

Ph.D THESIS SUMMARY

A PHARMACOLOGICAL STUDY OF THE TRPV1 CAPSAICIN RECEPTOR

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THEORETICAL BACKGROUND

As a result of long-standing contribution of Professor János Szolcsányi, member of Hungarian Academy of Sciences, there has been great history of investigation of capsaicin (N-vanilil-8-methyl-6-nonamide, spicy alkaloid of hot pepper) in our Department. According to notes, capsaicin has been widely used in folk medicine over 500 years to cure inflammations and sometimes painful conditions.

Capsaicin first excites some primary afferents causing burning pain, followed by desensitization (called analgesy) and at higher concentration it can be neurotoxic (Szolcsányi 1993, 2002, 2005; Sándor & Szállási, 2005).

Desensitization is subject of intensive research worldwide in a hope to design anti-inflammatory and pain reliever medicines. The term desensitization has two meanings: pharmacological desensitization means the decrease of receptor sensitivity against the agonist, while sensory or functional desensitization means diminution of responsiveness of nerve endings to natural stimuli. Morphological changes like swelling of mitochondria and neurodegeneration has also been observed. The decrease of excitatory effects and elimination of toxicity of any agents acting upon the TRPV1 receptors has primary importance in design of novel pain relievers.

The capsaicin-sensitive molecule was denoted by Caterina et al (1997) as VR1 (vanilloid receptor type 1). The receptor is ligand gated, nonselective cationic channel with $P_{Ca}:P_{Na}=9:1$, activated directly by vanilloids, painful heat (Szállási & Blumberg, 1999) as well as acidic pH (Tominaga et al., 1998). As proposed by Clapham et al (2003), the latest terminology accepted by IUPHAR (International Union of Pharmacology) the capsaicin receptor is called TRPV1 (transient receptor potential vanilloid 1).

Cloning of the rat TRPV1 capsaicin receptor (Caterina et al., 1997) was a real breakthrough in research and the molecule immediately generated high interests. Orthologues of TRPV1 receptor expressed in human (Hayes et al., 2000), rabbit (Gavva et al., 2002), guinea pig (Savidge et al., 2002), mouse (Correll et al., 2004) and dog (Phelps et al., 2005) were also cloned as well.

TRPV1 receptor is considered as a polymodal integrative protein to detect thermal and chemical stimuli acting upon a subclass of sensory receptors called polymodal nociceptors (Caterina et al., 2000) and having a critical role in inflammatory hyperalgesia (Davis et al., 2000). TRPV1 receptors identified in the preoptic area of hypothalamus are involved in central control of thermogenesis (Jancsó-Gábor et al., 1970. Szolcsányi et al., 1971).

Sensitization of the receptors by protons (pH shift to 6.4 or 5.9) decreases the heat threshold from 43 C° to 37 or 22 C° (Tominaga et al., 1998). A similar decrease of heat threshold of TRPV1 receptors under inflammation is caused by endogenous proinflammatory peptides and other factors released locally and acting through sensitizing mechanisms such as stimulation of protein kinases, hydrolysis of PIP₂, inhibition of interaction between TRPV1 and calmodulin. Sensitizing effects of bradikinin and nerve growth factor NGF are supposed to be mediated by hydrolysis of PIP₂ which removes inhibition of TRPV1 (Chung et al., 2001, Liu et al., 2005, Prescott & Julius, 2003).

Since some thermal sensitivity can still be observed in TRPV1 knockout mice, other thermal receptors should also be considered (Caterina et al., 2000, Davis et al., 2000). From the six members of the TRPV subfamily, 4 (TRPV1-4) are thermal sensitive ionic channels. Altogether over 30 members of the TRP family is known with explored functions and phylogenetics from *Caenorhabditis elegans* to *Homo sapiens*.

AIMS AND THEMATIC COMPONENTS

The main purpose was exploration of mechanisms to control changes in intracellular concentration of free calcium ions induced by activation of TRPV1 receptors. Pharmacological profile of TRPV1 receptor activation was studied in primary cultures of rat trigeminal ganglion cells, TRPV1 receptor transfected human fibrosarcoma (HT1080) cell line and in a variety of *in vivo* animal models.

The five main subjects are as follows:

- I. *In vitro* and *in vivo* pharmacology of the novel TRPV1 receptor antagonist SB366791
- II. *In vitro* study of effects of N-oleoyldopamine and its analogues
- III. Relative roles of protein kinase A and protein kinase C in modulation of the TRPV1 receptor
- IV. Elevation of intracellular calcium ion concentration after TRPV1 receptor activation under calcium free conditions
- IV. Co-investigation of mitochondrial membrane potential changes and the increase of intracellular calcium concentration induced by activation of TRPV1 receptors

METHODS

***In vitro* studied cells**

Primary cultures of cells isolated from rat trigeminal ganglia (TRG) as described before (Szöke et al., 2000).

Cell line expressing rat TRPV1 receptors (rTRPV1-HT5-1) made from HT1080 fibrosarcoma cells (Sándor et al., 2005).

