Peripheral Sensitization of Sensory Neurons

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 peripheral sites for therapeutic intervention. (Ethn Dis. 2010;20[Suppl 1]:S1-3–S1-6)

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

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 the viscerosensory afferents innervating the periphery and 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 peripheral sites for therapeutic intervention. (Ethn Dis. 2010;20[Suppl 1]:S1-3–S1-6)

PERIPHERAL SENSITIZATION OF SENSORY NEURONS

Victor V. Chaban, PhD, MSCR

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 excitability 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 (1:2000; 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 fluoroscen-conjugated tyramide (1:50; Tyramide Signal Amplification kit; NEN Life Science Products), and then washed in 0.1 M Tris buffer without saline, pH 7.5, and mounted on Superfrost slides.
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% CO2 incubator for 24 hrs, given fresh media without fetal bovine serum or phenol red and maintained in primary culture until used for experimental procedures. 

[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. Coverslips were mounted in 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 acetoxyethyl 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). Repeat- ed 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 phosphat-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...
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. 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. 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-
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. 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. 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%). 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