PERIPHERAL SENSITIZATION OF SENSORY NEURONS

Victor V. Chaban, PhD, MSCR

Introduction: Defining the sites and mechanisms of nociception is an important step in understanding and treating pain. During inflammation, increased nociceptive input from an inflamed organ can sensitize neurons that receive convergent input from an unaffected organ, but the site of visceral cross-sensitivity is unknown. This study examined the cellular responses to ATP and substance P stimulation in sensory neurons innervating visceral organs.

Methods: Lumbosacral dorsal root ganglia (L6-S1) were cut into slices and processed for substance P receptor expression using immunocytochemistry. Primary culture of dorsal root ganglion (DRG) neurons was used for [Ca2+]i measurement by videomicroscopy.

Results: DRG neurons express substance P receptors. Both brief addition of low dose adenosine triphosphate (ATP, 5 μM) and substance P (10 μM) significantly increased subsequent ATP stimulation at the same neuron.

Discussion: Sensitization of the DRG neurons innervating the different organs may be through the release of nociceptive transmitters such as ATP and/or substance P within the ganglion. Together, these experiments will increase our understanding of the important modulatory role of peripheral sensitization in nociceptive transmission and suggest potential periphepheral sites for therapeutic intervention. (*Ethn Dis*. 2010;20[Suppl 1]:S1-3–S1-6)

Key Words: DRG, Sensitization, ATP, Substance P, Calcium

From the Departments of Medicine and Biomedical Sciences, Charles Drew University of Medicine & Science, Los Angeles, California.

Address for reprint requests and correspondence to Victor Chaban, PhD; Charles Drew University of Medicine and Science; 1731 East 120th Street; Los Angeles, CA 90095; 323-357 3672; 323-357 3442 (fax); victorchaban@cdrewu.edu

INTRODUCTION

The cell bodies of primary visceral spinal afferent neurons are located in the dorsal root ganglia (DRG). Primary afferents transmit information about chemical or mechanical stimulation from the periphery to the spinal cord. Nociceptors are small-to-medium size DRG neurons whose peripheral processes detect potentially damaging physical and chemical stimuli. Adenosine triphosphate (ATP) has emerged as a putative signal for visceral pain. ATP is released by distention of the viscera and tissue damage.[1] Nociceptive C-fibers are activated by ATP and excitatory amino acids that are released by noxious stimuli from cells in target organs (paracrine action), from afferent terminals themselves (autocrine action[2]), or in sensory ganglia.[3] In our previous studies we observed that DRG neurons innervating viscera have a greater [Ca2+]i response to subsequent N-methyl-D-aspartic acid (NMDA) stimulation than somatic afferents.[4] This observation indicates that these neurons express receptors with higher permeability to Ca2+, which modulates transduction of nociceptive signals. Visceral DRG also express nociceptive ATP-sensitive purinergic (P2X3) and capsaicin-sensitive vanilloid (TRPV1) receptors.[5]

Sensitization of primary afferent neurons to stimulation may play a role in the enhanced perception of visceral sensation and pain. Chest pain from coronary heart disease, endometriosis, acute and recurrent/chronic pelvic pain in women or abdominal pain from irritable bowel syndrome are all visceral pain sensations that may result in part from sensitization.[6–9] Mechanisms of peripheral sensitization may involve increased transduction that is secondary to repeated stimulation or an increase in the excitability of the afferent nerves by molecules that decrease the excitation threshold. Sensitization can also develop in response to inflammation although basic mechanisms of this process are poorly understood. In this report, we studied if nociceptive mediators of inflammatory response such as substance P or repeated addition of low dose ATP can produce sensitization in a primary culture of lumbosacral DRG neurons.

