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Capsaicin modulates acetylcholine release at the myoneural junction

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ABSTRACT

Transient receptor potential (TRP) proteins are non-selective cation channel proteins that are expressed throughout the body. Previous studies demonstrated the expression of TRP Vanilloid 1 (TRPV1), capsaicin (CAP) receptor, in sensory neurons. Recently, we reported TRPV1 expression in mouse motor nerve terminals [MNTs; (Thyagarajan et al., 2009)], where we observed that CAP protected MNTs from botulinum neurotoxin A (BoNT/A). Phrenic nerve diaphragm nerve muscle preparations (NMP) isolated from isoflurane anesthetized adult mice were analyzed for twitch tension, spontaneous (mEPCs) and nerve stimulus evoked (EPCs) acetylcholine release. When acutely applied to isolated NMP, CAP produced a concentration-dependent decline of twitch tension and produced a significant decline in the amplitude of EPCs and quantal content without any effect on the mEPCs. The suppression of nerve stimulus evoked acetylcholine release by CAP was antagonized by capsazepine (CPZ), a TRPV1 antagonist. CAP did not suppress phrenic nerve stimulus evoked acetylcholine release in TRPV1 knockout mice. Also, CAP treatment, *in vitro*, interfered with the localization of adapter protein 2 in cholinergic Neuro 2a cells. Wortmannin, (WMN; non-selective phosphoinositol kinase inhibitor), mimicked the effects of CAP by inhibiting the acetylcholine exocytosis. Our data suggest that TRPV1 proteins expressed at the MNT are coupled to the exo-endocytic mechanisms to regulate neuromuscular functions.

1. Introduction

Transient receptor potential (TRP) vanilloid 1 (TRPV1), also known as capsaicin receptor, is a member of the TRP superfamily. TRPV1 is activated by various exogenous (capsaicin, resiniferatoxin, *etc.*) and endogenous (pH, temperature, endovanilloids, *etc.*) ligands (Caterina et al., 1997). Although reported to be exclusively expressed in the dorsal root ganglion neurons, recent studies have shown a widespread expression of TRPV1 in brain (Toth et al., 2005) spinal cord and in peripheral nociceptive neurons (Cui et al., 2006). At the peripheral terminals of primary nociceptors, TRPV1-mediated Ca^{2+} influx triggers the release of neuropeptides, which contribute to the development of neurogenic inflammation (Caterina and Julius, 2001). Capsaicin (CAP), a pungent principle from hot peppers of the capsicum family and resiniferatoxin are naturally occurring agonists that activate TRPV1 (Holzer, 1991; Szallasi and Blumberg, 1991). CAP activates the TRPV1 ion channel resulting in depolarization and excitation (Marsh et al., 1987) and the effects of CAP are blocked competitively by the antagonist capsazepine [CPZ; (Bevan et al., 1992)].

As early as 1982, the presence of CAP-sensitive afferent fibers in the skeletal muscles was recognized and it was shown that stimulation of these group IV afferents (nonmyelinated C fibers) by CAP caused reflex cardiovascular effects (Kaufman et al., 1982). At the frog neuromuscular junction, it was shown that cannabinoid and vanilloid receptor agonist arachidonyl-2-chloroethylamide (ACEA) and CAP increased the quantal content of stimulus evoked acetylcholine release and this effect was blocked by CPZ (Silveira et al., 2010). In a recent study, expression of TRPV1 receptor was demonstrated for the mouse MNT and that the receptor was sensitive to CAP (Thyagarajan et al., 2009). Although the existence of TRPV1 receptors in the mammalian motor nerve terminal (MNT) has been shown, the role it plays in the physiology of acetylcholine release has not been investigated. This work fills this knowledge gap by investigating the effect of CAP on the mouse MNT and analyzes the role of TRPV1 activation in the modulation of acetylcholine release at the MNT.

Abbreviations: AP-2, adapter protein 2; TRP, transient receptor potential; TRPV1, transient receptor potential vanilloid 1; $TRPV1^{-/-}$, TRPV1 knockout; MNT, motor nerve terminal; BoNT/A, botulinum neurotoxin type A; HRS, HEPES Ringer Solution; mEPC, miniature endplate currents; EPC, endplate current; CAP, capsaicin; CPZ, capsazepine; EDL, extensor digitorum longus; NMP, nerve muscle preparation; WMN, wortmannin.

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2. Material and methods

2.1. Animals

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee. Wild type C57BL/6 and TRPV1$^{-/-}$ mice (B6.129X1-*Trpv1*^{tm1Jul}/J) were obtained from Jackson Laboratories, CT, USA. Animals were housed in the research animal facility located in the School of Pharmacy and were given food and water *ad libitum*.

2.2. Muscle twitch tension

Diaphragm nerve muscle preparations (NMP) were isolated from adult male wild type or TRPV1 knockout (TRPV1$^{-/-}$) mice anesthetized with isoflurane. After assuring full anesthesia, the mice were killed by cervical dislocation. The procedure for removal of diaphragm muscle with the phrenic nerve, mounting in the tissue bath and recording nerve-elicited muscle twitches was according to that described earlier (Thyagarajan et al., 2009, 2010). The diaphragm with attached phrenic nerve was mounted in a glass chamber (Rodnoti Glass Technology, Inc., USA) filled with oxygenated (95% O_2–5% CO_2) normal Ringer solution (physiological solution; pH 7.4, 37 °C) containing (mM) NaCl (135), KCl (5), $MgCl_2$ (1), $CaCl_2$ (2), Na_2HPO_4 (1), $NaHCO_3$ (15) and glucose (5.5). The phrenic nerve was drawn into a suction electrode for indirect activation (1 Hz) of muscle twitches. One tendon of the muscle was tied to a Grass force transducer connected to a Digidata 1440 A (Molecular Devices Inc., USA) for acquisition of the mechanical response (grams of tension developed) in response to the nerve stimulation. Muscles were stretched to optimal length for force generation and equilibrated for 30 min prior to nerve stimulation at 1 Hz for data acquisition. CAP and CPZ were added to the physiological solution and concentrations were expressed as final bath concentration (μM). Data collected at 120 min period following the addition of different cumulative concentrations CAP was analyzed with pCLAMP 10 software. In experiment where CAP dose response was studied, no more than two concentrations of CAP were studied in one experiment ($n=4$ diaphragms (8 hemidiaphragms) obtained from 4 mice for each experiment).

