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Mechanism of capsaicin receptor TRPV1-mediated toxicity in pain-sensing neurons focusing on the effects of Na^+/Ca^{2+} fluxes and the Ca^{2+}-binding protein calretinin ☆

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ABSTRACT

Transient receptor potential vanilloid subtype 1 (TRPV1) receptor is a pain-sensing, ligand-gated, non-selective cation channel expressed in peripheral sensory neurons. Prolonged activation of TRPV1 by capsaicin leads to cell swelling and formation of membrane blebs in rat dorsal root ganglion (DRG) neurons. Similar results were obtained in NIH3T3 fibroblast cells stably expressing TRPV1. Here, we assessed the contribution of Ca^{2+} and Na^+ ions to TRPV1-mediated changes. Cell swelling was caused by a substantial influx of extracellular Na^+ via TRPV1 channels, causing concomitant transport of water. In the absence of extracellular Na^+, the membrane blebbing was completely inhibited, but Ca^{2+} influx did not change under these conditions. Na^+ influx was modulated by the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Elevation of $[Ca^{2+}]_i$ by ionomycin sensitized/activated TRPV1 channels causing cell swelling in TRPV1-positive cells. In the absence of extracellular Ca^{2+}, capsaicin caused only little increase in $[Ca^{2+}]_i$ indicating that the increase in $[Ca^{2+}]_i$ observed after capsaicin application is derived essentially from extracellular Ca^{2+} and not from internal Ca^{2+} stores. In the absence of extracellular Ca^{2+} also the process of cell swelling was considerably slower. Calretinin is a Ca^{2+} buffer protein, which is expressed in a subset of TRPV1-positive neurons. Calretinin decreased the amplitude, but slowed down the decay of Ca^{2+} signals evoked by ionomycin. Cells co-expressing TRPV1 and calretinin were less sensitive to TRPV1-mediated, capsaicin-induced volume increases. In TRPV1-expressing NIH3T3 cells, calretinin decreased the capsaicin-induced Ca^{2+} and Na^+ influx. Swelling and formation of membrane blebs resulted in impaired plasma membrane integrity finally leading to cell death. Our results hint towards a mechanistic explanation for the apoptosis-independent capsaicin-evoked neuronal loss and additionally reveal a protective effect of calretinin; we propose that the Ca^{2+}-buffering capacity of calretinin reduces the susceptibility of calretinin-expressing DRG neurons against cell swelling/death caused by overstimulation of TRPV1 channels. This article is part of a Special Issue entitled:12th European Symposium on Calcium.

1. Introduction

Transient receptor potential vanilloid type 1 (TRPV1) is one of the main pain-sensing receptors in sensory neurons [1,2]. TRPV1 forms a homo-tetramer with six trans-membrane segments per monomer; the N- and C-termini are intracellular. The cation pore is formed by P-loops of the four monomers and the P loop is the region between trans-membrane domains 5 and 6 [3]. When opened, both Na^+ and Ca^{2+} ions enter the cytoplasmic compartment through TRPV1 channels localized in the plasma membrane [4]. Functionally active TRPV1 channels are also present in the ER membrane ($TRPV1_{ER}$), depleting intracellular Ca^{2+} stores upon activation [5,6]. However, the sensitivity of $TRPV1_{ER}$ to exogenous activators is lower than that of the plasma membrane-embedded TRPV1 [7]. The channel can be activated by elevated temperature (>43 °C) [4], both acidic and basic pH [8], or by exo- and endovanilloids such as capsaicin (CAPS), resiniferatoxin (RTX) [4], N-arachidonoyl-dopamin [9] or anandamide [10]. On the contrary, TRPV1 is blocked with the channel blocker, Ruthenium Red (RuRed) [11] and with competitive antagonists such as capsazepine [12]. In the past, TRPV1 expression was assumed to be selective for pain-sensing sensory neurons [13]. However, TRPV1 expression was detected in various organs including brain, kidney, lung, testis, pancreas, spleen, liver, stomach, skin, muscle and in cell lines derived from those [14–16]. Interestingly, TRPV1 overstimulation leading to cytotoxicity was only reported for neurons suggesting that either I) expression levels of TRPV1 in non-neuronal tissues are low, II) neurons are particularly vulnerable or III) the physiological function of the TRPV1 channel is different in various organs [17]. One caveat of the above mentioned studies is that the authors did not consider that the samples used for the identification of TRPV1 might be "contaminated" with neuronal TRPV1-positive C and

