Neuropharmacology 75 (2013) 1–8

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Intrathecal P/Q- and R-type calcium channel blockade of spinal substance P release and c-Fos expression☆,☆☆

Tetsuji Terashima [a,b], Qinghao Xu [b], Shigeki Yamaguchi [a], Tony L. Yaksh [b,*]

[a] Department of Anesthesiology, Dokkyo Medical University, School of Medicine, Kitakobayashi 880, Mibu, Tochigi 321-0293, Japan
[b] Department of Anesthesiology, University of California, San Diego, 9500 Gilman Drive, San Diego, La Jolla, CA 92093-0818, USA

ARTICLE INFO

Article history:
Received 26 December 2012
Received in revised form
10 June 2013
Accepted 11 June 2013

Keywords:
Voltage sensitive calcium channels
SNX-482
ω-Agatoxin IVA
Neurokinin 1 receptor
c-Fos
Spinal cord

ABSTRACT

Intrathecal (IT) studies have shown that several voltage sensitive calcium channels (VSCCs), such as the L-, N- and T-type may play roles in nociception and that of these only the N-type regulates primary afferent substance P (SP) release. However, the actions of other VSCCs at the spinal level are not well known. We investigated the roles of spinal P/Q- and R-type VSCCs, by IT administration of R-type (SNX-482) and P/Q-type (ω-agatoxin IVA) VSCC blockers on intraplantar formalin-evoked flinching, SP release from primary afferents and c-Fos expression in spinal dorsal horn. Intraplantar injection of formalin (2.5%, 50 μL) produced an intense, characteristic biphasic paw flinching response. In rats with IT catheters, IT SNX-482 (0.5 μg) reduced formalin-evoked paw flinching in both phase 1 and 2 compared with vehicle. Intraplantar formalin caused robust neurokinin 1 receptor (NK1r) internalization (indicating SP release) and c-Fos expression in the ipsilateral dorsal horn, which were blocked by IT SNX-482. IT ω-agatoxin IVA (0.03, 0.125 and 0.5 μg) did not reduce formalin-evoked paw flinching or c-Fos expression at any doses, with higher doses resulting in motor dysfunction. Thus, we demonstrated that blockade of spinal R-type, but not P/Q type VSCCs attenuated formalin-induced pain behavior, NK1r internalization and c-Fos expression in the superficial dorsal horn. This study supports a role for Cav2.3 in presynaptic neurotransmitter release from peptidergic nociceptive afferents and pain behaviors.

1. Introduction

Voltage-sensitive calcium channels (VSCCs) facilitate calcium influx and play an important role in the regulation of neurotransmitter release, synaptic transmission and neuronal excitability (Catterall, 2000; Catterall and Few, 2008). The α1 is the pore-forming subunit and dictates the VSCC's major characteristics of pharmacology, electrophysiology and kinetic activity, representing the basis for the calcium channel subtype. Ten different α1 have been identified, namely Cav1.1–1.4, Cav2.1–2.3 and Cav3.1–3.3. These are distributed into five subgroups, L-(Cav1.1–1.4), P/Q-(Cav2.1), N-(Cav2.2), R-(Cav2.3) and T-type (Cav3.1–3.3). Based on their voltage activation properties, VSCCs are divided into two classes, high voltage-activated channels and low voltage-activated channels (Ertel et al., 2000). High voltage-activated channels include L-, P/Q-, N- and R-types. These are heteromeric complexes consisting of an α1 subunit along with auxiliary subunits such as α2δ, β and γ subunit. Low voltage-activated channels or T-type are activated by much more negative membrane potentials (Carbone and Lux, 1984; Nowycky et al., 1985) and are not known to interact with auxiliary subunits.

VSCCs are expressed in the dorsal root ganglion (DRG) and spinal cord dorsal horn (Murakami et al., 2001; Westenbroek et al., 1998; Yusaf et al., 2001), suggestive of their critical role in nociception. Intrathecal (IT) ziconotide (Prialt®), a selective N-type blocker is approved for treatment of severe chronic pain. Consistent with location of N-type VSCCs on peptidergic primary afferents, N-type VSCC blockade attenuates neurotransmitter release from primary afferents, as defined in in vitro and in vivo models (Evans et al., 1996; Maggi et al., 1990; Santicioli et al., 1992; Takasusuki and Yaksh, 2011). However, the role of other VSCCs subtypes in afferent neurotransmitter release remains unclear. We found

Abbreviations: CGRP, calcitonin gene-related peptide; GPCR, G protein-coupled receptor; DRG, dorsal root ganglia; IB4, isolectin B4; IT, intrathecal; NK1r, neurokinin 1 receptor; NMDA, N-methyl-D-aspartate; SP, substance P; trkA, tropomyosin receptor kinase A; TRPV1, transient receptor potential protein vanilloid 1; IR, immunoreactive; VSCCs, voltage-sensitive calcium channels.

