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Activation of the capsaicin-receptor TRPV1 by the acetaminophen metabolite *N*-arachidonoylaminophenol results in cytotoxicity

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ABSTRACT

Aims: The anandamide reuptake inhibitor *N*-arachidonoylaminophenol (AM404) and the reactive substance *N*-acetyl-*p*-benzoquinone imine (NAPQI) are both metabolites of acetaminophen and may contribute to acetaminophen-induced analgesia by acting at TRPV1 expressed in the peripheral or central nervous system. While NAPQI slowly sensitizes and activates TRPV1 by interacting with distinct intracellular cysteine residues, detailed properties of AM404 as an agonist of TRPV1 have not yet been reported on. We explored the effects of AM404 on recombinant human TRPV1 and in rodent dorsal root ganglion (DRG) neurons.

Materials and methods: HEK 293 cells expressing different isoforms of recombinant TRPV1 and rodent DRG neurons were employed for patch clamp and calcium imaging experiments. Cytotoxicity was assessed by propidium iodide and Annexin V staining on TRPV1-HEK 293 cells and with trypan blue staining on DRG neurons.

Key findings: AM404 activates hTRPV1 at concentrations > 1 μM and in a concentration-dependent manner. AM404 also potentiates TRPV1-mediated currents evoked by heat and anandamide. Moreover, AM404-evoked currents are potentiated by NAPQI. While the partly capsaicin-insensitive rabbit (o) TRPV1 fails to respond to AM404, AM404-sensitivity is restored by insertion of the capsaicin binding-domain of rat TRPV1 into oTRPV1. In DRG neurons, AM404-evoked calcium influx as well as cell death is mediated by TRPV1.

Significance: AM404 gates TRPV1 by interacting with the vanilloid-binding site, and TRPV1 is the main receptor for AM404 in DRG neurons. While direct activation of TRPV1 requires high concentrations of AM404, it is possible that synergistic effects of AM404 with further TRPV1-agonists may occur at clinically relevant concentrations.

1. Introduction

Acetaminophen is one of the most frequently used therapeutics world-wide. Although the mechanisms by which acetaminophen induces analgesia have been intensively studied, it is not clear which pharmacological properties are relevant.

The irritant receptor TRPA1 is activated by the reactive acetaminophen-metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), and this effect seems to mediate analgesia, hypothermia and airway inflammation [1–3]. We recently demonstrated that NAPQI also sensitizes and activates the capsaicin receptor TRPV1. However, a role of TRPV1 for acetaminophen-induced analgesia in rodents was previously reported to be due to activation of TRPV1 by AM404 [4,5]. AM404 is primarily known as an anandamide reuptake inhibitor, but also as an active metabolite of acetaminophen being produced in the CNS and as an agonist of TRPV1 [6,7]. Analgesia induced by intracerebroventricularly applied AM404 in mice indeed depends on TRPV1 [5], and acetaminophen-induced analgesia was suggested to be mediated by an inhibition of the calcium channel Cav3.2 resulting from an AM404-induced activation of TRPV1 [4]. Although the agonistic effect of AM404 on TRPV1 seems to of significant relevance for acetaminophen-analgesia, little is known about how AM404 interacts with and gate TRPV1. Except for the first finding from Zygmunt and colleagues demonstrating that 10 μM AM404 activates rat TRPV1 [7], the existing literature on the effects of AM404 at TRPV1 is restricted to a few studies employing calcium imaging techniques to study concentration-dependent activation of TRPV1 [4,7–11]. Furthermore, Roberts and colleagues demonstrated that high concentration of AM404 evokes TRPV1-mediated outward currents in rat DRG neurons [12].

Experimental studies on rodents have identified neurotoxic effects of clinically used dosages of acetaminophen [13]. This neurotoxic property of acetaminophen seems to be due to either induction of apoptosis or by production of reactive oxygen species [14,15]. Activation of TRPV1 was demonstrated to result in cell death in different types of cells [16–23]. While TRPV1 is primarily expressed in peripheral sensory neurons, it also seems to be expressed in restricted areas of the

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CNS [16,24,25]. Taking into regard that AM404 is supposed to activate TRPV1 in the CNS, it is possible that neurotoxicity of acetaminophen involves activation of TRPV1.

The aim of this in vitro study was to more closely explore the effects of AM404 on TRPV1 and to determine whether TRPV1 mediates AM404-induced cell death.

2. Methods

2.1. Cell culture and transfection procedures

HEK 239 cells with inducible hTRPV1 (kindly gifted by Dr. Peter Zygmunt, Lund, Sweden) were cultured in Dulbecco's modified Eagle medium Nutrient mixture F12 (DMEM/F12 Gibco/Invitrogen, Darmstadt, Germany) supplemented with 10% Fetal Bovine Serum (Biochrom, Berlin, Germany), blasticidin and zeocin (both Thermo Fischer Scientific, Braunschweig, Germany) under standard cell culture conditions with 5% CO_2 at 37 °C. When reaching 80% confluence, cells were detached using phosphate buffered saline (PBS, Lonza, Cologne, Germany) and transferred to 12-well cell culture dishes (Thermo Fischer Scientific, Braunschweig, Germany) or coverslips. hTRPV1-expression was induced by stimulation with standard culture medium containing tetracycline for 24 h. For transient expression in HEK 293 cells, cDNA for rat TRPV1 (rTRPV1, a kind gift from Dr. David Julius, San Francisco, CA, U.S.A.), rabbit TRPV1 and the o/rTRPV1-chimera (kind gifts from Dr. Narender Gavva, Amgen Inc., Thousand Oaks, CA, USA), hTRPV1 (kind gift by Dr. Peter Zygmunt, Lund, Sweden) together with 0.5 µg pEGFP (Clonetech, Palo Alto, U.S.A.) were transfected by using a nanofectin transfection kit (PAA, Pasching, Austria). All experiments were performed in accordance with the requirements of the local authorities (Gewerbeaufsicht in Niedersachsen, Germany).

