Spinal P2X receptor modulates reflex pressor response to activation of muscle afferents

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Spinal P2X receptor modulates reflex pressor response to activation of muscle afferents. Am J Physiol Heart Circ Physiol 288: H2238–H2243, 2005. First published December 22, 2004; doi:10.1152/ajpheart.01095.2004.—Static contraction of skeletal muscle increases blood pressure and heart rate. Previous studies suggested that the dorsal horn of the spinal cord is the first synaptic site responsible for those cardiovascular responses. In this study, we examined the role of ATP-sensitive P2X receptors in the cardiovascular responses to contraction by microdialyzing the P2X receptor antagonist pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) into the L7 level of the dorsal horn of nine anesthetized cats. Contraction was elicited by electrical stimulation of the L7 and S1 ventral roots. Blockade of P2X receptor attenuated the contraction induced-pressor response [change in mean arterial pressure (ΔMAP): 16 ± 4 mmHg after 10 mM PPADS vs. 42 ± 8 mmHg in control; P < 0.05]. In addition, the pressor response to muscle stretch was also blunted by PPADS (ΔMAP: 27 ± 5 mmHg after PPADS vs. 49 ± 8 mmHg in control; P < 0.05). Finally, activation of P2X receptor by microdialyzing 0.5 mM α,β-methylene into the dorsal horn significantly augmented the pressor response to contraction. This effect was antagonized by prior PPADS dialysis. These data demonstrate that blockade of P2X receptors in the dorsal horn attenuates the pressor response to activation of muscle afferents and that stimulation of P2X receptors enhances the reflex response, indicating that P2X receptors play a role in mediating the muscle pressor reflex at the first synaptic site of this reflex.

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Gao, Zhaohui, Valerie Kehoe, Lawrence I. Sinoway, and Jianhua Li. Spinal P2X receptor modulates reflex pressor response to activation of muscle afferents. Am J Physiol Heart Circ Physiol 288: H2238–H2243, 2005. First published December 22, 2004; doi:10.1152/ajpheart.01095.2004.—Statistical contraction of skeletal muscle increases arterial blood pressure and heart rate (HR) (7, 28). Neural signals from contracting skeletal muscle are generated by activating mechanically and metabolically sensitive nerve endings (receptors) located in the skeletal muscle and are subsequently carried to the central nervous system by thin group III and IV afferent fibers (20, 21). Together, activation of these receptors along with the reflex cardiovascular responses is referred to as the “exercise pressor reflex” (7, 20, 28). The majority of these thin fiber afferent fibers are known to make their first synapse in the superficial dorsal horn of the spinal cord. Studies have further demonstrated the neural transmitters/modulators that are involved in transmitting the exercise pressor reflex at this site (11–14, 23, 38–40).

Purinergic P2X receptors, ligand-gated ion channels that are activated by extracellular ATP, are selectively expressed by small- and medium-diameter sensory neurons (1, 3, 17, 33–35). ATP also has been shown to be responsible for the transmission of signals from the peripheral afferent nerve to the dorsal horn (1, 2, 4–6, 8). Previous studies by our laboratory (26) and others (15, 16) have shown that P2X receptor stimulation on the nerve ending of muscle afferents plays a role in mediating the autonomic adjustments to exercise. In this study, we hypothesized that ATP-sensitive P2X purinoreceptor would play a role in mediating the muscle pressor reflex at the dorsal horn of the spinal cord, a critical site responsible for the reflex cardiovascular responses to exercise. To test this hypothesis, we delivered the P2X receptor antagonist pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) into the dorsal horn using microdialysis methods. We then determined whether the cardiovascular response to static muscle contraction was blunted after P2X receptor blockade. We further determined whether the P2X receptor agonist α,β-methylene ATP would enhance the muscle reflex and whether the effect could be reduced by prior PPADS administration.

METHODS

All experimental procedures were approved by the Animal Care Committee of this institution and complied with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals.

General Procedures

Experiments were performed on nine anesthetized cats of either gender with weights of 4.5–5.8 kg. The animals were anesthetized initially with ketamine (25 mg/kg im) and then by inhalation of an isoflurane-oxygen mixture (2–5% isoflurane in 100% oxygen). A tube was inserted into the trachea via a tracheotomy to maintain an open airway, and a jugular vein and carotid artery were cannulated for drug administration and measurement of arterial blood pressure, respectively. The gaseous anesthetic was discontinued after α-chloralose (80 mg/kg) and urethane (200 mg/kg) were injected intravenously. Throughout the experiment, supplemental injections of α-chloralose (15 mg/kg) and urethane (40 mg/kg) were given if the cats exhibited a corneal reflex or withdrew a limb in response to a noxious stimulus. The respiratory activities were monitored by connecting a pneumotachograph (Fleisch) to a respiratory gas monitor (Datex-Ohmeda, Madison WI). Arterial blood gases and pH were also periodically checked (RapidLab 865 blood gas analyzer; Bayer, Tarrytown, NY) and maintained within normal limits (pH 7.35–7.45, PcO2 32–36 mmHg, PaO2 >80 mmHg) by adjusting the ventilator (model 661; Harvard Apparatus, South Natick, MA) or injecting 1 M sodium bicarbonate intravenously. Body temperature was continuously mon-
itoried with a rectal probe and was maintained between 37.5 and 38.5°C with a water-perfused heating pad and an external heating lamp.