METHODS

Immunocytochemistry

DRG neurons were collected from Long-Evans female rats. Twenty micron thick sections of DRG were treated with PBS containing 1% normal goat serum (NGS) and 0.1% Triton X-100 for 1 h, then incubated at 4°C overnight in a rabbit primary antibody for substance P (1:20000, IncStar, Stillwater, MN) in PBS containing 1% NGS. The sections were washed in PBS and then incubated in goat biotinylated anti-rabbit IgG antibodies for substance P (1:200; Vector Laboratories, Burlingame, CA) in PBS containing 1% NGS 1 hour at 4°C. After several rinses, tissue sections were incubated in streptavidin–HRP (1:100; Tyramide Signal Amplification kit; NEN Life Science Products, Boston, MA) for 30 min, then washed and incubated for 5 min in flourosceinconjugated tyramide (1:50; Tyramide Signal Amplification kit; NENLife Science Products), and then washed in 0.1 M Tris buffer without saline, pH 7.5, and mounted on Superfrost

Plus slides (Fisher Scientific, Pittsburgh, PA). Mounted sections were air-dried and coverslipped with Aqua Poly Mount (Polisciences, Inc, Warrington, PA) for analysis.

Animals

Healthy rats (1–2 month-old Long-Evans, Charles River Laboratories International, Inc. Wilmington, MA) were used without any special treatment. Veterinary care and all procedures were done in accordance with the guidance of the National Institute of Health and the Charles Drew University of Medicine and Science Policy on Humane Care and Use of Laboratory Animals.

Primary Culture of DRG Neurons

Lumbosacral adult DRGs (level L6-S1) were collected under sterile conditions and placed in ice-cold Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO). Adhering fat and connective tissue were removed and each DRG was minced with scissors and placed immediately in a medium consisting of 5 mL of DMEM (containing 0.5 mg/mL of trypsin (Sigma, type III), 1 mg/mL of collagenase (Sigma, type IA) and 0.1 mg/mL of DNAase (Sigma, type III) and kept at 37°C for 15 min with agitation. After dissociation of the cell ganglia, soybean trypsin inhibitor (Sigma, type III) was used to terminate cell dissociation. Cell suspension was centrifuged for 1 min at 1500 rpm and the cell pellet was resuspended in DMEM supplemented with 5% fetal bovine serum, 5% horse serum, 2 mM glutamine-penicillin-streptomycin mixture, 1 g/mL DNAase and 5 ng/ml NGF (Sigma). Cells were plated on Matrigel®-coated 15-mm coverslips (Collaborative Research Co., Bedford, PA) and kept at 37°C in 5% CO_2 incubator for 24 hrs, given fresh media without fetal bovine serum or phenol red and maintained in primary culture until used for experimental procedures.[4]

[Ca2+]i Fluorescence Imaging

For Ca2+ imaging analysis DRG neurons were loaded with fluorescence indicator dye Fura-2AM for 1 hour at 37°C in Hanks buffered saline solution (HBSS) containing 20 mM HEPES, pH 7.4.[4] Coverslips were mounted in fast-perfusion chamber P-4 (World Precision Instruments, Sarasota, Fl) and placed on a stage of Olympus IX51 inverted microscope. Observations were made at room temperature (20–22°C) with 20× UApo/340 objective. A fast superfusion system was used to perfuse the cells with HBSS and rapidly apply ATP and substance P. Fluorescence intensity at 505 nm with excitation at 334 nm and 380 nm was captured as digital images (sampling rates of 0.1–2 s). Regions of interest were identified within the soma from which quantitative measurements were made by re-analysis of stored image sequences using Slidebook® Digital Microscopy software. [Ca2+]i was determined by ratiometric method of Fura-2 fluorescence from calibration of series of buffered Ca2+ standards. ATP and substance P were acutely applied onto the experimental chamber. Repeated application of drugs was achieved by superfusion in a rapid mixing chamber into individual neurons for specific intervals (100–500 ms). Cells were perfused with experimental media (2 ml/min) using a Rainin® peristaltic pump.

Statistical Analysis

The amplitude of [Ca2+]i response represents the difference between baseline concentration and the transient peak response to drug stimulation. Significant differences in response to chemical stimulation were obtained by comparing [Ca2+]i increases during the first stimulation with the second or consequent stimulation. Statistical significance was determined using Student *t*-test or ANOVA and when appropriate followed by Tukey's post-hoc comparison (GraphPad Prism software, San Diego, CA). Differences of $P<.05$ were considered statistically significant.