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Aδ fibers that enmesh all internal organs. In addition, discordant results were obtained with two probably non-specific antibodies against TRPV1 resulting in different expression patterns, e.g. in the skin [18,19]. In line with the above concerns, Cre-recombinase expression driven by the TRPV1 promoter was mostly restricted to peripheral neurons in transgenic mice [20].

Depending on the concentration applied and the exposure time, TRPV1 agonists cause either desensitization [21] or selective degeneration of a distinct population of primary sensory neurons involved in the mediation of pain [22]. Systemic administration of CAPS results in the death of approximately 50% of sensory neurons in rat neonates [13] and in a loss of approximately 17% in adult rats [23]. RTX, an ultrapotent CAPS analogue, almost completely eliminates TRPV1-expressing afferent neurons in adult rats, when applied systemically [24]. Thus, selective ablation of pain-sensing neurons by administration of TRPV1 agonists may be effective in chronic pain treatment [25]; topical CAPS formulations have already been used for pain management [26]. The intrathecal administration of RTX went into a clinical trial to treat severe cancer-mediated pain [27]. However, the mechanisms by which TRPV1 agonists exert their cyto-/neuro-toxic effects are not fully understood.

Calretinin (CR) is a cytosolic Ca^{2+} buffer protein from the large family of EF-hand proteins, characterized by fast Ca^{2+}-binding kinetics [28] and may have additional functions, possibly as a Ca^{2+} sensor [29]. CR is expressed in specific populations of neurons in the central and peripheral nervous systems [30] and in certain cancer cells [31,32]. In neurons, CR was proposed to be implicated in neuroprotection. CR-expressing cortical neurons are selectively resistant to excitotoxicity *in vitro* [33] and ectopic CR expression delays the onset of cell death in glutamate-sensitive P19 cells [34] and protects N18-RE105 neuroblastoma-retina cells from glutamate-induced excitotoxicity [35]. In this study, we set out to investigate whether CR also has a protective effect against CAPS-induced, TRPV1-mediated "neurotoxicity." Beforehand we elaborated on the mechanisms of CAPS-induced cell death in an *in vitro* model system using TRPV1-expressing fibroblasts (NIH3T3). Activation of the TRPV1 channel led to entry of Ca^{2+} and mostly Na^{+}, the latter causing water influx and associated volume increase in TRPV1-expresing cells. The rapid volume increase caused a proportion of cells to lose plasma membrane integrity and to die by a necrotic, caspase-3/7-independent mechanism. Entry of Ca^{2+} via plasma membrane TRPV1 channels increased the effect on volume increase and cell death and the presence of CR attenuated the CAPS-mediated effects and consequently prevented/delayed cell death.

2. Materials and methods

2.1. Reagents

Thapsigargin, ionomycin (IONO), N-methyl-D-glucamine (NMDG) and rat nerve growth factor were purchased from Sigma Aldrich (St. Louis, MO, USA). Capsaicin (CAPS) and Ruthenium Red (RuRed) were purchased from Tocris Bioscience (Bristol, UK). Fluo-3-AM, FuraRed-AM, calcein-AM, CoroNa-Green-AM and pluronic acid were from Invitrogen (Carlsbad, CA, USA).

