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Differential cytotoxicity and intracellular calcium-signalling following activation of the calcium-permeable ion channels TRPV1 and TRPA1

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ABSTRACT

Several members of the transient receptor channel (TRP) family can mediate a calcium-dependent cytotoxicity. In sensory neurons, vanilloids like capsaicin induce neurotoxicity by activating TRPV1. The closely related ion channel TRPA1 is also activated by irritants, but it is unclear if and how TRPA1 mediates cell death. In the present study we explored cytotoxicity and intracellular calcium signalling resulting from activation of TRPV1 and TRPA1, either heterologously expressed in HEK 293 cells or in native mouse dorsal root ganglion (DRG) neurons. While activation of TRPV1 by the vanilloids capsaicin, resiniferatoxin and anandamide results in calcium-dependent cell death, activation by protons and the oxidant chloramine-T failed to reduce cell viability. The TRPA1-agonists acrolein, carvacrol and capsazepine all induced cytotoxicity, but this effect is independent of TRPA1. Activation of both TRPA1 and TRPV1 triggers a strong influx of external calcium, but also a strong calcium-release from intracellular stores most likely including the endoplasmic reticulum (ER). Activation of TRPV1, but not TRPA1 also results in a strong increase of mitochondrial calcium both in HEK 293 cells and mouse DRG neurons. Our data demonstrate that activation of TRPV1, but not TRPA1 mediates a calcium-dependent cell death. While both receptors mediate a release of calcium from intracellular stores, only activation of TRPV1 seems to mediate a robust and probably lethal increase in mitochondrial calcium.

1. Introduction

Thermo-sensitive and ligand-gated TRP receptors are unselective cation channels expressed in several tissues throughout the body. TRPA1 and TRPV1 are two closely related ion channels which are predominantly expressed in nociceptive sensory neurons mediating peripheral pain signalling [1,2]. Both ion channels can be activated by multiple noxious substances as well as by noxious temperatures, e.g. heat (TRPV1, TRPA1) and cold (TRPA1) [1–3]. Emerging evidence suggests that TRPV1 and TRPA1 are also expressed in several non-neuronal tissues, and they seem to play important roles in multiple processes including the regulation of cell viability and intracellular calcium homeostasis [1,4]. Activation of both TRPV1 and TRPA1 mediates a cationic influx through the open channel pores which displays a very high permeability for calcium ions [5,6]. TRPV1 is known to mediate cell death in both neuronal and non-neuronal cells, a property which is utilized when capsaicin is topically applied on the skin in order to alleviate focal neuropathic pain, e.g. inducing an ablation of TRPV1-expressing epidermal nerve fibers [5,7]. Furthermore, TRPV1 expressed in tumour cells, is discussed as a putative target for novel anticancer therapeutics inducing a TRPV1-mediated cell death [8,9]. Cytotoxicity mediated by TRPV1 seems to depend on an increase in intracellular calcium, but it is still unclear which mechanisms downstream of calcium are involved. Some studies suggest an apoptosis-like calcium-dependent, but caspase-independent mechanism [10,11]. Functional TRPV1 channels expressed in the ER were also reported to regulate cell viability by mediating ER stress [12]. TRPV1-mediated cell death also seems to be associated with a calcium-influx followed by dysfunction of mitochondria [13,14], and a recent report demonstrated that the calcium uniporter MCU and sodium/calcium transporter NCLX in the mitochondrial membrane mediate calcium overload and a consecutive cell death upon activation of TRPV1 [15]. In contrast to this quite extensive literature on the mechanisms of TRPV1-mediated cell death, it is less clear how or if at all activation of TRPA1

Abbreviations: DRG, dorsal root ganglion neuron; TRP, transient receptor potential; ER, endoplasmic reticulum; AITC, allyl isothiocyanate; HEK, human embryonic kidney; BCTC, 4-(3-Chloro-2-pyridinyl)-N-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide; BABTA, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis

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regulates cell viability as well. A recent study reported that the highly reactive warfare substance sulfur mustard induces cytotoxicity by activating TRPA1 [16]. TRPA1 was also reported to be crucially involved in ischemia-induced myelin damage by mediating influx of calcium [17], and mice lacking TRPA1 are partially protected from cuprizone-induced demyelination as a model of multiple sclerosis [18]. In sensory neurons however, TRPA1 was not yet reported to mediate neurotoxicity. In contrast, we recently reported that the common TRPV1- and TRPA1-agonist QX-314 induces cytotoxicity when activating TRPV1, but not TRPA1 [19]. Considering the fact that activation of both TRPV1 and TRPA1 results in a rapid and persistent calcium influx, it seems reasonable to assume that they should employ similar mechanisms to mediate a calcium-dependent cytotoxicity. In the present study we therefore aimed to further explore and compare intracellular calcium signalling and cytotoxic properties following activation TRPV1 and TRPA1. We measured changes in cytosolic and mitochondrial calcium levels together with analysis of cytotoxicity following activation of either TRPV1 or TRPA1 channels in HEK 293 cells expressing the human orthologues of TRPV1 and TRPA1 as well as rodent DRG neurons. We show that activation of TRPV1, but not TRPA1 mediates a calcium-dependent cell death. While both receptors mediate a release of calcium from intracellular stores including the ER, only activation of TRPV1 seems to mediate a robust and probably lethal increase in mitochondrial calcium.

2. Methods

2.1. Chemicals

Capsaicin, resiniferatoxin and anandamide (Sigma-Aldrich, Darmstadt, Germany) were dissolved in ethanol to a concentration of 10 mM and stored at −20 °C. AITC, acrolein and carvacrol were diluted in DMSO and stored at −20 °C. BCTC, HC-030031 and A967079 (Biotrend, Cologne, Germany) were dissolved in DMSO to a concentration of 10 mM and stored at 4 °C. zVAD-FMK, BAPTA and BAPTA-AM (all Sigma-Aldrich, Darmstadt, Germany) were freshly diluted to working concentrations immediately before experiments.

2.2. Cell culture

HEK-293 cells with a stable expression of hTRPV1 (a kind gift from Dr. Peter Zygmunt, Lund, Sweden), hTRPA1-HEK-293 cells and HEK–293 t cells were cultured in standard DMEM (D-MEM, Gibco, BRL Life Technologies, Karlsruhe Germany) with 10% FBS (Biochrom, Berlin Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Karlsruhe, Germany) and 2 mM Glutamax (Gibco, Karlsruhe Germany). 5 µg/ml blasticidin (PAA, Pasching, Austria) and 0.35% Zeocin (Invitrogen, Toulouse, France) were added for stable expression of hTRPV1 and for induction tetracycline (Sigma-Aldrich, Taufkirchen, Germany) 0.1 µg/ml was added to the medium 16–24 h prior to experiments. A stable hTRPA1-HEK 293 cell line was established by use of G418 800 µg/ml (both Sigma-Aldrich, Taufkirchen, Germany). Cells were cultivated at 37 °C and 5% CO_2 and prepared in petri dishes (Greiner Bio-One, Frickenhausen, Germany) or 12-well plates (Thermo Fisher Scientific, Schwerte, Germany) 12–24 h prior to the experiments. All experiments were performed in accordance with the requirements of the local authorities (Hannover, Niedersachsen, Germany).

Pretreatment with BAPTA, BAPTA-AM or zVAD FMK was performed for 30 min prior to incubation with cytotoxic substances. Substances for cytotoxicity experiments were added to the medium for 15 min. Supernatant was removed after 15 min and washed twice with PBS. The cell pellet containing non-adherent cells was resuspended with culture medium containing given back to the corresponding well and cells regenerated for 23 h and 45 min. Flow cytometry was performed 24 h after exposure to the respective substance. Due to the small cell count, cell death was assessed by trypan blue staining in DRG neurons.