RESULTS

DRG Neurons Express Substance P Receptors

For quantification of DRG neurons, 3–6 sections were analyzed from each ganglion from L1-S3 levels. A neuron was counted only when a distinct nucleus was present. The number of total and immunolabeled neurons per section was determined for substance P expression and a mean of immunolabeled cells was averaged across the sections. The mean number of immunolabeled cells was expressed as a percentage of labeled neurons per total number of neurons. We found that up to 25% of neurons were substance P-positive (Figure 1) in L6-S1 DRGs.

ATP-induced Ca2+ Flux in DRG Neurons *in vitro*

Lumbosacral DRG were collected from rats and cultured for 48–72 hrs on coverslips before being loaded with Fura-2 acetoxymethyl ester (Fura-2AM, Molecular Probes, Eugene, OR) by incubating cultures in a 5 μM solution for 1 hour at 37°C. Stimulation of small to medium size DRG neurons (< 35 μm in diameter) with 10 μM ATP caused transient [Ca2+]i increase (207.6 ±21.8 nM) in about 50% of neurons tested ($n=10$). Repeated ATP (10 μM) application by fast superfusion (~ 10 sec) produced reproducible [Ca2+]i responses (Figure 2A). The response to ATP was fully reversed after a 10–15 min washout with Hank's Balanced Salt Solution (HBSS; Gibco BRL buffered with 20 mM N-(2-hydroxyethyl) piperazine-N-(2-ethanesulfonic) acid: HEPES). Chelating extracellular Ca2+ with BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N′, N′-tetraacetic acid, 10 mM) eliminated ATP-induced [Ca2+]i responses. Pretreatment with the purinoreceptor antagonist phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) inhibited ATP responses: 5 μM produced a 70% inhibition and 10 μM completely blocked the ATP-induced [Ca2+]i (data

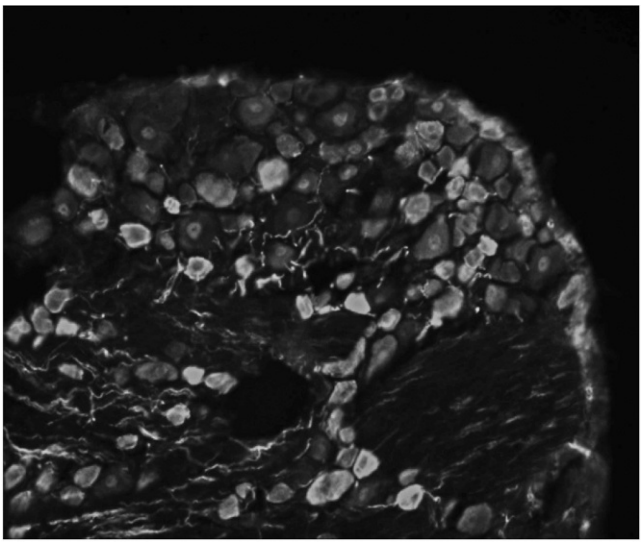

Fig 1. Substance P expression in DRG neurons in vivo using fluorescent microscopy. DRG sections were incubated in primary antibody for substance for substance P antibody (1:200; Vector Laboratories, Burlingame, CA)