Microfluorimetry

Measurement of intracellular concentration of free calciumions

Cells were loaded with 1 μ M with fluorescent, calcium indicator fura-2. The measurements were made at room temperature in dark using a small tissue chamber and an Olympus BX50WI upright epifluorescence microscope, x20 water immersed objective, a digital videocamera and computer. Fura-2 was excited with light at 340 and 380 nm and the emitted signal was monitored at 510 nm. The ratiometric fluorescence ($R=340/380$) was calculated on line with Axon Imaging Workbench 2.1 and processed with Microcal Origin 7.0.

Simultaneous measurement of intracellular concentration of free calciumions and relative membrane potential changes of mitochondria

Adaptation of this technique was my own task. Cells were loaded with two dyes, fura-2 (1 μ M) and rhodamin 123 (rhod-123, 26 μ M) for 30 min at 37°C. Three kinds of exciting lights (340, 380 and 488 nm) was used and the emitted light intensity was monitored using Imaging Workbench 4.0. The quenched rhod-123 fluorescence gets more intense when the charged dye accumulated in mitochondria is released back to the cytosol with depolarisation of the mitochondria (Emaus et al., 1986, White & Reynolds, 1995).

⁴⁵Ca²⁺ uptake experiments

I was also involved in adaptation of the technique of Wood et al (1988). One day before the experiment ~ 4000 rTRPV1-HT5-1 cells/well were plated onto 72 well Minitrays plates in 15 μ l medium. Next day the cells were incubated with the desired amount of the examined compound and 200 μ Ci/ml ⁴⁵Ca²⁺ isotope for 10 min at 37 °C. The radioactivity was measured in 2 ml scintillation liquid in a Packard Tri-Carb 2800 TR scintillation counter.

Radioimmune assay of substance P release from isolated rat tracheae

Tracheae from rat were preincubated in Krebs solution for 1 hour at 37°C. The solution was changed 3 times for 8 min (prestimulated-stimulated-poststimulated). Chemical stimulation with capsaicin (10^{-6} M) or electrical field stimulation (40 V, 0.1 ms, 10 Hz, 2 perc; 1200 impulzus) was performed to induce peptide release in the second fraction. SB366791 or the widely used competitive TRPV1 receptor antagonist capsazepine (CZP) was added into the incubation medium at the beginning of each 8 min period. In control experiments stimuli were applied in the absence of the antagonists. Concentrations of substance were determined by specific and sensitive radioimmunoassay (RIA) methods as described before (Németh et al., 1999).

***In vivo* experiments**

Scans of the joint were performed before and up to 35 min following topical application of 100 µg/kg capsaicin (100 µl) onto the joint surface. In other experiments, 20 min prior to capsaicin administration, animals received an intraperitoneal (i.p.) injection of either SB366791 (100 µg/kg or 500 µg/kg) or CZP (2 mg/kg).

Core body temperature of ♂ Wistar rats and Balb/c mice was measured with a rectal thermometer every 10 min for 1 hour. Capsaicin (300 µg/kg) s.c. injection produced a rapid fall in core body temperature. Pretreatment with SB366791 or capsazepine or in the control group with their solvent was performed i.p. 20 min before capsaicin administration. For investigation of the duration of activity, in separate groups of rats 500 µg/kg SB 366791 was injected i.p. 1 h and 2 h before capsaicin injection.

Effect of SB366791 or CZP was also studied upon (10 µg/ml, 50 µl) eyedropped capsaicin evoked wiping reflex (Szolcsányi et al., 1975). The antagonists were given i.p. 20, 60 or 120 min before capsaicin.

The noxious heat threshold of Wistar rats was determined by a computer-controlled increasing-temperature hot plate which has recently been validated (Almási et al., 2003). The animal was placed onto the plate the temperature of which was increased at a rate of 12 °C/min from 30 °C until the animal showed nocifensive behaviour (licking or lifting). Heat

allodynia was evoked by intraplantar (i.pl.) injection of RTX. One half of the group of animals was pretreated with the drug and the other with solvent i.pl. 5 min before RTX application. I.pl. treatment with either enzyme modulators alone failed to induce a significant alteration of the noxious heat threshold as assessed 5, 10, 15 and 20 min after drug administration.

I. IN VITRO AND IN VIVO PHARMACOLOGY OF THE NOVEL TRPV1 RECEPTOR ANTAGONIST SB366791

Introduction

The TRPV1 receptor can be considered as a pivotal molecular integrator of noxious chemical and thermal stimuli in the peripheral terminals of primary afferents involved in nociception and inflammation. Therefore, it has become a promising target for the development of a new generation of anti-inflammatory and analgesic agents (Szallási & Blumberg, 1999; Szolcsányi, 2002). Desensitisation of the C-fibre sensory neurones is the most intensively explored analgesic strategy in many companies (Novartis, Bayer, SmithKline, Johnson & Johnson, etc).

In biological and therapeutical respects the aim of pain relieving is to reduce the responsiveness of nociceptors. There are two possibilities on this point. One is to design a compound that can block the receptor without previous stimulation since absence of excitatory phase (or its low degree) means elimination of painful (burning) sensation. The other possibility is pharmacological inhibition of TRPV1 molecule with selective antagonists. This latter process would have a novel kind of analgetic action.

