Spinal P2X receptor modulates muscle pressor reflex via glutamate

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Li J, Lu J, Gao Z, Koba S, Xing J, King N, Sinoway L. Spinal P2X receptor modulates muscle pressor reflex via glutamate. J Appl Physiol 106: 865–870, 2009. First published August 8, 2009; doi:10.1152/japplphysiol.90879.2008.—Static contraction of skeletal muscle evokes reflex increases in blood pressure and heart rate. Previous studies showed that P2X receptors located at the dorsal horn of the spinal cord play a role in modulating the muscle pressor reflex. P2X stimulation can alter release of the excitatory amino acid, glutamate (Glu). In this report, we tested the hypothesis that stimulation of P2X receptors enhances the concentrations of Glu ([Glu]) in the dorsal horn, and that blocking P2X receptors attenuates contraction-induced Glu increases and the resultant reflex pressor response. Contracture was elicited by electrical stimulation of the L7 and S1 ventral roots of 14 cats. Glu samples were collected from microdialysis probes inserted in the L7 level of the dorsal horn of the spinal cord, and dialysate [Glu] was determined using the HPLC method. First, microdialyzing α,β-methylene ATP (0.4 mM) into the dorsal horn significantly increased [Glu]. In addition, contraction elevated [Glu] from baseline of 536 ± 53 to 1,179 ± 192 nM (P < 0.05 vs. baseline), and mean arterial pressure by 39 ± 8 mmHg in the control experiment. Microdialyzing the P2X receptor antagonist pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), dialyzed into the dorsal horn of the spinal cord, and dialysate [Glu] was determined using the HPLC method. In the control experiment, L7 stimulation increased [Glu] in the dorsal horn from baseline of 536 ± 53 to 1,179 ± 192 nM (P < 0.05 vs. baseline), and mean arterial pressure by 39 ± 8 mmHg in the control experiment. Microdialyzing the P2X receptor antagonist pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) attenuated the contraction-induced Glu increase (610 ± 128 to 759 ± 147 nM, P > 0.05) and pressor response (16 ± 3 mmHg, P < 0.05 vs. control). Our findings demonstrate that P2X modulates the cardiovascular responses to static muscle contraction by affecting the release of Glu in the dorsal horn of the spinal cord. NEURAL SIGNALS FROM CONTRACTING skeletal muscle are generated by activating mechanically and metabolically sensitive nerve endings (receptors) located in the skeletal muscle and subsequently carried to the central nervous system by thin group III and IV afferent fibers (22, 23). This neural processing increases arterial blood pressure and heart rate (HR) (9, 31). Together, activation of these receptors, along with the reflex cardiovascular responses, is referred to as the “exercise pressor reflex” (9, 22, 31). It is known that the majority of these thin-fiber afferent nerves makes their first synapse in the superficial dorsal horn of the spinal cord. Studies further demonstrate the neural transmitters/modulators involved in transmitting the muscle reflex are located here (15–18, 27, 42–44).

Purinergic P2X receptors are selectively expressed in small- and medium-diameter sensory neurons (1, 5, 21, 38–40). ATP has been shown to be responsible for the transmission of signals from the peripheral afferent nerve to the dorsal horn via P2X receptors (1, 2, 6–8, 10). A previous study has shown that activation of P2X receptors by microdialyzing α,β-methylene ATP (α,β-me ATP) into the dorsal horn significantly augments the cardiovascular responses to static muscle contraction (12). The P2X receptor antagonist, pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), dialyzed into the dorsal horn, attenuates the muscle reflex, suggesting that P2X receptors play a role in mediating the muscle pressor reflex at the dorsal horn of the spinal cord (12). However, the underlying mechanism needs to be investigated.

P2X receptors are localized on the central terminals of thin-fiber afferent nerves in the superficial dorsal horn of the spinal cord (38–40). There is strong evidence that presynaptic P2X receptors facilitate glutamate (Glu) release. For example, whole cell recordings from neurons in spinal cord slices have shown that ATP applied in bath solutions increases release of the Glu and synaptically induces currents (28). Furthermore, in a dorsal root ganglion-dorsal horn co-culture system, the application of ATP induces Glu release onto dorsal horn neurons (13, 14, 24). Another study (29) demonstrated that, when the P2X receptor antagonist PPADS was given during primary afferent stimulation, glutamatergic excitatory postsynaptic currents in the superficial dorsal horn neurons were inhibited. These findings indicate that released ATP at synapses is a positive modulator of glutamatergic transmission in the spinal cord via presynaptic P2X receptors.