☆ Preliminary data were presented in a poster at Neuroscience 2012 (Society for Neuroscience, New Orleans, Louisiana, USA, October 15, 2012).

☆☆ This work is attributed to 'Department of Anesthesiology, University of California, San Diego, California, USA'.

* Corresponding author. Tel.: +1 619 543 3597; fax: +1 619 543 6070.
E-mail addresses: youtei3@gmail.com (T. Terashima), q1xu@ucsd.edu (Q. Xu), shigeki@dokkyomed.ac.jp (S. Yamaguchi), tyaksh@ucsd.edu (T.L. Yaksh).

0028-3908/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuropharm.2013.06.018

previously that intrathecal L- and T-type blockers minimally affected intraplantar formalin-evoked release of substance P (SP) in vivo as measured by neurokinin 1 receptor (NK1r) internalization (Takasusuki and Yaksh, 2011). Those results suggested L- and T-type VSCCs were not involved in stimulus-evoked SP release. In the present study we focus on the role of P/Q- and R-types.

Cav2.1 (P/Q-type) channels contain the α1A subunit and are distributed in the nervous system including DRG and spinal dorsal horn (Catterall and Few, 2008; Kulik et al., 2004; Urban et al., 2005). P/Q-type VSCCs are inhibited by ω-agatoxin IVA, a 48- amino acid peptide isolated from the venom of funnel web spider, Agelenopsis aperta (Mintz et al., 1992). ω-agatoxin IVA blocks P-type with high affinity and Q-type with lower affinity (Adams, 2004). IT ω-agatoxin IVA reduces the number of flinches in formalin phase 2, but did not alter thermal escape latencies (Malmberg and Yaksh, 1994). Cav2.3 (R-type) channels contain the α1E subunit, and are detected in spinal cord and DRG (Murakami et al., 2001; Saegusa et al., 2000; Westenbroek et al., 1998; Yusaf et al., 2001). Cav2.3 knockout mice exhibited reduced pain behaviors in the formalin test (Saegusa et al., 2000). In another study, Cav2.3 was shown to participate in nerve injury-induced hypersensitivity (Matthews et al., 2007). Dense labeling of Cav2.3 was observed in the superficial layers of the dorsal horn (Saegusa et al., 2000). However, whether Cav2.3 is involved in primary afferent neurotransmitter release has not been established.

Here, we investigated intrathecal R- and P/Q-type channel blockers and spinal release of SP evoked by intraplantar formalin. Increase in local extracellular SP due to its release (induced by intraplantar formalin) from small peptidergic, transient receptor potential protein vanilloid 1 (TRPV1) (+) C-fibers evokes NK1r internalization (Kondo et al., 2005; Mantyh, 2002). Thus NK1r internalization provides a powerful tool to evaluate in vivo the effects of VSCCs blockade on afferent terminal release of SP. Furthermore, intraplantar formalin induces c-Fos protein expression in dorsal horn neurons, which receive input from small diameter Aδ and C primary afferents (Bullitt, 1990; Hunt et al., 1987; Long et al., 2012), causing c-Fos expression to be a useful index of spinal activation. This in vivo methodology allows us to assess the effects of R- and P/Q-type VSCCs antagonists upon SP release, dorsal horn neuron activation and assess the covariance of these effects with pain behaviors (flinching) at the corresponding drug doses.

2. Material and methods

2.1. Animals

Male Holtzman Sprague-Dawley rats (250–300 g; Harlan Indianapolis, IN) were individually housed in standard cages and maintained on a 12-h light/dark cycle (lights on at 07:00 h). Testing occurred during the light cycle. Food and water were available ad libitum to all rats in the study. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85–23, Bethesda, MD) and as approved by the institutional Animal Care and Use Committee of the University of California, San Diego. All efforts were made to minimize animal suffering, to reduce the number of animals used.

2.2. Intrathecal catheter implantation

Rats were implanted with a single intrathecal catheter for drug delivery (Malkmus and Yaksh, 2004; Yaksh and Rudy, 1976). In brief, rats were anesthetized by induction with 4% isoflurane in a mixture of air and 100% oxygen (1:1). Anesthesia was maintained with 2% isoflurane delivery by mask. The animal was placed on a stereotaxic head holder, a midline incision was made on the back of the occipital bone and the cisternal membrane was exposed by blunt dissection. A small opening was made on the cisternal membrane by a 22 gauge needle, and an 8.5 cm single-lumen polyethylene (OD 0.36 mm) catheter was inserted through the opening and passed into the intrathecal space to the level of the L2 to L3 spinal segments. The other end of the catheter was tunneled subcutaneously to exit through the top of the head, flushed with 10 μL of saline and plugged. Rats were given subcutaneously 5 mL of lactated Ringer's solution, to which was added 1.25 mg/mL of carprofen and recovered in a warmed chamber. Rats with any motor weakness or signs of paresis on recovery from anesthesia were euthanized immediately. Animals were allowed to recover for 5–7 days prior to other studies.