At the moment very few *in vivo* data are available on TRPV1 receptor antagonists, although there is a great need for potent and selective compounds in both basic research and drug development.

Results

1. In cultured trigeminal ganglion neurons, incubation with SB366791 for 5 min concentration-dependently inhibited the capsaicin-evoked (0.33 μM , 3 sec) Ca^{2+} influx, as measured by microfluorimetry. The inhibitory effect of the 0.5 μM concentration reached the level of significance, while 10 μM almost abolished the response. In the present model the IC_{50} value of this antagonist was 651.9 nM.

2. *In vitro* capsaicin evoked a 2-fold elevation of SP release from the nerve endings of the rat trachea, which was diminished in the presence of 100 and 500 nM SB366791 in a concentration-dependent manner. Electrical field stimulation induced a 3-4-fold increase of the outflow of SP, but this response was not influenced by 500 nM of the compound.

3. *In vivo* topical application of capsaicin caused a significant increase in knee joint blood flow with the maximal effect of the drug occurring 20 min after administration. Examination of the 20 min time point showed that this hyperaemic response to capsaicin was abolished by prior treatment with 500 µg/kg SB366791. Neither 100 µg/kg SB366791 nor CZP (2 mg/kg) had any significant effect on capsaicin responses in the joint.

4. In the hypothermia test, capsaicin-evoked decrease of rectal temperature was 1.68 ± 0.3 °C. Pretreatment with 500 µg/kg i.p. SB366791 20-60 min before capsaicin inhibited hypothermia by 40 %, but its 100 µg/kg dose and 2 mg/kg capsazepine were both ineffective. Neither SB366791 (2 mg/kg, i.p.) nor CZP (5 mg/kg, i.p.) inhibited capsaicin-evoked hypothermia in Balb/c mice.

5. Instillation of capsaicin into the left eye of the rat evoked 16.6 ± 2.13 wiping movements within 3 min. Pretreating the rats with 500 µg/kg i.p. SB366791 reduced the number of wiping movements by 58.9 %, 57.7 % and 35.5 % when administered 20 min, 1 hour or 2 hours before capsaicin instillation, respectively. Neither SB366791 (100 µg/kg) nor CZP (2 mg/kg) influenced this wiping behaviour.

Conclusions

SB366791 inhibited concentration-dependently the capsaicin-evoked release of the sensory neuropeptide SP from isolated rat tracheae and the capsaicin-induced Ca^{2+} influx in TRG cells with 651.9 nM IC_{50} .

Furthermore, we provided the first evidence that SB366791 was also effective *in vivo* in the rat. Capsaicin-evoked wiping movements and knee joint vasodilatation, as well as some fall in body temperature, were diminished by SB366791 in a similar 0.5 mg/kg dose. Capsazepine in the highest dose in which it is considered relatively selective (2 mg/kg) did

not induce significant inhibition in the *in vivo* models. However, neither antagonists could inhibit capsaicin-induced hypothermia in the mouse.

Based on these data SB366791 can be considered a more potent antagonist of the TRPV1 receptor than capsazepine in the rat, therefore it is a suitable antagonist for testing the role of TRPV1 receptors in different experimental conditions.

II. *IN VITRO* STUDY OF EFFECTS OF N-OLEOYLDOPAMINE AND ITS ANALOGUES

Introduction

TRPV1 is an integrator molecule gated by noxious heat, protons, capsaicin, resiniferatoxin and some synthetic vanilloid compounds. The endogenous ligand of the TRPV1 receptor, however, remained enigmatic. Several endogenous lipids including the endocannabinoid anandamide (Zygmunt et al., 1999) and lipoxygenase products like 12-(S)-hydroperoxyeicosatetraenoic acid (12-HPETE; Hwang et al., 2000) and N-arachidonyldopamine (NADA; Huang et al., 2002) have been proposed to serve as endogenous ligands for the TRPV1 capsaicin receptor under painful conditions, but their functional roles in this respect were questioned.

Recently, a novel endogenous lipid isolated from the brain, N-oleoyldopamine (OLDA) was reported to be 50 times more potent on the human TRPV1 receptor than on rat CB1 cannabinoid receptor and to produce heat hyperalgesia (Chu et al., 2003). Therefore, it seemed to be a more promising putative endogenous ligand for the capsaicin receptor.

The aim of the present work was to analyse the effect of OLDA and its analogues (*N-oleoylphenylethylamines*) synthesized by Central Chemical Research Institute of Hungarian Academy of Sciences *in vitro* on rTRPV1-transfected cell line (rTRPV1-HT5-1) and cultured trigeminal (TRG) neurons.

Results

1. In the first series of experiments Ca^{2+} uptake induced by N-oleoylphenylethylamines or capsaicin was analysed at the single cell level by a fluorescent imaging technique using fura-2 loaded TRG and rTRPV1-HT5-1 cells. Capsaicin (0.33 μM), OLDA (3 μM) or 3-MOLDA (10 μM) applied for 10-20 sec both to TRPV1-expressing HT5-1 cells or TRG neurons elicited Ca^{2+} transients, the magnitude of which was decreased upon repeated applications.