**Laminectomy**

The lower lumbar and upper sacral portions of the spinal cord were exposed. The dura was then opened. The L7 and S1 spinal ventral roots were carefully separated and cut close to the spinal cord. The peripheral ends of the transected L7 and S1 ventral roots were then placed on platinum bipolar stimulating electrodes, and the exposed spinal cord region was immersed in a pool of warm mineral oil (37°C).

**Microdialysis Procedures**

A Kopf carrier was used to vertically insert a microdialysis probe (model CMA 10, Bioanalytical Systems; 0.5-mm outer diameter, 1-mm membrane length) 3 mm into the L7 level of the dorsal horn of the spinal cord ipsilateral to the contracting leg. Probes were continuously perfused at a rate of 5 μl/min with artificial extracellular fluid (ECF) buffered to a pH of 7.4. ECF, made fresh for each experiment, contained 0.2% bovine serum albumin, 0.1% bacitracin, and the following ions (in mM): 6.2 K⁺, 134 Cl⁻, 2.4 Ca²⁺, 150 Na⁺, 1.3 P⁻, 13 HCO₃⁻, and 1.3 Mg²⁺.

**Experimental Protocol**

After the microdialysis probe was inserted, the cats were allowed to stabilize for at least 90 min after surgery. Blood pressure and HR responses to a static muscle contraction of the right triceps surae muscle were recorded. Muscle contraction was induced by electrical stimulation (3× motor threshold, 0.1-ms duration, 40 Hz) of the peripheral ends of the L7 and S1 ventral roots for 1 min.

**Microdialysis of PPADS into dorsal horn**. The purpose of this protocol was to examine the role of P2X receptor blockade in blood pressure and HR responses to muscle contraction by microdialyzing PPADS into the dorsal horn (n = 5) of the spinal cord at the L7. First, the control responses to contraction were determined during dialysis of ECF, and then 2.5–20 mM PPADS was dialyzed on the basis of previous reports (1, 2, 18, 31). Each concentration was dialyzed for 40 min, followed by muscle contraction. Finally, ECF was dialyzed after PPADS was discontinued to determine the recovery of the reflex responses. There was a 40-min rest period between bouts of muscle contraction.

In addition, the effect of PPADS on the cardiovascular responses evoked by a passive stretch of the triceps surae muscle was also examined. The muscle was stretched, and tension of ~9 kg was produced over 5–10 s by using a rack and pinion attached to the Achilles tendon of the cat. Muscle stretch was maintained for 1 min after a tension of 9 kg was achieved. Stretch was performed 40 min after microdialysis of ECF and 10 mM PPADS, respectively.

**Microdialysis of α,β-methylene ATP into dorsal horn**. The purpose of this protocol was to examine the role of P2X receptor stimulation in blood pressure and HR responses to muscle contraction by microdialyzing α,β-methylene ATP into the L7 level of dorsal horn (n = 4). Three interventions were performed in this protocol. First, cardiovascular responses to contraction were obtained at the end of 40 min of ECF dialysis. This served as a control. Second, induced responses were performed after 40 min of dialysis of 0.5 mM α,β-methylene ATP. Finally, 2.5 mM PPADS was dialyzed for 30 min, followed by 0.5 mM α,β-methylene ATP for 40 min. Muscle contraction was then conducted.

**Histological Examination**

At the end of each experiment, the spinal cord was removed, fixed in a solution of 10% phosphate-buffered formalin, and then stored at 4°C. After the tissue was adequately fixed, the tracks in the dorsal horn produced by the dialysis probe were examined. The results showed that dialysis probes were located in the dorsal horn. In three cats, 2% sky blue dye was dialyzed into the dorsal horn for 40 min. The rostrocaudal extent of staining was 1.5–2.0 mm and did not reach the ventral horn as reported previously (12).