2.3. EPC and mEPC measurement using two electrode voltage clamp

Diaphragm NMP were dissected from isoflurane-anesthetized mice after cervical dislocation as described above. The muscles were pinned to a Sylgard-lined plexiglass chamber, and bathed in HEPES Ringer Solution (HRS; 22–25 °C) containing (mM): NaCl (135), KCl (5), $MgCl_2$ (1), $CaCl_2$ (2), Na_2HPO_4 (1), HEPES (10, pH 7.4) and glucose (5.5). Spontaneous transmitter release [miniature endplate currents (mEPCs)] and nerve stimulus evoked endplate currents (EPCs) were recorded from the endplate region (−75 mV holding potential) using two-electrode voltage clamp technique as described previously (Thyagarajan et al., 2009, 2010). Two electrodes were inserted into the endplate membrane at interelectrode distance of approximately 50 μm. One electrode filled with 4 M potassium acetate served to deliver current while the other electrode filled with 3 M KCl recorded membrane voltage. The phrenic nerve was drawn into a suction electrode and stimulated supramaximally at 1 Hz or 20 Hz. The recordings of mEPCs and EPCs were obtained using Axoclamp 900A (Molecular Devices, Sunnyvale, CA) and analyzed offline for the EPC amplitude and mEPC amplitude and frequency. Prior to EPC recordings, 0.75 μM Conotoxin GIIIB was added to the HRS solution to block muscle contractions during the EPC recordings. Control recordings were made before application of CAP or CPZ. Collected data were analyzed with pCLAMP 10 software. Quantal content was calculated as ratio of the mean EPC amplitude to mean mEPC amplitude and represented as mean ± S.E.M.

2.4. Cell culture and transfection

Mouse cholinergic neuroblastoma (Neuro 2a) cells were cultured in Dulbecco's modified Eagle's F12 medium, pH 7.4, supplemented with 10% fetal bovine serum and antibiotics. Human embryonic kidney 293 (HEK293) cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. The rat TRPV1 tagged with the *myc* epitope on the N terminus in pCDNA3 vector was transfected using the Effectene reagent (Qiagen, Chatsworth, CA) in HEK293 cells or Neuro 2a cells. Transfected cells were exposed to Dulbecco's modified Eagle's medium (DMEM) containing 1 mg/ml G418 on day 3 and stable clones were obtained on day 21. Cells that stably expressed TRPV1 were maintained in G410 (250 μg/ml) and the medium was changed to normal DMEM (no G418) 48 h before the experiments.

2.5. Confocal microscopy and immunoblotting

In order to detect if TRPV1 is expressed at the mouse MNT, immunoblotting was performed on the phrenic nerve diaphragm NMP. HEK293 cells expressing TRPV1 channel protein and wild type HEK293 cells served as positive and negative controls, respectively. Diaphragm NMPs were used for immunoblotting experiments to detect fendogenous TRPV1 expression. Endplate-rich regions from the muscle NMP were stained for acetylcholine esterase (AChE) by a previously described method (Koelle and Horn, 1968) and modified by Gautron (Gautron, 1982). Acetylthiocholine was the substrate for the staining reaction. AChE labeling was performed at room temperature. After a 30 min reaction period, NMPs were washed with HRS. Endplate regions were cut under a stereo zoom microscope and flash frozen in liquid nitrogen. Tissues were homogenized in phosphate-buffered saline (PBS) containing 1% Nonidet 40 and complete protease inhibitor cocktail (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The homogenate was then centrifuged at 20,817*g* for 10 min at 4 °C, The crude lysate (100 μg) was resolved *via* SDS-polyacrylamide gel electrophoresis after boiling in 1× Laemmli. HEK293 cells that stably express TRPV1 were homogenized and resolved by SDS-PAGE similarly.

To demonstrate that CAP pretreatment modifies the subcellular distribution of AP-2 (adapter protein-2), TRPV1 stably expressing Neuro 2a cells were used. Two groups of TRPV1 expressing Neuro 2a cells were seeded on poly (L-lysine)-coated cover slips. The experimental group was incubated (at 37 °C) in HRS containing 1 μM CAP (see Fig. 6 legend) for 60 min and washed. The control group was incubated at 37 °C with vehicle only. After washing the coverslips with HEPES, cells were immunostained with AP-2-μ1 antibody (Santa Cruz Biotech, Inc, USA) followed by FITC (secondary antibody) and mounted with Vectashield on a slide and kept frozen at −20 °C before imaging with an LSM 710 confocal microscope. Images were saved and represented as TIFF files and analyzed with Image J software (National Institutes of Health, Bethesda, MD).

2.6. Chemicals and drugs

μ-Conotoxin GIIIB was obtained from Alamone Labs, Israel. CAP, capsazepine, wortmannin, and all other chemicals and drugs were obtained from Sigma, USA.