypical member, TRP, is deficient in a *Drosophila melanogaster* mutant exhibiting abnormal responsiveness to continuous light. The TRP superfamily in mammals has about 28 members, they can be divided into 6 subfamily, like ankyrin (TRPA), canonical (TRPC), melastatin (TRPM), polycystic (TRPP), mukolipin (TRPML) and vanilloid receptors. The TRPV1 is the first member of the TRPV subfamily, and there are now 5 different channels in this subfamily. Interestingly, other members of the family TRPV (TRPV2-6) can not be affected by capsaicin and resiniferatoxin (RTX). TRPV1 is predicted to have six transmembrane (TM) domains and a short, pore-forming hydrophobic stretch between the fifth and sixth TM domains. It is activated by capsaicin, by heat ( $>43^{\circ}\text{C}$ ), acidic conditions and various lipids. Many regions and amino acids in TRPV1 involved in specific functions have been identified since the cloning in 1997.

Like many other TRP channels, TRPV1 has a long amino terminus containing six ankyrin-repeat domains and a carboxyl terminus containing a TRP domain close to the sixth TM. The ankyrin repeats are known to bind to various cytosolic proteins. Calmodulin (CaM), has been reported to bind to the ankyrin repeat domain of TRPV1, which plays an important role in the  $\text{Ca}^{2+}$ -dependent receptor desensitization. Important roles for the sensitization of receptor by phosphorylation by protein kinase A (PKA), protein kinase C (PKC) and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CAMKII), were also described.

Intracellular  $\text{Ca}^{2+}$  level may be increased after the stimulation of TRPV1, and simultaneously decreasing activity of the receptor (desensitization) can be observed. During this period, activity of the receptor is regulated by reversible phosphorylation. The dephosphorylation can desensitize the receptor and reduce intracellular  $\text{Ca}^{2+}$  concentration. The role of protein phosphatase 2B (calcineurin) play a particularly important role in acute desensitization.

## **Physiological significance of TRPV1**

The TRPV1 was originally found in sensory C and A- $\delta$  fibres. It functions as a ligand-, proton- and heat-activated molecular integrator of nociceptive stimuli and hence represents a promising drug target for analgesia. Capsaicin not only causes pain, but also seems to exhibit analgesic properties, particularly when used to treat pain associated with diabetic neuropathies or rheumatoid arthritis. Activation of TRPV1 leads to central pain and to a local „sensory-efferent” effect. This paradoxical effect may relate to the ability of capsaicin to desensitize nociceptive terminals to capsaicin, as well as to other noxious stimuli following prolonged exposure. At the molecular level, an extracellular  $\text{Ca}^{2+}$ -dependent reduction of TRPV1 responsiveness upon continuous vanilloid exposure may lead to damage to the nerve terminal or depletion of calcitonin gene related peptide (CGRP) or substance P (SP). These peptides cause vasodilatation in different vascular beds such as mesenteric, hepatic, basilar, dural and meningeal arterioles.

However, TRPV1 expression has recently been identified in many cells in addition to sensory neurons. In particular, TRPV1 expression was detected in various cell types in the brain, and in the periphery, including arteriolar receptors responsible for vasoconstriction. Immunohistochemical studies have shown that TRPV1 is expressed in human skin epidermis and cultured epidermal keratinocytes. This was also supported by RT-PCR. The functional form of the receptor is also expressed in keratinocytes. Further investigations have shown that the epidermal keratinocytes play a role in the inflammatory processes of skin and regulation of inflammatory response. Pharmacology of the receptor can be important in the treatment of various skin diseases.

## **Expression of TRPV1 and the vascular effects of receptor stimulation**

Previous studies have also proposed that in certain circumstances, TRPV1 activation may lead to vasoconstriction in mesenteric, coronary, skeletal muscle and dural vessels, although the underlying mechanism remained obscure. A number of explanations have been proposed for the mechanism of constriction. In these latter cases, TRPV1-mediated substance P or endothelin release was suggested as possible mechanisms. However, our results suggest that TRPV1 is

functionally expressed in the smooth muscle layer of resistance arterioles and its activation can lead directly to an increase in intracellular  $\text{Ca}^{2+}$  levels, and vasoconstriction.

Research data indicate that the TRPV1 can be expressed in the vascular tissues and the effects of TRPV1 stimulation on the blood vessels suggested both dilation and constriction upon TRPV1 stimulation. 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 suggested cell type-specific differences in the capsaicin responsiveness. We concluded that the expression of TRPV1 in smooth muscle have important role in regulation of vessel diameter. It is interesting that in some of these cases, like in the case of rat mesenteric arteries, both vasoconstriction and vasodilation 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 the physiological effect of capsaicin stimulation is dominated by the active receptor population.

## **Pharmacology of TRPV1**

TRPV1 antagonists are in clinical trials for various conditions including dental pain, osteoarthritis, neuropathic pain, overactive bladder, chronic cough, rectal hypersensitivity, migraine, lower back pain and interstitial cystitis. Although some results of these trials are promising, they also revealed that TRPV1 antagonists can evoke serious hyperthermia. This hyperthermia is probably related to the involvement of TRPV1 in temperature regulation *in vivo*. However, the mechanism of this effect is not clear. Although some antagonists cause hyperthermia, others are without thermoregulatory effects in humans. This suggests that the TRPV1 responsible for analgesia is pharmacologically different from that involved in thermoregulation.