2.3. Dorsal root ganglion neuron culture

Adult wildtype C57BKL/6 mice or rat were euthanized by cervical dislocation. After surgical preparation and collection of ganglia, they were incubated for 60 min at 37 °C in DMEM (Invitrogen, Darmstadt, Germany) containing 0.6 mg/mL collagenase (type XI) and 3 mg/mL protease (both, Sigma Aldrich, Seelze, Germany). The ganglia were then gently triturated and dissociated neurons were plated in 24 well plates (Thermo Fischer Scientific, Braunschweig, Germany) with poly d-lysine (0.1 mg/mL for 30 min), and cultured (37 °C and 5% CO_2) in serum-free TNB-100 basal medium (Biochrom AG, Berlin, Germany), supplemented with penicillin/streptomycin 100 U/mL and 100 ng/mL nerve growth factor-7S (NGF, Alomone Laboratories, Jerusalem, Israel).

2.4. Flow cytometry

Cell viability was assessed by double staining with propidium iodide (PI) and FITC Annexin V (Annexin V) according to the protocol of the manufacturer (FITC Annexin V Apoptosis Detection Kit 1, BD Pharmingen, Heidelberg, Germany) and as described before [19]. Cells were detached using phosphate buffered saline (PBS) and washed together with the corresponding supernatant. Cell pellets were resuspended in Annexin V binding buffer (1x) and cell count was adjusted to 1×10^6/ml. 100 µl of the cell suspension were transferred into 5 ml polystyrene tubes (BD Pharmingen, Heidelberg, Germany). Staining was performed by adding 5 µl PI and 5 µl Annexin V. After incubation for 15 min at room temperature in the dark, 400 µl binding buffer (BD Pharmingen, Heidelberg, Germany) were added. Specimens were analysed within 1 h after staining by a Cytomics FC500 flow cytometer (Beckmann Coulter, Krefeld, Germany). 10000 cells per sample were measured. Experiments were repeated 3 times. Data was analysed with FlowJo version 10 (FlowJo LCC, Oregon, US). 10000 cells per sample were measured. Experiments were repeated 3 times.

2.5. Trypan blue staining

Cells were collected after exposure to agonists of TRPV1 or TRPA1 with supernatant from the 24 well plates and centrifugated. The supernatant was removed and the pellet was resuspended in 30–50 µl of PBS. 0.4% Trypan blue (Sigma-Aldrich, Seelze, Germany) was added to the cell suspension in a 1:1 ratio and solution was filled into a hemocytometer chamber (Brand, Wertheim, Germany). Following a two minute incubation period, 4 × 1 mm squares were counted within 30 min after staining with trypan blue. Blue stained cells were considered dead and unstained cells were considered viable. For assessment of viability, mean values for all cells and for unstained cells were calculated from the 4 chambers.

2.6. Calcium imaging

HEK 293 cells expressing hTRPV1 or hTRPA1 or DRG neurons of C57Bl/6 mice were cultured on Poly-L-Lysin coated coverslips. Cells were stained by 5 µM fura-2 AM and 0.02% pluronic (both from Biotium Inc., Fremont CA, USA) for about 45 min. Following a 10 min wash out period to allow fura-2-AM deesterification coverslips were mounted on an Axio observer D1 inverse microscope (Zeiss) with a 20 x objective. DRG neurons were constantly superfused with extracellular fluid (in mM: NaCl 145, KCl 5, $CaCl_2$ 1.25, $MgCl_2$ 1, Glucose 10, Hepes 10) using a software controlled 7-channel gravity driven common-outlet superfusion system [1]. Fura-2 was excited at 340 and 380 nm using a microscope light source and an LEP filter wheel (Ludl electronic products Ltd.). Images were exposed for 20 and 10 ms respectively and acquired at a rate of 1 Hz with a CCD camera (Cool SNAP EZ, Photometrics). Data were recorded and further analyzed using VisiView 2.1.1 software (all from Visitron Systems GmbH, Puchheim, Germany). Background was subtracted before calculation of ratios. Averaged