Diminishing responses as sign of desensitization were more pronounced in rTRPV1-HT5-1 cells than in TRG neurons. The time course of the effects of OLDA and 3-MOLDA on the TRPV1-transfected cells was slower than that evoked by capsaicin. 4-MOLDA (10 μ M, 20 sec) did not produce Ca-influx in either types of cells.

2. In $^{45}\text{Ca}^{2+}$ isotope uptake experiments OLDA was five times more potent than 3-MOLDA, but 50 times less potent than capsaicin in TRPV1-expressing HT5-1 cells. 4-MOLDA in itself did not cause $^{45}\text{Ca}^{2+}$ accumulation. However, preincubation with various concentrations (0.1-100 μ M) of 4-MOLDA for 15 min inhibited the effect of 100 nM capsaicin on rTRPV1-HT5-1 cells.

Conclusions

We reported that OLDA effectively activates and desensitizes the TRPV1 receptor and suggested that OLDA indeed should be considered as the most promising putative endogenous TRPV1 agonist identified so far. On both cell types examined, OLDA and 3-MOLDA showed slower kinetics of reactions than capsaicin. Thus, in rTRPV1-HT5-1 cells drugs caused more pronounced desensitisation than in TRG neurons. The EC_{50} values of RTX, capsaicin, OLDA and 3-MOLDA was 1.5 nM, 36 nM, 1.8 μ M and 9 μ M, respectively. 4-MOLDA induced calcium-signal in neither tests. However, the calcium accumulation effect of capsaicin was weakly blocked by pre-incubation with 4-MOLDA. From our preliminary experiments the IC_{50} value of 4-MOLDA is about 4 μ M.

Two OLDA-related molecules, the 3-MOLDA and 4-MOLDA differ from each other only in the position of a hydroxyl group being in the third or in the fourth position of the aromatic ring, respectively. One of the most interesting findings was that this difference makes 4-MOLDA an apparent antagonist in contrast to the agonistic effects of 3-MOLDA.

We also suggested that the position of a hydroxyl group in the fourth position of the aromatic moiety of capsaicin-type molecules is particularly important in their agonistic effect on the capsaicin receptor.

III. RELATIVE ROLES OF PROTEIN KINASE A AND PROTEIN KINASE C IN MODULATION OF THE TRPV1 RECEPTOR

Introduction

Like many ion channels, the function of the TRPV1 protein is modulated by phosphorylation/dephosphorylation processes. The function of the TRPV1 capsaicin receptor is subject to modulation by phosphorylation catalyzed by various enzymes including protein kinase C (PKC) and cAMP-dependent protein kinase A (PKA). Facilitation of TRPV1 receptor responsiveness to prostaglandins (PGE₂), anandamide, forskolin and glutamate by PKA activation has been revealed (Gu et al., 2003; De Petrocellis et al., 2001; Rathee et al., 2002; Hu et al., 2002). On the other hand bradykinin, PMA, ATP, nerve growth factor NGF have been shown to enhance the response of the TRPV1 receptor to capsaicin or other stimuli via activation of PKC (Vellani et al., 2001; Sugiura et al., 2002; Numazaki et al., 2002; Shu & Mendell, 2001).

The thermal responsiveness of TRPV1 receptors seems to be principally regulated by phospholipase C and protein kinase-related mechanisms as well (Tominaga et al., 2001; Chuang et al., 2001; Vellani et al., 2001).

The *in vivo* significance of the above findings at the level of peripheral nociceptors remains unknown. Relatively few studies have compared the effects of PKC *versus* PKA stimulation on TRPV1 receptor responsiveness in the same model. Finally, relatively few studies addressed the role of the non-stimulated, basal PKC and PKA activity on the sensitivity of TRPV1 receptor.

The aim of this part of my thesis was to compare systematically the significance of the basal and stimulated activity of PKC and PKA in TRPV1 receptor responsiveness *in vitro* by measurement of the intracellular calcium concentration in cultured TRG neurons and *in vivo* by determination of the behavioural noxious heat threshold in rats.

Results

We used two consecutive applications of a Ca²⁺ transient-inducing agent being either capsaicin (0.33 μM, 3 sec), RTX (1 nM, 7 sec) or high potassium (50 mM, 2 sec) separated by a period of washing with ECS (5 min, control) or drug solution (active treatment).

Upon repeated capsaicin, RTX or KCl exposures after 5 min, tachyphylaxis was observed as the drug evoked a smaller effect that corresponded to 92.6±4.1%, 59.3±6%, 93.8±2.9% of the first one, respectively.

1. Effects of protein kinase inhibitors on the capsaicin-induced Ca²⁺ accumulation

After incubation with *KT5720* (0.2 μM, 5 min), a potent cell-permeable selective inhibitor of PKA the second capsaicin-evoked Ca²⁺ accumulation response was significantly diminished to 33.7±5 % of the first one.