The most known antagonist of TRPV1 is capsazepine. This is non-selective antagonist because it could antagonize the voltage-gated  $\text{Na}^{+}$ ,  $\text{K}^{+}$  and  $\text{Ca}^{2+}$  channels as well as the human HCN1 channel. In our study we used AMG9810. Effects of these compounds have previously been

demonstrated on rat and human TRPV1 receptors. AMG9810 competitively inhibited the TRPV1-mediated constriction.

Vanilloid receptor ligands such as capsaicin have been divided into three structural regions, the A-, B- and C regions. The A-region plays an important role in the modulation of TRPV1 activity, accordingly both agonist and antagonist molecules can be synthesized by minor modifications of this region without major influences on the binding capacity of TRPV1. The most important roles of B- and C-region are the optimal orientation of A-region and binding of different agents to membranes, hence modification of those regions remarkably affects their binding to TRPV1.

Nonetheless, structure of the ultrapotent TRPV1 agonist resiniferatoxin (RTX) has underlined the potential existence of additional TRPV1 binding sites in C-region, which may result in three fold increase in their affinity to TRPV1. This capacity has been already utilized by insertion of a structure similarly to RTX into the C-region of experimental compounds.

### **The effects of anandamide**

After identification of the cannabinoid receptors, intense effort was made to identify their endogenous ligands. Arachidonylethanolamide (anandamide) was first identified in porcine brain as a substance which binds to cannabinoid receptors. It was followed by the identification of additional potential endogenous regulators like 2-arachidonyl glycerol (2-AG). It has since been shown that anandamide is not only an endogenous ligand for cannabinoid receptors (CB1 and CB2) but may also affect TRPV1 and other, recently identified targets. However, significant differences of effectiveness were found: in some experimental systems it was a complete, while in other conditions it was a partial agonist of TRPV1.

## **The synthesis and degradation of anandamide**

Although anandamide may be produced as a simple condensation product of arachidonic acid and ethanolamine, its *in vivo* synthesis seems to occur from the hydrolysis of N-acylphosphatidylethanolamine (NAPE) by a specific phospholipase D (NAPE-PLD).

Anandamide is a long-chain hydrocarbon molecule and its lipophilicity ensures that it passes through the cellular membrane. However, the anandamide transporters play an important role in intracellular transport and increasing the speed of degradation of anandamide. It is important to note that anandamide, in contrast to classical neurotransmitters, is not stored in the cells. Instead, it is synthesized upon receptor stimulation and immediately released. The effects of anandamide are readily terminated by intracellular hydrolysis, which is catalyzed by fatty acid amide hydrolase (FAAH).

Arachidonic acid is synthesized from linoleic acid and linolenic acid. Arachidonic acid generated for signaling purposes appears to be derived by the action of a phosphatidylcholine-specific cytosolic phospholipase A<sub>2</sub> (PLA<sub>2</sub>). Arachidonic acid release requires the activity of two other enzymes, such as phospholipase C (PLC) and diacyl-glycerol (DAG) lipase. DAG produced by PLC may be the substrate of DAG-lipase thus the precursor molecule of arachidonic acid.

## **Anandamide as an endovanilloid**

The primary enzyme responsible for anandamide metabolism is the fatty acid amide hydrolase. As has been mentioned, anandamide binding sites for both TRPV1 and CB receptors appear to be intracellular and the anandamide concentration is determined by its uptake and metabolism (this latter is catalyzed by FAAH). These factors are of particular importance in the stimulation of TRPV1, which requires substantial local (intracellular) concentrations of anandamide. Indeed, inhibition of FAAH activity significantly increases intracellular anandamide concentrations and also anandamide efficacy at TRPV1. The affinity of anandamide for TRPV1 binding seems to be similar or about fivefold weaker than for capsaicin. In general, efficacy of anandamide on TRPV1 appears to be tissue, expression system, and species dependent.

Accordingly, anandamide is a partial activator of TRPV1 when the receptor expression is low, while it is a full agonist when receptor expression is high. In addition, the intrinsic efficiency of anandamide is apparently regulated.

### **The vasoactive effects of anandamide**

Anandamide evokes vasodilation in various arteriolar beds of different species. Its effectiveness ranges from robust to slight to evoke relaxation in conduit arteries. Although stimulation of cannabinoid and vanilloid receptors can evoke vasodilatation, in many cases anandamide mediated vasodilatation was found to be independent of these receptors.

## **2. AIMS OF THE STUDY**

Our scientific aims were:

1. Characterization of vascular TRPV1:
  - identification the TRPV1 expressing vascular cell types,
  - studying the mechanism of TRPV1 mediated vascular constriction,
  - pharmacological characterization of vascular TRPV1,
  - determination of pharmacological differences between the vascular and neuronal TRPV1.
  
2. Characterization of the vascular effects of the endovanilloid anandamide:
  - studying the mechanism of anandamide induced vasodilatation,
  - characterization of the role of TRPV1 activation/desensitization in the mechanism of vasodilatation.



### **3. METHODS**

Animal care and experimental procedures complied with NIH guidelines and were approved by the Ethical and Experimental Animal Research Committee of the University of Debrecen. Anesthesia was induced by intraperitoneal injection of sodium pentobarbital (50 mg/kg).