2.3. Voltage-sensitive calcium channel (VSCC) blockers on formalin-induced paw flinching behavior

Formalin-induced flinching behavior was analyzed by a paw movement detection system (Yaksh et al., 2001). Briefly, a soft metal band was placed around the left hind paw and secured with a drop of adhesive. Animals were allowed to acclimate in individual acrylic glass chambers for 30 min before experimental manipulation. Rats were intrathecally treated with saline, SNX-482 (0.5 μg) or ω-agatoxin IVA (0.03, 0.125 and 0.5 μg) in a volume of 10 μL, followed by 10 μL of saline. 10 min after the IT injection, rats received a subcutaneous injection of 50 μL of formalin (2.5%) into the dorsal side of the banded paw. Rats were immediately, placed into test chambers and paw flinching assessed over the ensuing 60 min interval with an automated device (Department of Anesthesiology, University of California, San Diego, CA) for 60 min.

2.4. VSCC blockers on formalin-induced NK1r internalization and c-Fos expression

Rats with catheter received IT saline or antagonist ten minutes before intraplantar formalin (50 μL, 2.5%) injection. For NK1r internalization, rats were transcardially perfused 10 min after formalin. For c-Fos expression, rats were perfused 120 min after formalin.

2.5. Tissue preparation and immunohistochemistry

Anesthetized rats were transcardially perfused with NaCl (0.9%) followed by paraformaldehyde (4%) in 0.1 M phosphate buffer (PB), pH 7.4. The lumbar spinal cord was removed and post-fixed in paraformaldehyde (4%) overnight. After cryoprotection in 30% sucrose, coronal sections (30 μm) were cut on a sliding microtome (HM 450; Thermo Scientific, Kalamazoo, MI).

For NK1r, sections were incubated in a rabbit anti-NK1r polyclonal antibody ab6 (Abcam, Eugene, OR, 1:3000) and mouse anti-NeuN MAB377 (Millipore, Temecula, CA, 1:500) overnight at room temperature. After the sections were rinsed in PBS, they were incubated for 2 h at room temperature in a goat-anti-rabbit secondary antibody conjugated with Alexa 488 (Invitrogen, Carlsbad, CA, 1:500) to identify NK1r and a goat-anti-mouse secondary antibody conjugated with Alexa 555 (Invitrogen, Carlsbad, CA, 1:500) to identify NeuN. All sections were finally rinsed and mounted on glass slides and coversliped with ProLong mounting medium (Invitrogen, Carlsbad, CA).

Immunohistochemistry of c-Fos was performed using the avidin-biotin complex (ABC) method. In short, free-floating spinal cord sections were treated with 3% hydrogen peroxide (Sigma, St. Louis, MO) for 10 min and then incubated in primary antibody solution containing 0.5% Triton X-100, 10% goat serum in phosphate buffered saline, and anti-c-Fos (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, 1:10,000) overnight at room temperature. After sections were rinsed in PBS, incubated with a biotinylated goat-anti-rabbit secondary antibody (BA-1000, Vector laboratories, Burlingame, CA, 1:500) for 120 min. Sections were incubated in ABC solution for 1 h (PK-6100, Vector laboratories, Burlingame, CA) and subsequently DAB substrate solution (SK-4100, Vector laboratories, Burlingame, CA) for an appropriate amount of time. Following mounting and dehydration, slides were coversliped with DPX (Electron Microscopy Science, Hatfield, PA).

2.6. Quantification of NK1r internalization and c-Fos expression

The amount of NK1r internalization was quantified using a standard method (Abbadie et al., 1997a; Kondo et al., 2005; Mantyh et al., 1995). NK1r-positive neurons in lamina I/II on both sides of the dorsal horn were counted using an Olympus BX-51 fluorescence microscope (Olympus Optical, Tokyo, Japan) at ×60 magnification. Neurons with 10 or more endosomes in their soma and the conterminous proximal dendrites were deemed as having internalized NK1r. The person counting the neurons was blinded to the experimental treatment. In each segment, three to four randomly selected sections were counted. The sum of the number of NK1r-positive neurons with and without NK1r internalization taken from a segment were used to calculate the percentage of NK1r internalization to represent the particular spinal segment for a given animal. Four animals per each treatment group were used for statistical analysis. The ratio of cells showing NK1r internalization versus all NK1r-positive cells was reported.

We quantified the c-Fos-immunoreactive (IR) neurons under the Olympus BX-51 microscope at ×10 magnification in the ipsilateral and contralateral spinal dorsal horn. The number of c-Fos-IR neurons in lamina I/II and lamina III/V was determined by a blinded observer. Means counted from six to nine randomly selected sections at L3–L6 level spinal cord were employed for each animal. At least one spinal section was selected for each spinal segment. Five animals per treatment group (for the ω-agatoxin IVA study, two to five animals) were used for statistical analysis. Light microscopic images were taken using MagnaFire SP (Optronics, Goleta, CA) and processed by Photoshop CS5 (Adobe, San Jose, CA).