The Vanilloid Receptor: Localization, Function, and Its Potential as a New Target for the Treatment of Pain

Summary of PhD Thesis

László J. Kárai, M.D.

Department of Neurology
Faculty of Medicine, University of Szeged
Hungary
2006

INTRODUCTION

The vanilloid receptor 1 (TRPV1, VR1) is a ligand-gated, cation channel that is activated by heat, acid and vanilloids, such as capsaicin, a principal ingredient in hot peppers. The vanilloid receptor belongs to the transient receptor potential (TRP) channel superfamily of ion channels and is expressed almost exclusively in a subset of sensory neurons in the dorsal root and trigeminal ganglia that belong mainly to the C and A∂-type of primary afferent nociceptive fibers. These fibers create a dense three-dimensional meshwork in our body, which instantaneously notifies us about harmful stimuli, but can also cripple those of us who suffer from chronic inflammatory conditions, neuropathic or malignant metastatic disease associated pain. There are reports on the expression of TRPV1 in the central nervous system and at peripheral sites such as the transitional epithelium of the urinary bladder, or keratinocytes, but the presence or the function of the receptor at those sites are not well characterized. In the sensory ganglia, TRPV1 positive neurons are responsible for the transduction of noxious stimuli such as heat, acidic pH, vanilloid agonists and also inflammation and the transmission of associated action potentials to the spinal cord, as confirmed by experiments on the TRPV1 gene knockout mouse. Recent evidence suggests posttranslational modification of the receptor, mainly by protein kinases C and/or A. These modifications sensitize the receptor, leading to increased calcium flux through the plasma membrane of nociceptive sensory neurons. The original electrophysiological findings localized the receptor to the cell membrane, new studies, however, reveal a second population of intracellularly localized receptors. The potential function of the intracellular TRPV1, such as at the endoplasmic reticulum (ER), has important implications for the precise regulation of free intracellular calcium. The regulation of this divalent cation plays a pivotal role in protecting the cell body and the peripheral ending from overactivation and calcium cytotoxicity.

Current pain therapies rely on non-steroidal anti inflammatory drugs (NSAIDs), morphine and morphine analogs, local or generalized anesthetics. Studies with the ultrapotent TRPV1 agonist resiniferatoxin (RTX) offer a new method for management of severe pain. Pain-sensing neurons and nerve endings can be incapacitated by overactivation of the TRPV1 receptor. During this process, toxic amounts of extracellular calcium enter the cell, incapacitating cell functions and eventually leading to the reversible destruction of the nerve ending or elimination of the neuron. Experiments with the cloned TRPV1 receptor in cell

lines heterologously expressing TRPV1 and in dorsal root ganglion cultures provided a new level of understanding of how the receptor mediated cell deletion is working and can be used as the basis for new treatment strategies aimed at pain relief. Due to the specific expression pattern of the vanilloid receptor among dorsal root ganglion sensory neurons, resiniferatoxin treatment is highly selective. Peripheral administration of the ultrapontent vanilloid agonist RTX has a reversible, transient (up to three weeks), but profound effect on pain sensation. Intraganglionic or intrathecal applications which place the drug close to the neuronal perikarya, causes irreversible changes leading to deletion of vanilloid-sensitive neurons and providing long lasting and effective pain therapy. Proprioception or epicritic sensations remain intact, as neurons responsible for these sensations do not express the receptor.

Aims of the thesis

The thesis has two main parts. The first describes the basic cellular and molecular events occurring during activation of the vanilloid receptor. The second part focuses on possible therapeutic exploitation of the restricted and specific expression pattern of TRPV1 in different pain-associated conditions. Severe pain is still managed by opioids, sodium channel blockers or non-selective neuroablative treatments. Although, they can provide relief, their serious side effects, or *non-selective* complications, such as tolerance, impaired consciousness, and compromised motor, bladder or bowel function, can limit their effective use and erode quality of life. TRPV1 is one of the most important transducers and integrators of nociceptive signaling and understanding its molecular science can provide better pain-treatment options that preserve epicritic and kinesthetic sensations, motor control and consciousness.