#### **Experimental animal models**

The experiments were performed on male Wistar rats and on male mice (control C57BL/6J and TRPV1<sup>-/-</sup> knockout mice). Rats (WKY/NCrl) were maintained on a standard laboratory food and water *ad libitum*.

#### **Preparation of cannulated skeletal muscle arterioles**

Arterioles were isolated from skeletal muscle (*musculus gracilis*) of the rat and arteriolar diameter was measured. Briefly, arterioles were kept in a physiological saline solution at an intraluminal pressure of 80 mmHg until the development of spontaneous myogenic response (constriction to intraluminal pressure). Changes in intraluminal arteriolar diameter were measured after the various treatments.

#### **The effects of TRPV1 agonists**

Changes in diameter to TRPV1 agonists were tested with cumulative application of (capsaicin, 0.1 nM-1 mM; RTX, 1 pM-10 nM; JYL-273, 0.1 nM-1 mM; MSK-195, 0.1 nM-3 mM; JYL-79, 3 pM-10 mM; JYL-1511, 1 nM-1 mM). The specificity of agonist responses was tested by the application of the TRPV1 antagonist AMG9810. Arterioles were isolated from wild-type and TRPV1 knockout mice as detailed for the rat. Changes in diameter to TRPV1 agonists were tested by measuring responses to cumulative doses of capsaicin (0.1 nM-30 mM).

## **Determination of antagonist equilibrium dissociation constant**

EC<sub>50</sub> of capsaicin was calculated in the absence (designated as A) or in the presence of AMG9810 (designated as A'), then  $\log((A/A')-1)$  values were plotted as a function of the logarithm of AMG9810 concentration. Data were fitted by linear regression, and the antagonist equilibrium dissociation constant was obtained from the *x*-intercept.

## **Parallel measurement of vascular diameter and intracellular Ca<sup>2+</sup> concentrations**

Skeletal muscle arterioles were isolated and cannulated from the gracilis muscle of the rat, as mentioned above. After the arteries had been mounted in the tissue chamber, the physiological buffer was supplemented with 1% BSA and 5 mM Fura-2AM fluorescent Ca<sup>2+</sup> indicator dye for 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<sup>2+</sup> concentrations (IncyteIm2 instrument) by recording images (cut off >510 nm) excited alternatively by 340 and 380 nm light. Images were recorded every 2-5 s and evaluated offline.

## **Isolation of smooth muscle cells from canine coronary arteries**

For these measurements smooth muscle cells were isolated from coronary arterioles of adult beagle dogs. After the adherence of the cells to the glass coverslips placed in the wells the media was changed to DMEM containing 1% BSA and 5 mM fura2-acetoxymethyl ester for 2 hour at room temperature. The cover slips were then placed in a suitable chamber for intracellular Ca<sup>2+</sup> concentration measurements. These measurements were started by washing the cells with Dulbecco's PBS (DPBS) three times, and the measurements were performed in DPBS. The fluorescence of individual cells was measured with an InCyt Im2 fluorescence imaging system. 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 mM capsaicin and then with 100 mM KCl. Data were analysed with the InCyt 4.5 software and further processed with Excel and Prism 5.0 software.

## Measurements of eye wiping

One drop (10  $\mu$ l) of agonists (capsaicin, 1  $\mu$ M; RTX, 10 nM; JYL-273, 1 mM; MSK-195, 1 mM; JYL-79, 1 mM; JYL-1511, 1 mM) was put into the right or left conjunctiva of the rat (single treatment for each rat). The number of eye wipes was counted for 60 s. In the control group, the same volume of solvent was administered in a similar manner.

## Effects of anandamide on skeletal muscle arterioles

Skeletal muscle arterioles were isolated and cannulated from the gracilis muscle of the rat, as mentioned above. In some cases arteriolar responses were studied after endothelium denudation. The endothelium of the arterioles was removed by perfusion of the vessels with air. Endothelium denudation was confirmed by the loss of dilation in response to acetylcholine and by the maintenance of dilation in the presence of the NO donor sodium nitroprusside. Acute effects of anandamide (0.1-30  $\mu$ M), arachidonic acid (1  $\mu$ M), capsaicin (1  $\mu$ M, TRPV1 agonist) and WIN55-212-2 (1  $\mu$ M, CB agonist) on arteriolar diameter were tested for 20 min with arteriolar diameter being measured every 10 s. In some cases, arteries were pretreated with baicalein (1  $\mu$ M), indomethacin (10  $\mu$ M), PPOH (20  $\mu$ M) or URB-597 (1  $\mu$ M) before the addition of anandamide (1 or 30  $\mu$ M). After arteries were treated for 20 min as described above, they were then incubated with PSS alone for 40 min (washout of the original stimulus and a time-dependent action of its metabolites followed for 40 min, referred to as “regeneration” in the text). Arteriolar diameter was determined before addition of the drugs, after 20 min treatment, and finally, after the 40 min regeneration period. Effects of the treatments on the myogenic response were also determined.

## Immunohistochemical procedures

Rat skeletal muscle (*gracilis muscle*) was dissected from Wistar rats and embedded in Tissue-Tek O.C.T compound. Cryostat sections (thickness 10  $\mu$ m) were placed on adhesive slides

and fixed in acetone. The slices were stained with anti-FAAH (dilution: 1:50) and with anti-smooth muscle actin antibodies.