**Data Acquisition and Analysis**

Arterial blood pressure was measured with a pressure transducer (model P23 ID; Statham, Oxnard, CA) connected to an arterial catheter. Mean arterial pressure (MAP) was obtained by integrating the arterial signal with a time constant of 4 s. HR was derived from the arterial pressure pulse. The calcaneal bone of one hindlimb was cut, allowing the Achilles tendon to be connected to a force transducer for measurement of developed tension during electrically stimulated muscle contraction. The pelvis was stabilized in a spinal unit (Kopf Instruments, Tujunga, CA), and the knee joints were secured by attaching the patellar tendon to a steel post. All measured variables were continuously recorded on an eight-channel chart recorder (model TA 4000; Gould Instruments, Valley View, OH). These variables also were sampled with an iMac computer that was equipped with a PowerLab data-acquisition system (ADInstruments, Castle Hill, Australia).

Control values were determined by analyzing at least 30 s of the data recorded immediately before a given muscle contraction/stretch. The peak response of each variable was determined as the peak change from the control value. Experimental data (MAP, HR, tension) were analyzed using one-way ANOVA with repeated measures. A Tukey post hoc test was utilized as appropriate. All values are expressed as means ± SE. For all analyses, differences are considered significant if P < 0.05. All statistical analyses were performed using Sigma Stat for Windows (version 2.03; SPSS Science Products).

**RESULTS**

**Effect of P2X Blockade on Muscle Pressor Reflex**

The artificial ECF was dialyzed continuously into the dorsal horn to obtain the control MAP and HR responses to muscle contraction before dialysis of 2.5, 5, 10, and 20 mM PPADS. Baseline MAP and HR were 124 ± 16 mmHg and 130 ± 7 beats/min, respectively. Those basal values were not altered by the dialysis of PPADS (P > 0.05). Furthermore, there were no significant differences in baseline MAP and HR before each bout of contraction. Muscle contraction significantly increased MAP and HR compared with basal values before contraction. However, the increases for MAP and HR were smaller after dialysis of PPADS (ΔMAP: 42 ± 8 mmHg in control vs. 16 ± 4 mmHg after 10 mM PPADS, P < 0.05; Fig. 1). There were no significant differences in developed tension among the interventions. This result suggests that P2X blockade within the dorsal horn of the spinal cord attenuates the reflex responses to muscle contraction.

In addition, muscle stretch also increased MAP and HR. Dialysis of 10 mM PPADS into the L7 level of the dorsal horn blunted the pressor and HR responses at 40 min of the perfusion (Fig. 2). Baseline values for MAP were 114 ± 12 mmHg for ECF control and 110 ± 8 mmHg for PPADS (P > 0.05 vs. control).

**Effect of P2X Activation on Muscle Pressor Reflex**

The basal MAP and HR before muscle contraction were 121 ± 11 mmHg and 127 ± 10 beats/min for ECF control. Those values were not altered by the dialysis of α,β-methylene ATP (P > 0.05). There were no significant differences in
baseline MAP and HR before each bout of contraction. Forty minutes after P2X receptor agonist α,β-methylene ATP was dialyzed, the pressor response evoked by muscle contraction was significantly enhanced (Fig. 3). Developed tensions by contraction were similar (Fig. 3).

Figure 3 further shows that the augmented pressor response produced by α,β-methylene ATP was antagonized by prior dialysis of PPADS, suggesting that the effect of α,β-methylene ATP was mediated via P2X.

**DISCUSSION**

The purpose of this study was to determine whether ATP-sensitive P2X receptors within the dorsal horn of the spinal cord, the first synaptic site of the muscle pressor reflex, play a role in mediating the reflex. The results of this study show that blockade of P2X receptors within the dorsal horn abolished the pressor response to static contraction and passive muscle stretch (Figs. 1 and 2). Moreover, our data also show that P2X...
stimulation enhanced the reflex response and that the effect was antagonized by prior administration of PPADS, a selective receptor blocker (Fig. 3). Thus spinal ATP and P2X purinoceptors play an important role in the processing of muscle afferent inputs emanating from the stimulation of group III and IV thin fiber muscle afferents.

P2X purinoceptors are localized on the central terminals of thin fiber afferent nerves in the superficial dorsal horn of the spinal cord (33–35). This presynaptic localization suggests that the receptors may play a role in controlling neurotransmitter release. There is some evidence that presynaptic P2X receptors facilitate glutamate release. For example, whole cell recordings from neurons in spinal cord slices have shown that ATP applied in a bath potentiated both glutamate- and synaptically induced currents (24). Furthermore, in a dorsal root ganglion-dorsal horn coculture system, ATP application induced glutamate release onto dorsal horn neurons (10, 22). Another study (27) demonstrated that when P2X receptor antagonist PPADS was given during primary afferent stimulation, glutamatergic excitatory postsynaptic currents in superficial dorsal horn neurons were inhibited. Consistent with these in vitro results, in vivo data in the mouse spinal cord have shown that hyperalgesia evoked by intrathecal applied α,β-methylene ATP was antagonized by the exocytosis inhibitor botulinum neurotoxin B and N-methyl-D-aspartate (NMDA) glutamate receptor antagonist (32). Together, these findings indicate that synaptically released ATP is a positive modulator of glutamatergic transmission in the spinal cord via presynaptic P2X receptors.