METHODS

Molecular Studies, Electrophysiology and Imaging Methods

To obtain TRPV1-specific mRNA, 100 DRGs were rapidly removed from 12 adult Harlan Sprague-Dawley rats. Total RNA was isolated with the TRI REAGENT (Molecular Research Center Inc., Cincinnati, OH). A fragment, comprising the sequence between the XbaI and AflIII sites of rat TRPV1, was amplified first by the Access RT-PCR system (Promega) and then cloned into the BlueScript vector (Stratagene). The missing 5'- sequence was added likewise with the SacI and XbaI sites. At the 5' ends of the N- and C-terminal fragments, the SacI and AflIII sites were incorporated with forward primers AGATCTCGAGCTCAAATGGAACAACGGGCTAGCTTAGACTC and CTGTATTCCACATGTCTGGAGCTGTTCAAGTTC, respectively. As reverse primers

ACTGAGTCCCGGGCGCTGATGTCTGCAGGCT and ACACAGTCGACTTTCTCCCCTGGGACCATGGAATCCTT were used, in which the XbaI and SalI sites were incorporated, respectively. The SacI-AflIII sites and the RT-PCR generated AflIII-SalI fragments were triple-ligated into a SacI and SalI cut pEGFP-N3 vector (CLONTECH). The immediate early promoter of the cytomegalovirus in the pEGFP-N3 vector was employed to produce the full-length TRPV1 with the eGFP tag. Rat TRPV1 with the short, 12-amino acid ε-tag (KGFSYFGEDLMP) was constructed in a vector, pεMTH, driven by the metallothionein promoter. The basal activity of the pεMTH promoter was used in NIH 3T3 cells to produce a TRPV1-εMTH cell line, yet prevent toxicity from long term, high level expression of TRPV1.

For patch clamp studies, TRPV1eGFP-expressing COS7 and HEK293 cells were voltage-clamped in Krebs buffer containing (in mM) NaCl (124), KCl (4.9), KH_2PO_4 (1.2), $MgSO_4$ (2.4), $CaCl_2$ (2.5), $NaHCO_3$ (25.6), and glucose (10), using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Recordings were carried out with patch electrodes (2–10 MV) filled with 10 mM HEPES buffer (pH7.4) containing (in mM) CsCl (120), tetraethylammonium chloride (20), $CaCl_2$ (1), $MgCl_2$ (2), EGTA (10), ATP (4), and GTP (0.5).

COS7, NIH 3T3, and HEK293 cells were seeded on 25-mm coverslips and transfected with 1 μg each of the plasmid constructs, cultured for 24 h post-transfection at 35 °C, then mounted in a 1-ml chamber and examined with a MRC-1024 Bio-Rad confocal microscope. To study the two- and three-dimensional distribution of fluorescent chimeric proteins, each *x*-*y* plane was scanned over 1 s and at 0.2-μm increments in the *z* axis mode. To label different subcellular compartments of live cells fluorescently, the ER marker eGFP-KDEL (CLONTECH) was transiently transfected in COS7 and NIH 3T3 cells or the ER tracker Blue-White vital dye was used (Molecular Probes). To label mitochondria, MitoTracker Red was employed (Molecular Probes). The dye was incubated for 30 min at a 250 nM concentration; the cells were then washed with HBSS supplemented with 1 mM $CaCl_2$ and 0.8 mM $MgCl_2$, buffered with 15 mM HEPES (pH 7.4). Loading of human or rat DRG neurons for calcium imaging with Indo 1-AM or Fluo-4 AM was performed similarly. Labeled cells were imaged with a MRC-1024 Bio-Rad confocal system or with an upright microscope (Olympus BX60) illuminated with an arc lamp. The fluorescent signal was intensified (Model KS-1380, Videoscope International, Washington, D. C.) before capture by a CCD camera (Pulnix, Sunnyvale, CA). Images were digitized and integrated (2 frames/image) on a Macintosh

computer running Synapse 3.7, an image acquisition and analysis program (Synergy Research, Silver Spring, MD). Neurons were outlined, and fluorescent intensities were analyzed. Fluorescence intensity values in the non-zero pixels within each slice were averaged (F) and plotted as normalized fluorescence intensities ($\delta F/F0$) against time. Analyses of the traces and area measurements were performed by the Kaleidograph software (Synergy Software, Reading, PA). The Indo-1 imaging was performed by the MRC-1024 Bio-Rad confocal system. To quantitate the fluorescence ratio, perikarya of the cells were marked with the graphic tools of the LaserSharp software in the field of a 40x objective of the Bio-Rad confocal system. Ratiometric imaging was performed at 10-s intervals with an UV laser, and the ratio of fluorescence intensity emitted at 405 and 485 was calculated.