## **Data analysis and statistical procedures**

Statistical differences were evaluated by Student's *t*-test by comparing values before and after treatments (paired) or comparing eye wipes of vehicle-treated rats with those of TRPV1 agonist treated rats (unpaired). Statistical analysis was made by GraphPad 5.0 using analysis of variance (repeated measures ANOVA) with Dunnett's post hoc test to detect significant differences from control values. P-values <0.05 were considered to be significant.

## 4. RESULTS

### **The vascular effects of TRPV1 activation**

Application of the TRPV1-specific agonist capsaicin (1  $\mu$ M) resulted in a substantial constriction (decrease of arteriolar diameter from  $210\pm 11$   $\mu$ m to  $91\pm 17$   $\mu$ m,  $n=7$ ,  $P < 0.01$ ) of skeletal muscle arterioles, which was similar to noradrenaline (10  $\mu$ M, decrease of arteriolar diameter to  $68\pm 9$   $\mu$ m,  $n=7$ ). In contrast, the endothelium-dependent vasodilator acetylcholine evoked dilatation (increase in arteriolar diameter to  $240\pm 20$   $\mu$ m,  $n=7$ ,  $P=0.03$ ).

### **Specific effects of the TRPV1 antagonist AMG9810**

The vast majority of published data suggest that vascular TRPV1 stimulation produces a dilatation. It was therefore necessary to test the TRPV1 specificity of this capsaicin mediated contractile responses. First, a competitive antagonist of TRPV1 was applied. AMG9810 antagonized capsaicin-mediated contractions in a dose-dependent manner. Moreover, the potency of AMG9810 determined in these assays (177 nM) was in agreement with its potency determined in other TRPV1-specific systems (87.3 nM). The TRPV1 selectivity of these capsaicin-mediated contractile responses was also tested in TRPV1 knockout (TRPV1<sup>-/-</sup>) mice. The potency of capsaicin (EC<sub>50</sub>) was 137 nM and efficacy was 73% (decrease in diameter from  $69\pm 8$   $\mu$ m to  $24\pm 3$   $\mu$ m,  $n=6$ ) in arteries from wild-type mice, while the same capsaicin treatments were without vasoconstrictive effects in TRPV1<sup>-/-</sup> mice ( $n=5$ ).

### **The mechanism of TRPV1 mediated constriction**

The potential mechanism of TRPV1-mediated constrictions was evaluated. Activation of TRPV1 results in an increase in intracellular Ca<sup>2+</sup> concentrations in many TRPV1-expressing cell types and this contributes to the physiological effects. To detect capsaicin-mediated changes in intracellular Ca<sup>2+</sup> concentrations, a Ca<sup>2+</sup>-imaging system was applied. Simultaneous measurement of intracellular Ca<sup>2+</sup> concentration and vascular diameter (outer diameter in this case) of cannulated

rat arterioles isolated from the gracilis muscle of the rat was performed. The capsaicin-evoked vasoconstriction was paralleled by an increase in intracellular  $\text{Ca}^{2+}$  concentration. Moreover, both vascular diameter and intracellular  $\text{Ca}^{2+}$  concentration increased in a dose-dependent manner, with potency in the nanomolar range (note maximal responses at 1  $\mu\text{M}$ ). To identify the TRPV1-expressing cell type, arteriolar smooth muscle cells were isolated from canine coronary arteries and changes in intracellular  $\text{Ca}^{2+}$  concentrations to capsaicin (1  $\mu\text{M}$ ) and to KCl (100 mM) treatments were tested. The capsaicin-mediated increase in intracellular  $\text{Ca}^{2+}$  concentrations in the cells responding to capsaicin (10 out of 28 cells) was similar (increase in 340/380 ratio from  $0.69 \pm 0.10$  to  $0.93 \pm 0.17$ ) to the increase evoked by depolarization (100 mM KCl, 340/380 ratio was  $1.04 \pm 0.20$ ).

## **Pharmacological properties of the vascular TRPV1**

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

RTX was tested under the same conditions. Surprisingly no vascular effects were detected upon application in a concentration range from 1 pM to 10 nM. Moreover, no effects were detected

upon application of 10 nM for 20 min. However, capsaicin (1  $\mu$ M) was without effect after 40 min regeneration, suggesting complete tachyphylaxis of arterial TRPV1 upon the otherwise ineffective RTX treatments.

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

MSK-195 had a potency of 120 nM and an efficacy of  $71\pm 11\%$  (n=5). Application of 1  $\mu$ M MSK-195 for 20 min resulted in a transient decrease in arterial diameter (decrease from  $235\pm 19$   $\mu$ m to  $155\pm 25$   $\mu$ m at 90 s, n=6). However, the kinetics of this acute desensitization were slower than that for capsaicin, since the original arteriolar diameter was not restored during the 20 min incubation (arterial diameter after 20 min incubation was  $193\pm 25$ ,  $P=0.03$  vs. before treatment, n=6). Similar to all the agonists mentioned above, MSK-195 also evoked a complete tachyphylaxis of capsaicin-sensitive vascular TRPV1.