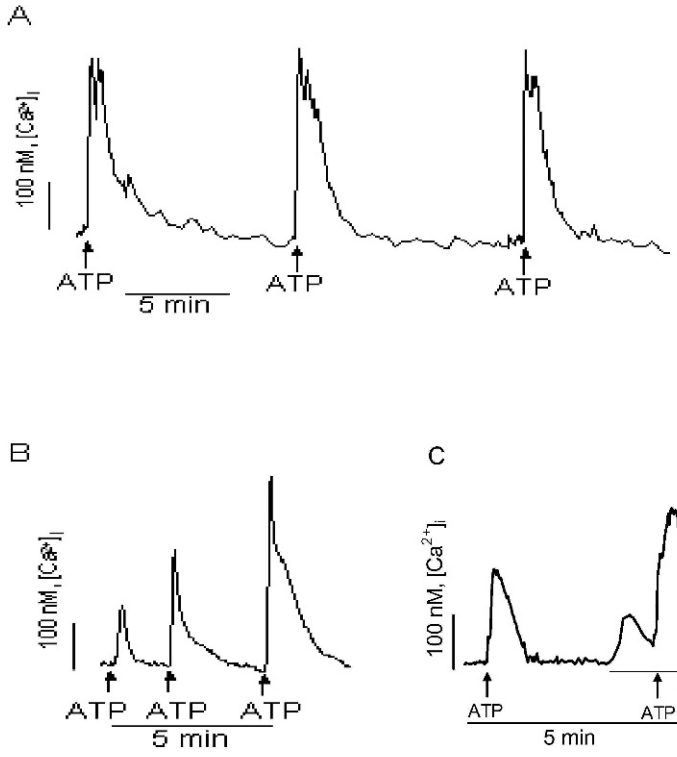

Fig 2A, B, C. Sensitization of DRG neurons in vitro using ratiometric Ca2+ videomicroscopy. A) Repeated ATP (10 μM) stimulation after 10 min wash-out with experimental medium produced reproducible [Ca2+]i responses in DRG neurons (time and [Ca2+]i indicated by bars). The trace is from a single neuron and shows features of typical responses with rapid rise after stimulation (indicated by arrow) followed by a slower recovery; B) Sensitization of [Ca2+]i response to repeated stimulation with ATP (5 μM) added within 5 min interval; C) Substance P (SP; 10 μM) increases ATP-induced [Ca2+]i flux suggesting sensitization

not shown). These results indicate that ATP-induced Ca2+ flux is dependent on activation of P2X receptors and plasma membrane Ca2+ channels which sensitize the DRG cells to repeated ATP stimulation.

Sensitization of DRG Neurons by ATP and Substance P

When a repeated ATP (5 μM) stimulation was used within 5 min the [Ca2+]i showed an increasing response (n=4) (Figure 2B). Similarly, pre-treatment with substance P (10 μM) augmented the subsequent [Ca2+]i response from 183±17.6 to 249±23.8 induced by ATP (n=6) (Figure 2C). Thus, substance P or increasing amounts of ATP that signal nociception and sensitize response to ATP may be a good model to study cellular mechanism of peripheral sensitization.

DISCUSSION

ATP plays vital roles in cellular metabolism and as an extracellular signal. ATP transduces noxious stimuli by activating purinergic, ATP-gated P2X receptors on primary afferent fibers. Opening of P2X channels results in membrane depolarization sufficient to trigger action potentials and Ca2+ influx through defined voltage-gated calcium channels (VGCC) associated with nociception. According to this theory, pain of tissue irritation (mechanical distortion or inflammation) is due to ATP activation of high threshold nociceptors.[10] The predominant ATP receptor in small diameter nociceptive DRG neurons is the P2X3. Thus, P2X3-null mice have reduced pain-related behavior in response to noxious stimuli.[11,12] Significantly, inflammation dramatically alters purinoception by causing a several fold increase in ATP-activated currents, alters the voltage dependence of P2X receptors, and enhances the expression of P2X receptors increasing neuronal hypersensitivi-

ty.[13] The inflammatory process produces a number of mediators which activate nociceptors by interacting with ligand-gated ion channels or by sensitizing primary afferents. One mechanism for sensitization involves phosphorylation of ion channels and receptors including P2X and substance P. Inflammation does not change the percentage of total cells responding to ATP but sensitizes the ATP response and increases the expression of P2X2 and P2X3.[13] Thus, the increased sensitivity during the inflammation is due to an increase in ATP responses suggesting that a small amount of ATP would evoke depolarization sufficient to elicit action potentials in DRG neurons.[13] This pathological response arises from sensitization of DRG neurons to external stimuli. Previous studies have demonstrated that a large percentage of visceral afferent fibers are substance P-positive (50%).[14,15] Capsaicin treatment abolished virtually all the immunoreactive fibers suggesting that these afferents also express nociceptive TRPV1 receptors.

DRG neurons in vitro are a well-accepted model to examine primary afferent response to nociceptive and anti-nociceptive signals. It should also be noticed that nociceptive systems implicated in the etiology of many pain-associated disorders may be complicated by input of other signaling pathways such as endogenous opioids and others. Within the context of our hypothesis, modulation of nociceptive response depends on the type of pain, its durations and the involvement of other anti-nociceptive mechanisms.