Primary DRG neuron cultures

DRG neuron-enriched cultures were prepared from embryonic rats (E16). Human DRG tissue was obtained from Advanced Bioscience Resources Inc. (Alameda, California, USA). Ganglia were collected in cold Lebowitz medium (Invitrogen Corp., Carlsbad, California, USA). After digestion with 0.125% trypsin at 37°C for 25 minutes, cells were seeded on coverslips coated with poly-D-lysine. Culture medium contained DMEM with 20 mM HEPES, 7.5% FBS, 7.5% horse serum, and 50 μg/ml nerve growth factor. After the first day, 5 mg/ml uridine, 2 mg/ml FUDR (Sigma-Aldrich, St. Louis, Missouri, USA) was added to inhibit non-neuronal cell division.

Histology and Immunohistochemistry

Human and rat dorsal root ganglia were fixed in S.T.F. (Streck Laboratories, INC. Omaha, NE) for 24 hours and paraffin processed. Five μm sections were stained with H&E or used for immunohistochemistry. Primary antibodies used were anti-rat and anti-human TRPV1, (1:1,000) (Affinity Bioreagents), CGRP (1:2,500) and mouse monoclonal N52 (1:2,000) (Chemicon International). In short, following deparaffinization, sections were blocked with 10% normal goat serum (S-1000, Vector Laboratories, Inc., Burlingame, CA). Epitope unmasking was performed with Target Retrieval Solution (S1700, Dako, Carpinteria, CA) at 95˚C for 20 minutes. Visualization of the immunocomplexes was done by the Vectastain Elite Rabbit IgG and the Peroxidase Substrate Kits (PK-6101 and SK-4100, Vector Laboratories, Inc., CA). Control sections for assessment of non-specific binding were processed in an identical way except for omission of the primary or secondary antibodies.

In vivo experiments

Procedures for rat studies followed the NIH Guidelines for the Care and Use of Laboratory Animals, and were approved by the National Institute of Dental and Craniofacial Research Animal Care and Use Committee. A protocol to treat dogs with RTX was approved by the University of Pennsylvania School of Veterinary Medicine Institutional Animal Care and Use Committee prior to the start of the study.

RTX injections and sensory testing in rats

For intraplantar RTX injection, unanesthetized adult male Sprague–Dawley rats (200–400 g) were gently restrained and RTX (0.0625–10.0 μg, LC Laboratories) or vehicle (0.25% Tween 80 in phosphate buffered saline, 0.05% ascorbic acid) was injected (i.pl., 100 μl) into the mid-ventral surface of the hindpaw.

The trigeminal microinjection was performed in male Sprague-Dawley rats (300 g, n = 54). Rats were anesthetized with ketamine/xylazine and mounted in a stereotaxic frame. A 10-μl Hamilton syringe (Hamilton Company, Reno, Nevada, USA) was positioned at 2.5 mm posterior and 1.5 mm lateral to the bregma. The skull was drilled, and the needle was advanced until it gently touched the base of the skull; the needle was retracted 0.1 mm, and RTX (20 or 200 ng) was injected in a volume of 2 μl over 10 minutes. A pharmacological-grade preparation of RTX was formulated as follows: PBS, pH 7.2, containing 0.1 μg/μl RTX, 0.05% ascorbic acid, and 7% Tween 80. Vehicle contained no RTX. The Pharmaceutical Development Section, Clinical Center, NIH, performed characterization of this preparation.

For the intrathecal injection rats (n = 58) were anesthetized with isoflurane, and RTX (10–200 ng in 30 μl over 10 minutes) or vehicle was administered by inserting a 20-gauge guide catheter into the L4/L5 interspace. A PE-10 catheter was advanced 1 cm into the intrathecal space. The criterion for the intrathecal placement was withdrawal of clear cerebrospinal fluid.