Pretreatment with either *chelerythrine chloride* (*chelerythrine*, 10 μM, 5 min) a selective, cell-permeable inhibitor of PKC, or *staurosporine* (1 μM, 5 min), a rather nonspecific protein kinase inhibitor affecting both PKC and PKA, led to a slight and statistically nonsignificant decrease in the magnitude of the capsaicin-evoked response to 70.9±9.8% and 49.7±7.3%, respectively.

2. Effects of protein kinase inhibitors on RTX-induced Ca²⁺ accumulation

Pretreatment with *KT5720* (0.2 μM, 5 min) reduced the Ca²⁺ accumulation evoked by the second application of RTX to 34.2±2.8 %, its inhibitory effect being significantly higher than that of RTX tachyphylaxis.

Effect of neither *chelerythrine* (10 μM, 5 min; 75.5±20%) nor *staurosporine* (1 μM, 5 min; 54.7±7%) was significantly different from RTX-evoked tachyphylaxis.

3. Effects of protein kinase activators on the capsaicin-induced Ca²⁺ accumulation

Dibutyl cyclic adenosine-5'-monophosphate (*dbcAMP*, 200 μM) an analogue of PKA, applied for 15 s significantly increased the capsaicin-evoked Ca²⁺ transients to 156.7±27.2%.

Phorbol 12-myristate 13-acetate (*PMA*, 1 μM, 10 s) a selective activator of PKC, also induced a significant 148.1±27.4% facilitation of the capsaicin-evoked Ca²⁺ accumulation.

Forskolin (0.1 μM, 30 s) a selective activator of PKA through stimulation of adenylyl cyclase, insignificantly enhanced the capsaicin response augmenting it to 123.8±14.7%.

4. Effects of protein kinase inhibitors and activators on the high potassium-induced Ca²⁺ accumulation

High potassium-elicited Ca²⁺ signals were also examined as a control to determine whether the depolarization-induced and TRPV1 receptor-mediated Ca²⁺ uptake were affected in the same way by protein kinase inhibitors and activators.

Neither *KT5720* nor *chelerythrine*, nor *staurosporine* decreased significantly the KCl-evoked Ca^{2+} response, since it remained 99.9 ± 7.6 %, 91.6 ± 1.9 % and 97.3 ± 2 % of the control value, respectively. Similarly, *dbcAMP* and *PMA* failed to alter significantly the KCl-evoked Ca^{2+} accumulation, their effect being a slight reduction to 88.3 ± 2 % and 90.3 ± 2.7 %, respectively.

5. Effects of protein kinase inhibitors and activators on the RTX-induced drop of the noxious heat threshold

Intraplantar (i.pl.) injection of $0.5 \mu\text{M}$ RTX ($100 \mu\text{l}$) solution resulted in a marked drop of the noxious heat threshold as measured with the increasing-temperature hot plate.

This response was significantly diminished by i.pl. applied *KT5720* ($0.2 \mu\text{M}$, $50 \mu\text{l}$) and *staurosporine* ($1 \mu\text{M}$, $50 \mu\text{l}$) with percentage inhibition values being 62% and 43%, respectively. *Chelerythrine* ($5 \mu\text{M}$, $50 \mu\text{l}$) failed to significantly alter the effect of RTX.

I.pl. *forskolin* ($2.5 \mu\text{M}$, $50 \mu\text{l}$) and *PMA* ($1.6 \mu\text{M}$, $50 \mu\text{l}$) pretreatment significantly facilitated the heat threshold drop evoked by RTX ($0.05 \mu\text{M}$, $100 \mu\text{l}$) resulting in an enhancement to 222% and 218%, respectively.

Conclusions

KT5720 alone, a selective inhibitor of PKA, reduced the calcium transients induced by capsaicin or RTX in trigeminal sensory neurons (TRG). Neither *chelerythrine* nor *staurosporine* managed to alter significantly either of these responses, although the former one inhibited almost completely the effect of *PMA* in the *in vitro* assay. *Staurosporine* might had sufficient inhibitory effect on PKA in cutaneous nociceptors, but only less in trigeminal neurons and that is why it was able to diminish significantly the drop of the noxious heat threshold evoked by intraplantar RTX injection. Activator(s) of PKA and PKC significantly enhanced the effect of capsaicin in the calcium uptake assay and that of RTX in the heat allodynia model.

None of the protein kinase inhibitors or activators altered the calcium transients evoked by high potassium, verifying that their effects are mediated on the level of TRPV1 receptor.

The results provide evidence that basal activity of PKA is involved in the maintenance of TRPV1 receptor responsiveness to capsaicin and RTX both in somata of cultured TRG neurons *in vitro* and in peripheral terminals of dorsal root ganglion neurons *in vivo*, whereas

such a role for PKC is negligible. Upon stimulation above the resting level, however, both PKA and PKC are able to enhance the effects of TRPV1 receptor agonists.

IV. ELEVATION OF INTRACELLULAR CALCIUM ION CONCENTRATION AFTER TRPV1 RECEPTOR ACTIVATION UNDER CALCIUM FREE CONDITIONS

Introduction

Calcium ions pass through TRPV1 channels along their concentration gradient from outside to inside. Raising of free calcium ion concentration in the cytosol is crucially involved in desensitization and tachyphylaxis, which depend on calcium entry (Cholewinski et al., 1993, Garcia-Hirshfield et al., 1995, Koplas et al., 1997, Mandadi et al., 2004).