JYL-79 was found to be the most potent vascular TRPV1 agonist ( $EC_{50}=3.9$  nM, n=8). Its efficacy was  $36\pm 8\%$  (n=8). It also evoked a transient vasoconstriction when applied at a concentration of 1  $\mu$ M (decrease of vascular diameter from  $228\pm 13$   $\mu$ m to  $127\pm 12$   $\mu$ m at 100 s, n=5). The desensitization of the receptor was not complete at the end of the 20 min incubation (vascular diameter at 20 min was  $204\pm 13$   $\mu$ m,  $P=0.046$  vs. before treatment, n=5). Moreover, no response to capsaicin (1  $\mu$ M) was observed after 40 min regeneration (n=5).

To estimate the threshold of TRPV1 stimulation, which causes desensitization and tachyphylaxis of vascular TRPV1, a partial agonist (JYL-1511) was applied. Its efficacy as an agonist was about 17%, and its potency was 3 nM in a CHO cells overexpressing rat TRPV1 (CHO-TRPV1) cell line. JYL-1511 was without effects on the vascular diameter in the concentration range 1 nM-1  $\mu$ M (n=6). Application of 1  $\mu$ M for 20 min was also without effect. A partial inhibition (tachyphylaxis) of the capsaicin response (1  $\mu$ M) was noted after 40 min regeneration (decrease of vascular diameter from  $244\pm 14$   $\mu$ m to  $209\pm 17$   $\mu$ m,  $P=0.02$ , n=6).

According to these data, the agonism of JYL-1511 appears to be  $10\pm 5\%$  and its antagonism is  $70\pm 11\%$  at the vascular TRPV1.

## **Differences between the vascular and neuronal TRPV1 populations**

Sensory neuronal activation was also tested here by the use of the eye wiping assay. JYL-1511 did not evoke significant effects, while all of the other agonists increased the number of eye wipes.

## **Anandamide induced vasodilatation**

Anandamide mediated effects were tested on isolated skeletal muscle resistance arteries from the rat. Anandamide ( $0.1\ \mu\text{M}$ ) was without effect upon 20 min treatment or after 40 min regeneration while higher doses showed vasoactivity. In particular,  $1\ \mu\text{M}$  anandamide evoked a transient dilation;  $30\ \mu\text{M}$  evoked a transient constriction followed by dilation at the end of the incubation (20 min) and this dilation was further increased over the 40 min regeneration period (incubation in PSS alone).

## **Effects of anandamide on vascular myogenic tone**

We hypothesized that the dilations observed under our *in vivo* conditions, where intraluminal pressure is not constant, may be related to a blunted myogenic response. To test this hypothesis in detail, the myogenic response of these arterioles was determined at the beginning of the experiments (control), at the end of the 20 min treatment+40 min regeneration in the presence of extracellular  $\text{Ca}^{2+}$  (active diameter), and in the absence of extracellular  $\text{Ca}^{2+}$  (passive diameter).  $0.1\ \mu\text{M}$  anandamide was without effect on the myogenic response, while higher doses of anandamide ( $1$  and  $30\ \mu\text{M}$ ) effectively antagonized the myogenic response. These findings suggested the existence of an anandamide sensitive pathway which may evoke dilation mediated by the synthesis and release of dilative agents, or alternatively, may impair the vascular myogenic response.



## **Role of the endothel in anandamide induced vasodilatation**

We therefore examined the behavior of endothelium denuded arterioles. First, the effect of denudation was tested by the application of acetylcholine. Acetylcholine responses were blunted after denudation (increase in arteriolar diameter from  $180\pm 8$  to  $242\pm 11$   $\mu\text{m}$  in the case of control,  $P<0.05$ , compared to no change ( $173\pm 14$  to  $188\pm 10$   $\mu\text{m}$ ) in the case of endothelium denuded arterioles,  $n=5$ ). In contrast, smooth muscle function was not altered significantly by the procedure as shown by nitroprusside mediated dilation ( $n=5$ ) or by the unaltered myogenic response of the arteries ( $n=11$ ). Having confirmed that we were able to successfully denude the arterioles, we compared the effects of  $30$   $\mu\text{M}$  anandamide on the denuded arterioles and on those with an intact endothelium. Although anandamide mediated effects were biphasic in both cases (intact endothelium, versus endothelium denuded arteries), sustained dilation upon anandamide treatment was not present in endothelium denuded arteries. Nonetheless, a similar impairment of myogenic response was noted in the endothelium denuded arteries as it was observed for intact arteries.

## **Role of the anandamide degradation in dilatation**

Inhibition of the metabolism of anandamide to arachidonic acid ( $1$   $\mu\text{M}$  URB-597, fatty acid amide hydrolase, FAAH inhibitor) completely prevented the effects of  $30$   $\mu\text{M}$  anandamide on the myogenic response, although it did not affect the acute responses to anandamide, under constant pressure. Application of  $1$   $\mu\text{M}$  arachidonic acid evoked a vasoconstriction similarly to the previous treatments but was without any apparent additional effects on endothelium denuded arterioles.

## **Functional anandamide receptors**

Next, an effort was made to identify the anandamide receptors involved in the dilation of these resistance arterioles. The TRPV1 agonist capsaicin ( $1$   $\mu\text{M}$ ) evoked a transient vasoconstriction during the  $20$  min treatment, but was without effects on the arteriolar diameter at the end of the treatment ( $20$  min) or after the regeneration period. The CB1 and CB2 receptor agonist WIN55-212-2 ( $1$   $\mu\text{M}$ ) was without significant effects. These observations suggested a

TRPV1 and cannabinoid receptor independent pathway in the acute dilation evoked by anandamide.