In this study we investigated the mechanisms of sensitization of DRG neurons in response to application of substance P in ATP-evoked Ca2+-transients. Our data suggest that either sub-threshold dose of ATP or pretreatment with substance P produce enhanced Ca2+ response leading to the sensitization. To our knowledge, this is a first report of this observation. Sensitization accounts for a dramatically lowered nociceptive threshold to mechanical manipulation of the inflamed area. Within the context of the cross-sensitization hypothesis, inflammation sensitizes non-inflamed viscera that are innervated by the same DRG. Cross-sensitization occurs as a result of intra-DRG release of sensitizing mediators such as ATP or substance P3 which we modeled in this report in cultured DRG by repeated ATP or substance P and ATP stimulation. Visceral nociception and nociceptor sensitization appear to be regulated by ATP and substance P, thus, the DRG is an important site of visceral afferent convergence and cross-sensitization.

Implications for Improving Health Disparities

In the United States, pain accounts for nearly 20% of all primary health care visits. Over the last decade, the medical literature has carefully documented the undertreatment of all types of pain by physicians especially in vulnerable populations in medically underserved areas that received almost no attention from the medical community. Our data can potentially lead to better understanding the basic mechanisms of pain perception and to the discovery of new therapeutic intervention for pain-associated disorders.

ACKNOWLEDGMENTS

This research was supported by NIH Grant NS 063939-01.

REFERENCES

1. Burnstock G. Purine-mediated signalling in pain and visceral perception. *Trends Pharmacol Sci*. 2001;22(4):182–188.
2. Burnstock G. P2X receptors in sensory neurones. *Br J Anaesth*. 2000;84(4):476–488.
3. Matsuka Y, Neubert JK, Maidment NT, Spigelman I. Concurrent release of ATP and substance P within guinea pig trigeminal ganglia in vivo. *Brain Res*. 2001;915(2):248–255.
4. McRoberts JA, Coutinho SV, Marvizon JC, et al. Role of peripheral N-methyl-D-aspartate (NMDA) receptors in visceral nociception in rats. *Gastroenterol*. 2001;120(7):1737–1748.
5. Chaban VV. Visceral sensory neurons that innervate both uterus and colon express nociceptive TRPv1 and P2X3 receptors in rats. *Ethn Dis*. 2008;18(2 Suppl 2):S2-20–S2-24.
6. Giamberardino MA. Recent and forgotten aspects of visceral pain. *Eur J Pain*. 1999;3(2):77–92.
7. Mayer EA, Naliboff BD, Chang L. Basic pathophysiologic mechanisms in irritable bowel syndrome. *Dig Dis*. 2001;19(3):212–218.
8. Berkley KJ, Cason A, Jacobs H, Bradshaw H, Wood E. Vaginal hyperalgesia in a rat model of endometriosis. *Neurosci Lett*. 2001;306(3):185–188.
9. Wesselmann U, Lai J. Mechanisms of referred visceral pain: uterine inflammation in the adult virgin rat results in neurogenic plasma extravasation in the skin. *Pain*. 1997;73(3):309–317.
10. Bodin P, Burnstock G. Purinergic signalling: ATP release. *Neurochem Res*. 2001;26(8–9):959–969.
11. Cockayne DA, Hamilton SG, Zhu QM, et al. Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature*. 2000;407(6807):1011–1015.
12. Zhong Y, Dunn PM, Bardini M, Ford AP, Cockayne DA, Burnstock G. Changes in P2X receptor responses of sensory neurons from P2X3-deficient mice. *Eur J Neurosci*. 2001;14(11):1784–1792.
13. Xu GY, Huang LY. Peripheral inflammation sensitizes P2X receptor-mediated responses in rat dorsal root ganglion neurons. *J Neurosci*. 2002;22(1):93–102.
14. Green T, Dockray GJ. Characterization of the peptidergic afferent innervation of the stomach in the rat, mouse and guinea-pig. *Neuroscience*. 1988;25(1):181–193.
15. Papka RE, Storey-Workley M, Shughrue PJ, et al. Estrogen receptor-alpha and beta- immunoreactivity and mRNA in neurons of sensory and autonomic ganglia and spinal cord. *Cell Tissue Res*. 2001;304(2):193–214.