Others assume that in addition to the plasmamembrane-bound receptors, TRPV1 receptors located in the ER can also release calcium directly from the pool stored in the ER. The increase of calcium ion concentration induced by agonists given in calcium free solutions supports the theory of calcium release from intracellular stores (Olah et al., 2001, Marshall et al., 2003, Eun et al., 2001, Liu et al., 2003).

We examined how the two kinds of cells used in our laboratory respond to capsaicin given in calcium free solution.

Results

Fura-2 microfluorescence changes was measured using 3 kinds of ECS solutions. One contained 1 mM calcium-chloride as usual and served as control. Calcium was omitted from solution type I, while solution type II contained no calcium and had 3 mM EGTA.

Capsaicin (1 μ M, 10 sec) produced significantly larger peak calcium increase in rTRPV-HT5-1 cells ($R=0.62 \pm 0.3$) as compared to TRG neurons ($R=0.377 \pm 0.02$). Proportion of capsaicin sensitive cells was $\sim 70\%$ in both groups (*Fischer test*).

In nominally calcium free bath (solution type I) the capsaicin-induced calcium changes were significantly reduced in both kinds of cells. The peak R dropped to 0.029 ± 0.003 in rTRPV-HT5-1 and to 0.026 ± 0.006 in TRG cells, but the ratio of capsaicin sensitive cells was about half of the control, $\sim 35\%$ in both cell types (*Fischer test*).

Adding EGTA to the bath (solution type II) abolished the capsaicin-evoked responses completely.

Conclusions

Under control condition, 1 μM capsaicin induces near twice as big average R value in transfected cells as in TRG neurones. In nominally calcium free solution (type I) the mean R values remained below 0.03 both kinds of cells, which can well be attributed to contamination of calcium ions in the bath. 1 μM capsaicin plus 3 mM EGTA (type II) did not lead to detectable signals in either cell types. Ratios of apparently capsaicin sensitive cells were similar in the two kinds of cells in all cases.

This observation is in good agreement with previous data obtained on sensory neurons (Cholewinski et al., 1993, Garcia-Hirshfield et al., 1995, Koplas et al., 1997, Mandadi et al., 2004), but contradicts some recent observations where the vanilloid receptor was found on the endoplasmatic reticulum of sensory neurons and was able to release calcium from intracellular stores (Olah et al., 2001, Marshall et al., 2003, Eun et al., 2001, Liu et al., 2003).

Differences in methods, composition of fluids (concentration of EGTA and capsaicin) might explain why we failed to support these latter data. However it seems important that, in contrast with other cell lines, activation of intracellular TRPV1 receptors appears negligible in rTRPV-HT5-1 cells.

V. CO-INVESTIGATION OF MITOCHONDRIAL MEMBRANE POTENTIAL CHANGES AND INCREASE OF INTRACELLULAR CALCIUM CONCENTRATION INDUCED BY ACTIVATION OF TRPV1 RECEPTORS

Introduction

Szöke et al. reported before (2002 *a* and *b*) that newborn rats treated by capsaicin or anandamide lost their nociception in 3 weeks time and no neurogenic inflammation could be induced in them. Later the number of small, type B sensory neurones was decreased and selective swelling of mitochondria was demonstrated.

The prolonged and strong desensitization of polymodal nociceptors may be one of the functional changes associated with the permanent morphological signs of capsaicin treatment, but the casual relationships are unclear.

In order to understand the exact role of mitochondria both in cell physiology and pathophysiology after TRPV1 receptor activation we used simultaneous measurements of capsaicin-evoked (0.33-10 μM) changes in intracellular calcium level and mitochondrial membrane potential in TRG neurons and a TRPV1-transfected cell-line. Simultaneous

measurement of relative mitochondrial membrane potential ($\Delta\Psi_m$) and calcium level ($\Delta[Ca^{2+}]_c$) in the cytosol may reveal functional changes of mitochondria due to activation of TRPV1 receptors. Optical properties of fura-2 and the strongly polar rhod-123 stains allow such kind of study in experiments controlled by Imaging Workbench 4.0. Responses to the protonophore FCCP, which completely dissipates mitochondrial potential, provided references in some experiments.

Results

From point 1. to 3. we deal with signals induced by the first capsaicin application (0.33 or 3.3 μ M, 10 sec). FCCP (5 μ M, 20 sec) was given at 3-5 min after last capsaicin exposures at the end of each experiment to evoke a gradual increase in both signals in cells stimulated previously with capsaicin.

1. Rapid onset of both fura-2 (indicated the $\Delta[Ca^{2+}]_c$) and rhod-123 (indicated the $\Delta\Psi_m$) responses were followed either short or prolonged decay. In average, the fura-2 signal preceded the rhod-123 one by 0.96 sec. The responses were quantified with peak R values and the duration of responses at 50% level of the peak (*halfwidth*). Some responses were *transient* showing less than 200 sec halfwidth others were *sustained* which failed to recover to the 50% level within 200 second.