### **Anandamide derived arachidonic acid metabolism**

To identify this pathway, the dilation evoked by 30  $\mu\text{M}$  anandamide (the dose which effectively dilated the arteries in 20 min) was further investigated. Inhibition of the metabolism of anandamide to arachidonic acid (1  $\mu\text{M}$  URB-597) completely prevented anandamide mediated dilation. Arachidonic acid treatment (1  $\mu\text{M}$ ) resulted in dilation similar to that for anandamide in the presence of endothelium. The effects of these treatments on vascular diameter after 40 min regeneration were also tested. Again, no dilative effect of TRPV1 or CB1 and CB2 activation was found, while anandamide mediated dilation was lost upon the inhibition of the anandamide metabolizing enzyme FAAH (URB+ANA) and was mimicked by arachidonic acid. Effects on the myogenic response were also studied in detail. Stimulation of TRPV1 by capsaicin (1  $\mu\text{M}$ ) and of cannabinoid receptors by WIN55-212-2 (1  $\mu\text{M}$ ) were without effects on the myogenic response. On the contrary, 30  $\mu\text{M}$  anandamide evoked a loss of myogenic response which was completely prevented by the FAAH inhibitor URB-597 (1  $\mu\text{M}$ ), or by endothelium denudation. Arachidonic acid (1  $\mu\text{M}$ ) evoked a loss of myogenic response similar to that by 30  $\mu\text{M}$  anandamide in the presence of endothelium. Based on these data, we hypothesized that the vascular dilation induced by anandamide was at least partly mediated by arachidonic acid resulting from its hydrolysis by FAAH and reflected the loss of myogenic response caused by this arachidonic acid. To test this hypothesis, 1  $\mu\text{M}$  anandamide (the lowest dose found to be effective at antagonizing the myogenic response) was used and the main pathways responsible for the synthesis of vasoactive agents derived from arachidonic acid were selectively inhibited. 1  $\mu\text{M}$  anandamide evoked a transient constriction if the lipoxygenase pathway (baicalein, 1  $\mu\text{M}$ ) or cytochrome P450 (PPOH, 20  $\mu\text{M}$ ) was inhibited. This vasoconstriction was similar to that found in the case of higher doses of anandamide (30  $\mu\text{M}$ ). Nonetheless, vascular diameter was generally not significantly different at the end of the treatments, nor were there any vascular effects after 40 min regeneration, except in

the case of inhibition of cyclooxygenase by indomethacin (10  $\mu\text{M}$ ), which unmasked a fast and robust dilation evoked by 1  $\mu\text{M}$  anandamide.

An effort was also made to identify the pathways involved in the mediation of anandamide evoked loss of the myogenic response. Baicalein did not abolish anandamide mediated loss of the myogenic response. In contrast, inhibition of cytochrome P450 by PPOH (20  $\mu\text{M}$ ) or of cyclooxygenase by indomethacin (10  $\mu\text{M}$ ) completely prevented the anandamide mediated loss of the myogenic response.

### **Expression of fatty acid amide hydrolase (FAAH) in skeletal muscle arterioles**

Finally, the vascular localization of the anandamide metabolizing enzyme FAAH was studied. FAAH expression was detected by immunohistochemistry in the smooth muscle layer of the same arteries which were isolated for the physiological measurements.

## 5. DISCUSSION

### The vascular TRPV1

Here we report an analysis on the pharmacological properties of vasoconstrictive 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. Previously, TRPV1 was shown to be expressed in vascular smooth muscle cells and it was suggested that activation of TRPV1 is directly linked to intracellular  $\text{Ca}^{2+}$  elevations in smooth muscle. Indeed, in the present study we found that a decrease in arteriolar diameter was paralleled by an increase in intracellular  $\text{Ca}^{2+}$  concentrations in the vascular wall; moreover, direct intracellular  $\text{Ca}^{2+}$  concentration measurements revealed, for the first time, the presence of functional TRPV1 in isolated arteriolar smooth muscle cells. Vasoconstriction in response to TRPV1 stimulation was reported decades ago and this effect was confirmed later. 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. This suggested the involvement of different receptors or different pharmacology for TRPV1-mediated vascular dilatation and constriction. Although the mechanism of the vasoconstrictor effects of TRPV1 agonists were generally not investigated in detail, it was suggested that TRPV1-induced vasoconstriction is probably mediated by endothelin-1 or substance P release from sensory neurons. We have recently shown that stimulation of TRPV1 in skeletal muscle arterioles results in a substantial vasoconstriction.

## **The specificity of capsaicin mediated constriction**

The TRPV1 specificity of capsaicin-mediated arteriolar vasoconstriction was ultimately proven here. Most importantly, capsaicin-mediated responses were absent in TRPV1 knockout mice. Moreover, an effort was also made to investigate the potential mechanisms. Intracellular  $\text{Ca}^{2+}$  concentration measurements showed a capsaicin mediated increase in the arteriolar wall as well as in isolated arteriolar smooth muscle cells. 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  $\text{Ca}^{2+}$  concentrations and to vasoconstriction.