2.2. Cell cultures and plasmids

NIH3T3 cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum, 100 U/ml penicillin-streptomycin (PEN-STREP). TRPV1-expressing NIH3T3 cells were a kind gift from Dr. Zoltan Olah, Szeged, BRC. In those cells, the metallothionein promoter is used to drive the expression of full-length rat TRPV1 with a short 12 amino acid ε-tag [5]. This promoter is slightly leaky. Without any induction, it provides a stable, permanent expression of TRPV1 protein [5]. All cultures were maintained in a humidified atmosphere at 37 °C and 5% CO_2. For the generation of a NIH3T3 and TRPV1/NIH3T3 cell line stably expressing a fusion protein termed eGFP-CR consisting of the enhanced green fluorescent protein (eGFP) as the N-terminal part and full-length human calretinin as the C-terminal part, the retroviral expression vector pLVTHM (Addgene plasmid 12247 [36]) was used. The GFP cassette in pLVTHM was replaced with the eGFP-CR cDNA (B. Schwaller and J. Antonov, unpublished). Briefly, the eGFP-CR insert was synthesized by PCR using the primers *PmeI*-eGFP (5′-GTTTAAACCGCCACCATGGTGAGCAAGGGC-3′) and *SpeI*-CR (5′-ACTAGTTTACATGGGGGGCTCGCTGCA3′) using the plasmid pEGFP-CR as template. The PCR fragment was subcloned into pGEM-T-easy (Promega). pGEM-T-easy was digested with *Pme*I and *Spe*I and the PCR amplicon inserted into the unique sites of the pLVTHM to produce the final pLVTH-eGFP-CR. The CMV-mRFP-CR plasmid coding for the monomeric red fluorescent protein (mRFP) was produced by inserting the CR sequence into the pDsRed-Monomer-C1 plasmid (Clontech, Mountain View, CA). Briefly, *Hind*III and *Kpn*I restriction endonuclease sites were incorporated into the CR PCR fragment amplified from pRSV-CR [34] using the forward 5′-GAGAAAGCTTTAGCTGGCCCGCAG CAGC-3′ and reverse 5′-GAGAGGTACCTTACATGGGGGGCTCGCTGCA-3′ primers, respectively. After digesting the PCR fragment with these enzymes, the size-separated DNA insert was ligated in pDsRed-Monomer-C1 at the compatible *Hind*III and *Kpn*I sites. The mRFP-CR-expressing NIH3T3 cells were generated by transient transfection using the TransIT®-2020 transfection reagent (Mirus Bio, Madison, WI, USA) following the manufacturer's instructions. All plasmids were verified by DNA sequencing. Lentivirus was produced by the calcium phosphate transfection method using HEK293T cells and three plasmids: expression plasmid (pLVTHM-eGFP-CR), envelope plasmid pMD2G-VSVG (Addgene plasmid 12259) and packaging plasmid (pCMV-dR8.91), a kind gift from Prof. D. Trono (EPFL, Lausanne). Viral supernatants were collected after 48 h and 72 h, filtered, aliquoted and frozen at −80 °C [37]. After infection of NIH3T3, HeLa or TRPV1/NIH3T3 cells, clones showing high fluorescence intensity, i.e. high protein expression levels, were selected for further experiments.

2.3. Primary DRG cultures

DRG primary cultures were prepared from embryonic rats (E15). Embryos were removed from the uterus and placed in Petri dishes containing Krebs-Ringer buffer (in mM: 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2. $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2 glucose; pH 7.4). The cords were dissected and DRGs were removed. The tissue was digested in 0.05% trypsin at 37 °C for 10 min and dissociated cell cultures were maintained in DMEM containing 5% horse serum and 100 ng/ml nerve growth factor to promote neuronal survival and differentiation. After 2 days *in vitro* (DIV2) primary DRG cultures were analyzed using confocal microscopy (Leica TCS SP5).

2.4. Cobalt histochemistry

CAPS-induced activation of plasmalemmal TRPV1 was assessed by cobalt uptake. Cells seeded on glass coverslips at approximately 50% confluence were washed with physiological salt solution (0.9% NaCl) and then incubated for 10 min in the same solution containing 5 mM $CoCl_2$ and one of the following substances: 1) no additive (control); 2) CAPS (5 μM); 3) IONO (5 μM); 4) CAPS (5 μM) and Ruthenium Red (20 μM). Cells were washed 3-times with physiological salt solution. Cobalt taken up by activated TRPV1 channels was precipitated by adding a 2.5% β-mercaptoethanol solution in physiological salt solution for 5 min. Cells were fixed in 4% paraformaldehyde solution and mounted. Pictures from the slides with cobalt precipitate were taken with a light microscope DM RBE (Leica, Wetzlar, Germany).