The sensory tests of the trigeminal nerve were performed in 30 rats after TG microinjection of RTX using the capsaicin-induced eye-wipe response. Capsaicin solution (0.01%), with saline (50 μl) was dropped into the cornea, and eye wipes were counted for 1 minute. Efferent functions of the trigeminal nerve were tested in rats (n = 7) at 7 days after injection. Rats were anesthetized with ketamine/xylazine and hair removed with Nair (Carter-Wallace Inc., New York, New York, USA). After washing thoroughly with water, 5% CAP cream, containing 0.05% ascorbic acid, was applied evenly to the entire head and shoulder

region. After 15 minutes, the left femoral vein was catheterized and a 0.5-ml bolus of Evans Blue (Sigma-Aldrich) (20 mg/kg in saline) was injected. Radiant-heat thermal testing was performed on unrestrained rats (n =58), the endpoint recorded was latency (seconds) to paw withdrawal. Thermal intensity was set to produce a baseline withdrawal latency of 8–10 seconds. A 10-second withdrawal latency corresponds to a temperature at the surface of the paw of 45.2°C. Mechanical sensory stimulation included a pinch with toothed forceps, the application of graded von Frey filaments, and assessment of pinprick hyperalgesia. Assessment of locomotion and proprioception was performed in the intrathecally injected (vehicle and RTX) rats on an accelerating Rota-Rod (4–40 rpm in 5 min; model 7750; Ugo Basile Biological Research Apparatus, Comerio, Italy).

Intrathecal RTX injections in dogs

Dogs were maintained under general anesthesia with isoflurane and oxygen. The cisterna magna was penetrated with a 20-gauge 4-cm spinal needle. When the needle is properly placed, the cerebrospinal fluid flows freely. A single dose of 1 μg/kg of RTX was injected over 10 minutes, followed by 0.2 ml of sterile saline; any arousing effects were controlled with intravenous fentanyl. The effectiveness of intrathecal RTX injection on noxious thermal pain sensation as well as pain associated with cancer and arthritis were tested. The changes in pain related behaviour in dogs suffering from cancer and arthritis were scored by the owners with the Visual Analogue System (VAS), with zero designated as no pain and 100 the worst pain imaginable.

RESULTS AND DISCUSSION

Molecular and Imaging Data

The vanilloid receptor belongs to the Transient Receptor Potential family of ion channels (TRP). It has six transmembrane domains, an intracellular N-terminal portion with multiple (3) ankyrin repeats and an intracellular C-terminal domain. The functional channel is formed as a tetramer with other TRPV1 molecules. Plasmid constructs were generated from rat dorsal root ganglia using the ε epitope of PKC epsilon (εMTH) and the enhanced green fluorescent protein (eGFP). These constructs show the same biological and electrophysiological characteristics as the wild type receptor. The eGFP labeled TRPV1 was essential in the confocal studies to decipher the localization of the vanilloid receptor and to understand the cellular processess taking place during activation with vanilloid agonists.

Direct visualization of TRPV1-eGFP disclosed sub-cellular localization to the plasma membrane ($TRPV1_{PM}$) including the microvilli, and to the endoplasmic reticulum ($TRPV1_{ER}$) as confirmed by overlap with the vital ER-Tracker dye and studies with the KDEL-eGFP plasmid. Upon activation with the potent vanilloid agonist resiniferatoxin (RTX) we observed an abrupt vesiculation of the plasma and ER membranes together with a change in shape of the mitochondria, which "rounded up" into spherical particles in contrast to their normal filiform contour. The fragmentation of intracellular organelles coincided with the sudden elevation of intracellular calcium levels ($[Ca^{2+}]_i$) observed during Fluo-4 and Indo-1 imaging experiments. This substantial and prolonged increase in $[Ca^{2+}]_i$ led to calcium cytotoxicity and ultimately to the death of the cells as confirmed by nuclear propidium iodide uptake by vanilloid sensitive cells and neurons.

Although, the ER localization of TRPV1 may not be surprising, due to protein synthesis in general, finding functionally active receptor at this site intrigued us. Ca^{2+}-imaging studies in “zero” ($<10^{-7}$ M) extracellular calcium levels together with blockade of the plasma membrane localized receptor ($TRPV1_{PM}$) by ruthenium red (RR) revealed calcium fluxes originating from intracellular stores in vanilloid sensitive DRG neurons upon administration of RTX. This vanilloid regulated pool almost entirely overlaps with the thapsigargin (TG) sensitive intracellular calcium store which functionally defines the ER pool. The ER-localized TRPV1 pool ($TRPV1_{ER}$) was evident along the neuronal axonal and dendritic arborizations. Total depletion of this store with RTX compromises ER functions and terminates in cell death in vanilloid-sensitive neurons. Signalling through $TRPV1_{PM}$, and $TRPV1_{ER}$ can regulate meticulously the Ca^{2+}-homeostasis within the nociceptive neurons including the peripheral endings. This fine regulation is of paramount importance since over-activation, as in a chronic pain state, can lead to calcium overload and result in peripheral nerve terminal inactivation. These experiments also highlight the critical role of the ER during vanilloid agonist treatment and provide further evidence to Berridge’s “neuron-in-neuron” concept, which confers the ER with different and crucial signalling functions in addition to its role in protein synthesis.