Next, the effects of pharmacological TRPV1 inhibition on this response were tested. The TRPV1 antagonist AMG9810 (previously tested on exogenous and sensory neuronal TRPV1) inhibited this capsaicin-evoked arteriolar constriction in a competitive fashion. These findings suggest that TRPV1 antagonists developed as analgesic agents may also interfere with skeletal muscle blood perfusion by inhibiting vascular TRPV1.

Nonetheless, the major goal of this present work was to investigate the structure-activity relationship of TRPV1 agonists for the vascular TRPV1 in functional assays. Our results confirmed that TRPV1 stimulation by capsaicin evokes a substantial constriction in isolated cannulated skeletal muscle arteries. Here, a series of commercially available agonists were also tested in addition to capsaicin. Significant differences in potency, efficacy and desensitization were found. It was observed that some of the TRPV1 agonists (such as RTX and JYL-273) were able to desensitize vascular TRPV1 without any apparent vascular effects. This behaviour of resiniferatoxin in the vascular diameter assay is not unprecedented; a very similar action ('desensitization' to capsaicin without prior activation) has been demonstrated for pulmonary chemoreflex. One hypothesis for this desensitization is that low level activation of TRPV1 with certain structures may be sufficient to evoke complete tachyphylaxis, without increasing the intracellular  $\text{Ca}^{2+}$  concentrations to those levels needed to induce vasoconstriction. Alternatively, tachyphylaxis may be the reason for the irreversible activation of TRPV1 by RTX leading to a sustained Ca influx. To measure the level of activation needed to evoke tachyphylaxis a partial

agonist (JYL-1511) was used. Its partial antagonism was confirmed on vascular receptors (about 10% agonism and 70% antagonism), and its application resulted in significant tachyphylaxis, suggesting a role for desensitization rather than sustained  $\text{Ca}^{2+}$  influx in this system.

Taken together, these results suggest the vascular smooth muscle-located receptor and also the TRPV1 responsible for eye irritation upon capsaicin treatment *in vivo* seem to have different ligand selectivity for desensitization from that of the TRPV1 expressed in CHO cells. It was observed that the kinetics of acute desensitization were different in the case of agonists evoking vascular constriction. With capsaicin, complete acute desensitization was observed, while for other agonists, JYL-79, MSK-195, only a partial desensitization was observed. The fact that different agonists evoked responses with different durations suggests that TRPV1 agonists may be tailored to desired duration of vascular effects.

## **The vascular effects of anandamide**

An important aim of the study was to investigate the mechanism of anandamide evoked vasodilation in skeletal muscle resistance arteries of the rat. The importance of anandamide metabolism *in vivo* has recently been recognized in different systems, highlighted by the fact that the anandamide level is 15 fold higher in FAAH knockout mice, accompanied with decreased nociception, but no obvious hemodynamic differences compared to wild type littermates. It should be noted, however, that detailed examination of *in vivo* myogenic reactivity and autoregulatory ability is yet to be performed. A similar effect was observed by inhibition of FAAH by URB-597 (the same inhibitor which was used here), which evoked decreased inflammatory responses. In these inflammatory pain models, the analgesic effects of FAAH inhibition were related to the elevated level of anandamide and presumably to higher stimulation of the analgesic cannabinoid receptors. Anandamide was found to be rapidly metabolized in vascular preparations and it was suggested that its metabolism may limit anandamide effectiveness on vascular cannabinoid receptors. Moreover, vascular effects of anandamide breakdown products were published more than a decade ago, suggesting significant metabolism of anandamide to arachidonic acid and to

prostaglandin E<sub>2</sub> in cerebral arterioles, contributing to indomethacin (a cyclooxygenase inhibitor) dependent dilatations. These findings were confirmed in bovine coronary artery rings by experiments showing that inhibition of anandamide conversion prevented anandamide mediated relaxation and the relaxation was not affected by cannabinoid receptor inhibition. Our data confirm these observations in small arteries isolated from rat skeletal muscle (*gracilis muscle*).

A significant novelty of the present study is to relate the effects of anandamide to the myogenic response of resistance arterioles. Cannulated resistance arterioles develop a myogenic constriction upon elevated intraluminal pressures (myogenic response), a process which has a prominent role in the regulation of blood distribution in vivo. The effect of anandamide and its breakdown on the spontaneous myogenic response was addressed for the first time. To this end, the effects of anandamide were evaluated after a 20 min application followed by a 40 min washout in order to observe the long-term effects of metabolism. This protocol may eliminate other more direct effects of anandamide which could complicate analysis, revealing its vasodilatative actions via metabolism.

Our data showed that anandamide antagonized the myogenic response, which was mediated by its FAAH dependent breakdown, independently of endothelium. This finding was further investigated regarding the potential mechanisms. Arachidonic acid had effects similar to anandamide on the myogenic response in arterioles with intact endothelium. Functional data suggested that the metabolism of anandamide to arachidonic acid was responsible for the myogenic response. Correspondingly, expression of FAAH (the metabolizing enzyme which was inhibited by URB) was localized to the smooth muscle cell layer of the gracilis arteries. Furthermore, stimulation of the known anandamide receptors (vanilloid and cannabinoid receptors) was without effect on the myogenic response.