2. Frequency distribution of halfwidth values on histogram had two peaks both in TRG and in rTRPV1-HT5 cells, independently of the capsaicin concentration. Percent ratio of the transient and sustained responses were similar in the two kinds of cells at 0.33 μ M capsaicin only. In contrast 3.3 μ M capsaicin produced significant differences in the duration of responses observed in the two kinds of cells.

3. Application of capsaicin (for 10 sec) elicited calcium influx (R) more or less associated with loss of $\Delta\Psi_m$ which was compared with responses to the finally applied mitochondrial protonophore, FCCP (5 μ M, 20 sec). Since FCCP could collapse $\Delta\Psi_m$ completely hence FCCP evoked $\Delta\Psi_m$ was considered 100 %.

Capsaicin affected *TRG neurons* in a concentration-dependent manner: the higher concentration was applied the higher R and $\Delta\Psi_m$ were measured. Capsaicin at 3.3 μ M was able to cause almost threefold R and more than twofold $\Delta\Psi_m$ as well. High concentration of

capsaicin (3.3 μM) induced more than three times larger capsaicin/FCCP fura2 ratio than that evoked by 0.33 μM capsaicin (518% versus 166%) in case of sensory neurons. Capsaicin evoked (0.33 and 3.3 μM) fura2 signals in sensory neurons associated with rhod123 waves of about 26 % and 70% of the FCCP evoked, respectively.

Unlike TRG, *in rTRPV1-HT5-1* cells the ratio of capsaicin/FCCP signals could not be calculated, since FCCP did not cause further deltapssim if cells once were exposed by capsaicin beforehand.

4. From point 4. we investigated the parallel changes in both signals in dual stained cells induced by repeated capsaicin (0.33 or 3.3 μM , 10 sec) treatments each followed by 3-5 min washing periods.

In TRG neurons the degree of desensitisation turned significant just at higher concentrations of capsaicin when the second peak is about 30 % and the third is nearly 15 % of the first one. Small amount of capsaicin (0.33 μM) is weakly able to depolarize the mitochondria inner membrane on 3 consecutive applications in sensory neurons.

In contrast to TRG, *in rTRPV1-HT5-1* cells first exposure of 0.33 μM capsaicin proved to be already potent enough to evoke twice as large fura2 and rhod123 signals as that evoked by the highest concentration of capsaicin in cultured trigeminal cells. To our surprise the higher concentrations of capsaicin did cause as large dissipation of $\Delta\Psi_m$, parallel with significantly attenuated calcium influx than induced by 0.33 μM capsaicin. In transfected cells capsaicin induction could only be repeated twice and neither the last (third) application of capsaicin nor the final FCCP test could induce further $\Delta\Psi_m$, as we demonstrated previously.

Conclusions

We were the first to study with paired microfluorescence technique the capsaicin-evoked intracellular calcium and mitochondrial membrane potential changes comparatively in TRG neurons and in TRPV1 receptor transfected cells.

The data reveal strong, but complicated relationships between the peak magnitudes and the durations of the two kinds of fluorescence changes in both cell types. Both transient and sustained components were observed in a large number of cells irrespective of their native or cloned nature, concentration of capsaicin, or sequential number of capsaicin applications. rTRPV1 transfected HT5-1 cells and trigeminal neurons reacted to capsaicin in a fairly similar way, but only when they were exposed to small concentration of capsaicin. There was a trend to find sustained fura2 responses more frequently with higher (μM) concentrations of

capsaicin, or to second, third applications in TRG neurons. However halfwidth values of rTRPV1-HT5-1 cells given at first capsaicin exposure (3.3 μ M) differed significantly from that observed at 0.33 μ M capsaicin and strikingly differed from corresponding measurements in TRG neurons, as well.

Repeated exposures of TRG neurons to capsaicin in the nanomolar range (0.33 μ M) induced desensitization without causing conspicuous changes of mitochondrial membrane potential. Depolarization of the mitochondria follows the enhanced cytosolic free calcium level (the fura-2 signal raised consistently before the rhod-123 signal) and unlike rTRPV1-HT5-1 cells, the mitochondrial depolarizing effect of higher concentration of capsaicin is remarkably increased in TRG cells. In rTRPV1 transfected HT5-1 cells first exposure of 0.33 μ M capsaicin was sufficient to dissipate mitochondrium membrane potential completely. No change in mitochondrial potential was observed in large TRG neurons or human fibrosarcoma cells which were not transfected with rTRPV1.

SUMMARY OF NEW FINDINGS

1. SB366791 acts as a reliable TRPV1 antagonist both *in vivo* and *in vitro*. Therefore, it can be a good starting point in the development of a novel kind of pain reliever.

2. OLDA effectively activates and desensitizes the TRPV1 receptors. Therefore OLDA can serve as an endogenous ligand of TRPV1 receptors. Related molecules like 3-MOLDA and 4-MOLDA act in the opposite ways on the TRPV1 receptors since 4-MOLDA seems to be an antagonist in contrast to the agonist 3-MOLDA. A hydroxyl group on fourth position of the aromatic moiety of vanilloid compounds may be particularly important in the activation of the receptor.