Hand et al. (11) previously reported that static muscle contraction increased the extracellular concentration of glutamate in the dorsal horn. It was also found that microdialysis of antagonists to NMDA/non-NMDA glutamate receptors into the dorsal horn significantly attenuated the reflex cardiovascular responses to muscle contraction (12, 13, 37). These findings support the role played by glutamate and its receptors in processing the muscle reflex within the dorsal horn. In the present study, our data show that dialyzing PPADS into the dorsal horn blunted the reflex pressor response to contraction. We speculate that presynaptic P2X receptor blockade inhibited glutamate release and the muscle pressor reflex evoked by muscle stimulation was attenuated (Fig. 4). Conversely, dialysis of α,β-methylene ATP into the dorsal horn facilitated glutamate release and augmented the reflex response. Important questions raised by the current findings are as follows: 1) Does the spinal cord extracellular ATP concentration rise with muscle contraction? and 2) When P2X blockers are given within the dorsal horn during muscle contraction, is glutamate release attenuated? Finding the answers to these questions requires additional studies.

It has been reported that postsynaptic P2X receptors exist on dorsal horn neurons. Iontophoretic ATP administration in vivo excited the neuronal cells of the dorsal horn (9, 29, 30). Thus postsynaptic P2X receptors also may play a potential role in spinal transmission of neural signals in the processing of thin fiber afferents. This postsynaptic mechanism also supports our observations that blockade of P2X receptor within the dorsal horn attenuated the pressor response induced by stimulation of muscle afferent fibers (Fig. 4). However, studies have shown that only a small proportion of the dorsal horn has purinergic synaptic input. For example, only <5% of the tested lamina II neurons showed ATP-mediated excitatory postsynaptic current in response to dorsal root stimulation (1). Another study found that a residual current in superficial dorsal horn neurons of spinal cord slices could not be detected after the glutamatergic component of excitatory postsynaptic current evoked by afferent fiber stimulation was blocked (27). Thus a presynaptic mechanism of ATP activation of P2X receptors may play a major role in the processing of thin fiber muscle afferent signals. Nonetheless, either presynaptic action of ATP at P2X receptors enhancing glutamate release or ATP excitatory effects via postsynaptic P2X receptors on second-order neurons is consistent with our findings in this report (Fig. 4).

Fig. 3. Effect of microdialyzing α,β-methylene ATP into the dorsal horn on the peak MAP and HR responses during 1-min muscle contraction. Prior dialysis of PPADS (30 min) selectively antagonized the effect of P2X stimulation. α,β-Methylene ATP was dialyzed for 40 min before evoked contraction. Values are means ± SE (n = 4). *P < 0.05, significant difference vs. ECF control.

Fig. 4. Schematic depicting the possible mechanisms of P2X receptors modulating the reflex muscle response in the dorsal horn of the spinal cord. The pre- and postsynaptic roles of P2X receptors are shown. Stimulation of thick fiber muscle afferent increases glutamate release in the dorsal horn and mediates reflex muscle responses. Presynaptic P2X blockade attenuates glutamate release and inhibits the response. P2X receptors also exist on the dorsal horn neurons. Postsynaptic P2X blockade also may attenuate the pressor response seen when muscle afferents are engaged. NMDA, N-methyl-D-aspartate.
Finally, whole recordings from cultured neurons of superficial dorsal horn have shown that ~50% of the cells utilize ATP as an excitatory transmitter acting at P2X-sensitive P2X receptor (19). Those neuronal cells corelease the inhibitory neurotransmitter GABA with ATP (19). Wilson (36) reported that administration of muscimol (a GABA receptor agonist) in the dorsal horn abolished the reflex pressor response to stimulation of muscle afferent fibers. Thus P2X receptor-mediated activation of inhibitory interneurons by synthetically released ATP could inhibit transmission of neural signals from thin fiber activation within the dorsal horn. Speculatively, a fine balance of excitatory and inhibitory components of ATPergic transmission exists under normal conditions. It is also likely that this balance could shift under pathological conditions, such as heart failure. Currently, we know that P2X-mediated reflex pressor responses are augmented in rats in which heart failure is induced by myocardial infarctions (25).

In conclusion, spinal P2X purinoceptors play a role in mediating the reflex cardiovascular responses to activation of muscle afferent fibers. ATP and P2X receptors are likely involved in the processing of this neural signal in the superficial dorsal horn, and ATP plays a role as a neurotransmitter/modulator.

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