The exact pathway downstream of anandamide derived arachidonic acid leading to dilation remained elusive. A prominent role of cytochrome P450 epoxygenase and cyclooxygenase enzymes were found, suggesting a complex interplay between these systems. It is conceivable that both epoxyeicosatrienoic acids produced by cytochrome P450 epoxygenase and prostacyclins produced by cyclooxygenase may attenuate the myogenic response.

## 6. CONCLUSIONS

Our results indicate that TRPV1 (a nonspecific  $\text{Ca}^{2+}$  channel) activation leads to an increase in intracellular  $\text{Ca}^{2+}$  concentrations in isolated coronary smooth muscle cells and in the wall of isolated skeletal muscle arteries, resulting in vasoconstriction. The pharmacological profile of the vascular TRPV1 differs from that of the TRPV1 population responsible for sensory irritation. Arteriolar TRPV1 was inhibited by a competitive TRPV1 antagonist developed as an analgesic suggesting that vascular TRPV1 activation may represent a side effect of TRPV1 antagonists when used as analgesics *in vivo*. Moreover, vascular TRPV1 may be a new therapeutic target for the regulation of tissue blood distribution.

On the other hand, anandamide mediated vascular effects are independent of vanilloid and cannabinoid receptors in the rat gracilis artery. We propose that anandamide is metabolized to arachidonic acid in vascular smooth muscle cells. Anandamide derived arachidonic acid synthesis may directly lead to dilation (in an endothelium dependent manner) or to a blunted myogenic response (in an endothelium independent fashion) which may also contribute to physiological vasodilatation. Furthermore, these findings also suggest that anandamide synthesized in cells like neurons or activated macrophages may diffuse to the adjacent vascular beds and trans-activate the arachidonic acid pathway in the vascular wall by its FAAH mediated breakdown. This transactivation of the arachidonic acid pathway may result in an impaired vascular autoregulation and in a consequent vasodilatation affecting tissue blood distribution. Moreover, the concentration-dependent effects of anandamide observed here may suggest a significant role not only in local blood flow/distribution but also in certain vascular pathophysiological states, such as inflammation, or hypotension associated with sepsis.



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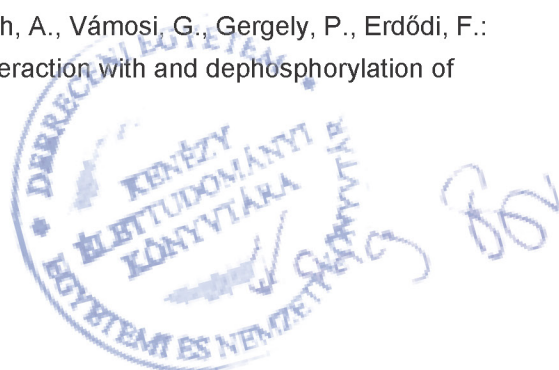
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### List of publications related to the dissertation

1. **Czikora, Á.**, Lizanecz, E., Boczán, J., Daragó, A., Papp, Z., Édes, I., Tóth, A.: Vascular metabolism of anandamide to arachidonic acid affects myogenic constriction in response to intraluminal pressure elevation.  
*Life Sci.* 90 (11-12), 407-415, 2012.  
DOI: <http://dx.doi.org/10.1016/j.lfs.2011.12.016>  
IF:2.527 (2011)
2. **Czikora, Á.**, Lizanecz, E., Bakó, P., Rutkai, I., Ruzsnavszky, F., Magyar, J., Pórszász, R., Kark, T., Facskó, A., Papp, Z., Édes, I., Tóth, A.: Structure-activity relationships of vanilloid receptor agonists for arteriolar TRPV1.  
*Br. J. Pharmacol.* 165 (6), 1801-1812, 2012.  
DOI: <http://dx.doi.org/10.1111/j.1476-5381.2011.01645.x>  
IF:4.409 (2011)

### List of other publications

3. Koložsvári, B., Bakó, É., Bécsi, B., Kiss, A., **Czikora, Á.**, Tóth, A., Vámosi, G., Gergely, P., Erdődi, F.: Calcineurin regulates endothelial barrier function by interaction with and dephosphorylation of myosin phosphatase.  
*Cardiovasc. Res. Epub ahead of print (2012)*  
DOI: <http://dx.doi.org/10.1093/cvr/cvs255>  
IF:6.064 (2011)



4. Szántó, M., Rutkai, I., Hegedűs, C., **Czikora, Á.**, Rózsahegyi, M., Kiss, B., Virág, L., Gergely, P., Tóth, A., Bai, P.: Poly(ADP-ribose) polymerase-2 depletion reduces doxorubicin-induced damage through SIRT1 induction.

*Cardiovasc. Res.* 92 (3), 430-438, 2011.

DOI: <http://dx.doi.org/10.1093/cvr/cvr246>

IF:6.064

5. Kark, T., Bagi, Z., Lizanecz, E., Pásztorné Tóth, E., Erdei, N., **Czikora, Á.**, Papp, Z., Édes, I., Pórszász, R., Tóth, A.: Tissue-specific regulation of microvascular diameter: Opposite functional roles of neuronal and smooth muscle located vanilloid receptor-1.

*Mol. Pharmacol.* 73 (5), 1405-1412, 2008.

DOI: <http://dx.doi.org/10.1124/mol.107.043323>

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