Dorsal root ganglion (DRG) neurons were prepared from neonatal Sprague Dawley rats or adult wildtype C57/BL6 mice. 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) or glass coverslips coated with poly D-lysine (0.1 mg/mL for 30 min). Cells were 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).

2.2. Patch clamp recordings

Whole-cell patch clamp experiments were performed with a HEKA Electronics USB 10 amplifier and the Patchmaster Software (HEKA Electronics, Lambrecht, Germany) as described previously [23]. Patch pipettes were pulled from borosilicate glass pipettes with a resistance of 3–5 MΩ and filled with pipette solution containing [mM]: KCl 140, $MgCl_2$ 2, EGTA 5, HEPES 10 with pH adjusted to 7.4 by KOH. Standard extracellular solution contained [mM]: NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 1.2, HEPES 10 and glucose 10: pH 7.4 adjusted by NaOH. Calcium free extracellular solution contained [mM]: NaCl 140, KCl 5, $MgCl_2$ 2, EGTA 5, HEPES 10 and glucose 10: pH 7.4 adjusted by NaOH. We visualized the transfected cells by EGFP-fluorescence prior to the recording. One cell per dish was recorded and currents were filtered at 2 kHz and sampled at 10 kHz. Cells reaching an initial 1 GΩ seal with < 50 pA leak current at − 60 mV were measured.

The heat stimulus was delivered using a multichannel, gravity-driven system incorporating rapid-feedback temperature control. In this system, a platinum-covered glass capillary, positioned < 100 µm from the cell under study, was used as a common outlet. The Software Fitmaster (HEKA Electronics, Lambrecht, Germany) and Origin software (Origin 7.0273, Origin Lab, Northampton, MA) were used for data analysis.

2.3. Calcium imaging

Cells were pre-incubated with 4 µM Fura-2-AM and 0.02% pluronic. Following wash out to allow Fura-2-AM deesterification, cells were mounted on an inverse microscope with a 20 × objective (Axio observer D1, Zeiss). Cells were superfused with extracellular solution containing [in mM] NaCl 145, KCl 5, $CaCl_2$ 1.25, $MgCl_2$ 1, glucose 10, HEPES 10, pH adjusted to 7.4 using NaOH, using a gravity-driven superfusion system. Fura-2 was excited using a microscope light source and an LEP filter wheel (Ludl electronic products Ltd., Hawthorn, U.S.A.) to switch between 340 and 380 nm wavelengths. Images were exposed for 20 and 40 ms respectively and acquired at a rate of 1 Hz with a CCD camera (Cool SNAP EZ, Photometrics, Tucson, U.S.A.). Data were recorded using VisiView 2.1.1 software (Visitron Systems GmbH, Puchheim, Germany). Background fluorescence was subtracted before calculation of ratios. Test substances were applied at various concentrations for different time spans appropriate to the corresponding experiment. Capsaicin stimuli were used as positive control to identify ion channel-expressing cells. Means (± S.D.) of ratio F340/380 nm for regions of interest are presented. Microsoft Excel 2010 (Microsoft, Redmond, WA, USA), Origin 7.0273 and Origin 8.5.1 (Origin Lab, Northampton, MA, USA) were used for data analysis.

2.4. Flow cytometry

We assessed cell viability 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, Germany) as we described before [23]. After induction of expression of hTPRV1, AM404 was added for 15 min or 24 h. The supernatant was removed after 15 min and cells were washed twice with PBS. The cell pellet containing non-adherent cells was resuspended with culture medium containing tetracycline and given back to the corresponding well. Cells were allowed regenerate for 24 h before flow cytometry was performed. Cells were detached using phosphate buffered saline (PBS) and washed together with the corresponding supernatant. Cell pellets were resuspended in Annexin V binding buffer (1 ×) 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). Data were analysed with Flowjo version 10 (FlowJo LCC, Oregon, US). 10,000 cells per sample were measured. All experiments were repeated three times.

2.5. Trypan blue staining

DRG neurons were collected after 24 h exposure to AM404 with supernatant from the 24 well plates and centrifuged. Supernatant was removed and the pellet was resuspended in 30–50 µl of PBS. 0.4% Trypan blue (Sigma, Seelze, Germany) was added to the cell suspension in a 1:1 ratio, and solution was filled into a haemocytometer chamber (Brand, Wertheim, Germany). After a 2 min incubation period, 4x1mm squares were counted within 30 min after staining with trypan blue. Blue stained cells were considered dead, unstained cells were considered viable. For assessment of viability, mean values for all cells and for unstained cells were calculated from the 4 chambers and subsequent viable.