3. Basal activity of PKA, unlike PKC, is involved in the maintenance of resting TRPV1 receptor responsiveness both in somata of trigeminal sensory neurons and in peripheral terminals of dorsal root ganglion neurons. However, stimulation of either PKA or PKC above the resting level can lead to an enhanced TRPV1 receptor function at both sites.

4. rTRPV1-HT5-1 cell line does not respond with intracellular calcium ion level elevation to 1 μ M capsaicin in calcium free extracellular media, similarly to cultured TRG neurons. In our opinion ER membrane contains just a few functioning TRPV1 receptors and those can only be activated with toxically high concentrations of TRPV1 agonists.

5. A direct coupling could be demonstrated between TRPV1 activation and membrane potential changes of mitochondria in two kinds of TRPV1 receptor expressing cells exposed to capsaicin. On the basis of time courses the two cell types behaved rather similarly especially with nanomolar concentration of capsaicin. Depolarization of the mitochondria follows the enhanced cytosolic free calcium level and at higher capsaicin concentrations (at 3.3 μ M) the mitochondrial depolarizing effect of capsaicin is increased in cultured trigeminal neurons, in contrast to TRPV1-transfected HT5-1 cells.

Since no irreversible mitochondrial damage were observed under the present conditions in TRG neurons, we concluded that *in vivo* mitochondrial impairment induced by capsaicin might be a secondary cellular response in trigeminal cells and not its direct effect on the mitochondria.

6. These data indicate that the rTRPV1-HT5-1 cell line can serve as a useful and promising model of capsaicin sensitive sensory neurons even when complex cellular responses like tachyphylaxis, mitochondrial function and calcium homeostasis are involved.

7. Introduction the previously mentioned new methods to the Department of Pharmacology and Pharmacotherapy of the University of Pécs may give good opportunity to improve on some drugs effecting the TRPV1 capsaicin receptor. Results of this thesis mean excellent basis for starting the high throughput screening (HTS) of the potent compounds *in vitro*.

PUBLICATIONS USED AS A BASIS FOR THE PRESENT THESIS

Angelika Varga, József Németh, Árpád Szabó, Jason J. McDougall, Chunfen Zhang, Krisztián Elekes, Erika Pintér, János Szolcsányi, Zsuzsanna Helyes: Effects of the novel TRPV1 receptor antagonist SB366791 *in vitro* and *in vivo* in the rat
Neuroscience Letters **385**, pp. 137-142, 2005 (*IF*: 2.019)

Angelika Varga, Kata Bölcskei, Éva Szőke, Róbert Almási, Gábor Czéh, János Szolcsányi, Gábor Pethő: Relative roles of protein kinase A and protein kinase C in modulation of TRPV1 receptor responsiveness in rat sensory neurons *in vitro* and peripheral nociceptors *in vivo*
Neuroscience 2006 (in print, *IF*: 3.456)

János Szolcsányi, Zoltán Sándor, Gábor Pethő, **Angelika Varga**, Kata Bölcskei, Róbert Almási, Zsuzsanna Riedl, György Hajós, Gábor Czéh: Direct evidence for activation and desensitization of the capsaicin receptor by N-oleoildopamine on TRPV1-transfected cell, line in gene deleted mice and in the rat
Neuroscience Letters **361**, pp. 155-158, 2004 (*IF*: 2.019/2=1.0095)

Zoltán Sándor, **Angelika Varga**, Péter Horváth, Barbara Nagy, Zoltán Szilvássy, János Szolcsányi: Construction and analysis of a new stable cell line expressing the rat TRPV1 receptor
Cellular and Molecular Biology Letters **10**, pp. 499-514, 2005 (*IF*: 0.495)

Angelika Varga, Zoltán Sándor, Zsolt Balla, Gábor Czéh, János Szolcsányi: Relative changes of intracellular calcium level and mitochondrium membrane potential simultaneously induced by capsaicin
(közlésre előkészített kézirat)

Published abstracts related to the thesis: 6

Oral presentations related to the thesis: 9

Posters related to the thesis: 17

Impact factor of publications related to the thesis: 6.9795

PUBLICATIONS NOT RELATED TO THE THESIS

Balázs Jakab, Zsuzsanna Helyes, Kata Bölcskei, **Angelika Varga**, Árpád Szabó, Katalin Sándor, Krisztián Elekes, Rita Börzsei, Erika Pintér, József Németh, János Szolcsányi: Examination of the novel TRPV1 receptor antagonist JYL1421 (SC0030) *in vitro* and *in vivo* in the rat

Eur J Pharmacol. **517**, pp. 35-44. 2005 (*IF*: 2.432)

Rudolf Gesztelyi, Judit Zsuga, Agnes Cseppento, Agnes Bajza, **Angelika Varga**, Judit Szabo, József Szentmiklosi: Special sensitization pattern in adenisine-induced myocardial responses after thyroxine-treatment

J. Pharmacol. Sci. **91**, pp. 295-304, 2003 (*IF*:1.419)

Published abstracts not related to the thesis: 2

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