RTX induced reversible and irreversible analgesia, “molecular neurosurgery”

The aforementioned basic cellular and molecular studies suggested a novel approach for therapeutic intervention in patients with intractable pain problems. By exploiting the specific expression of TRPV1 in nociceptive neurons and their selective susceptibility to vanilloid agonists, especially the ultrapotent RTX, it was possible to literally kill pain-sensing primary neurons yet leave other somatosensory neurons intact. Vanilloid-sensitive cells

respond to RTX application with an initial Ca^{2+}-signal followed by neuronal death. The treatment is selective; only the vanilloid-sensitive neurons are eliminated. On the basis of these cellular and in vitro findings we developed a new model system in rats and dogs applicable to intractable pain in humans. The intervention according to the clinical scenario can be reversible or irreversible and be applied in an anatomically targeted way. If RTX is injected to the periphery it leads to *transient* nerve terminal inactivation, which is followed by regeneration of the fibers and return of the sensation in approximately three weeks. *Irreversible* effect (permanent analgesia) can be obtained by intraganglionic or intrathecal administration of RTX (close to the neuronal cell body), which results in selective neuronal death. Other sensory modalities (touch, pinprick, high-threshold heat) together with locomotion, tested on an accelerating Rota-Rod, remained intact. The histological and immunohistochemical results from the treated tissues correleted well with the behavioral data: selective damage of peripheral nerve fibers or selective destruction of a subpopulation of dorsal root ganglion neurons were observed. TRPV1 negative (large, myelinated) neurons remained intact even long after the treatment (more than a year). Both peripheral and central administration of the drug were remarkably effective in the carrageenan-induced inflammatory hyperalgesia model. These findings strongly correlate with the data obtained from the TRPV1-knockout mice. The treatment can be targeted to the desired anatomical area and it remained localized as was experienced during the intraganglionic and intrathecal injections. The canine model provides a transitional bridge between non-clinical rodent data and eventual human trials, and reinforces the idea that the treatment will be safe and effective. The selectivity of RTX in live-cell imaging of human DRG neurons provides a second type of translational observation between basic in vitro observations and human clinical application.

Taken together, these studies represent a progressive series of steps from investigations of a heterologously expressed cloned ion channel, through rat DRG neurons, to experimental rodent and clinical veterinary models, to human cells, that explore the clinical potential of selective nociceptive neuronal deletion for pain control. The cell deletion approach may provide an important alternative to present treatments, and its eventual use will help to improve the quality of life in patients with unrelenting pain.

THE THESIS IS BASED ON THE FOLLOWING PUBLICATIONS

Karai L, Brown DC, Mannes AJ, Connelly ST, Brown J, Gandal M, Wellisch OM, Neubert JK, Olah Z, Iadarola MJ. Deletion of vanilloid receptor-1 expressing primary afferent neurons for pain control. J Clin Invest. 2004 May;113(9):1344-52.

Karai LJ, Russell JT, Iadarola MJ, Olah Z. Related Articles, Links
Vanilloid receptor 1 regulates multiple calcium compartments and contributes to Ca^{2+}-induced Ca^{2+} release in sensory neurons. J Biol. Chem. 2004 Apr 16;279(16):16377-87.

R.M. Caudle, **L. Karai**, N. Mena, B.Y. Cooper, A.J. Mannes, F.M. Perez, M.J. Iadarola, Z. Olah Resiniferatoxin Induced Loss of Plasma Membrane in Vanilloid Receptor Containing Cells Neurotoxicology. 2003 Dec; 24(6):895-908.

Neubert JK, **Karai L**, Jun JH, Kim HS, Olah Z, Iadarola MJ (2003)
Peripherally Induced Resiniferatoxin Analgesia. Pain. 2003; 104(1-2): 219-228.

Olah Z, Szabo T, **Karai L**, Hough C, Fields RD, Caudle RM, Blumberg PM, Iadarola MJ. Ligand-induced dynamic membrane changes and cell deletion conferred by vanilloid receptor 1. (2001) J. Biol. Chem., Vol. 276, Issue 14, 11021-11030