Therefore, in this report, we first determined the concentrations of Glu ([Glu]) in the dorsal horn using microdialysis methods when P2X receptors were activated. We further determined whether blocking P2X receptors with PPADS attenuated the [Glu] and the reflex cardiovascular responses to muscle contraction. We hypothesized that P2X modulates the reflex cardiovascular responses by affecting the release of Glu in the dorsal horn of the spinal cord.

METHODS

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

Experiments were performed on 14 anesthetized male cats (body weight: 4.6–6.0 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). An endotracheal tube was inserted into the trachea via a tracheotomy and attached to a ventilator (model 683, Harvard, South Natick, MA). A jugular vein and common carotid artery were cannulated using PE-90 of polyethylene catheters 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 ventilator was set with a tidal volume of 20 ml/stroke and a rate of 20–30 strokes/min. A respiratory gas monitor (Datex-Ohmeda, Madison, WI) was used to monitor volumes of O₂ and CO₂. 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 Torr; PO₂ >80 mmHg; HCO₃⁻: 20–25 mmol/l) by adjusting the ventilator or injecting sodium bicarbonate intravenously. Body temperature was continuously monitored with a rectal probe and was maintained between 37.5 and 38.5°C by a water-perfused heating pad and an external heating lamp.

Laminectomy and Measurement of Muscle Tension

The lower lumbar and upper sacral portions of the spinal cord were exposed. The dura was then opened. The L7 and S₁ ventral spinal 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).

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 joint secured by attaching the patellar tendon to a steel post.

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 dorsal horn of the spinal cord ipsilateral to the contracting leg. The probe was then attached to a perfusion pump (model 102, CMA) and continuously perfused at a rate of 5 μl/min with artificial extracellular fluid (ECF) buffered to pH 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 P⁵⁻, 13 HCO₃⁻, and 1.3 Mg²⁺. At the end of each protocol, the dialysate was collected in 250-μl microcentrifuge tubes, immediately sealed, and stored at 4°C. After the tissue was adequately fixed, the tracks in the dorsal horn produced by the dialysis probe were examined. In six cats, 2% sky blue dye were 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 (16). We have confirmed that dialysis probes were positioned in the dorsal horn in all animals that were included for data analysis in this experiment.

Experimental Protocol

After the microdialysis probe was inserted, the cats were allowed to stabilize for at least 90 min. Blood pressure and HR responses to a static muscle contraction of the triceps surae muscle were recorded. The muscle contraction was induced by electrical stimulation (three trains of supramaximal stimuli, 100 Hz for 10 sec) every 30 sec. The arterial signal with a time constant of 4 s. HR was derived from the arterial pressure pulse. All measured variables were continuously recorded on an eight-channel chart recorder (Gould Instruments, model TA 4000, Valley View, OH). These variables were also sampled by a personal computer that was equipped with PowerLab data-acquisition system (ADInstruments, Castle Hill, Australia). The tension-time index was calculated by integrating the area between the tension trace during muscle contraction and the baseline level using the PowerLab software and was expressed as kilogram times seconds.

Control values were determined by analyzing at least 30 s of the data immediately before a given muscle contraction. Experimental data (MAP, HR, time-tension index and Glu) were analyzed using one-way ANOVA with repeated measures. Tukey post hoc tests were utilized as appropriate. All values were expressed as means ± SE. For all analyses, differences were considered significant if P < 0.05. All statistical analyses were performed using SPSS for Windows version 15.0 (SPSS Sci.).

RESULTS

P2X Activation Increased [Glu] in Dorsal Horn

As reported previously (15, 25, 26), the level of [Glu] stabilized ~120–180 min after insertion of dialysis probes into the nerve tissues. [Glu] was 2,246 ± 632, 1,224 ± 250, 847 ± 198, and 615 ± 151 nM 60, 40, 20, and 10 min before starting of the first protocol, respectively. Figure 1 shows that dialyzing α,β-me ATP into the dorsal horn of the spinal cord increased the [Glu] in six cats. Furthermore, ECF was dialyzed after discontinuing α,β-me ATP to determine the postintervention (Fig. 1).

In addition, 2.5 mM of PPADS was dialyzed for 20 min and followed by 0.4 mM of α,β-me ATP for 10 min in a subset of the experiment. The dialysate from 10-min collection of
αβ-me ATP dialyzing was analyzed for Glu. The prior dialysis of PPADS significantly attenuated the effects of αβ-me ATP. The Glu was 614 ± 76 nM during αβ-me ATP with prior dialysis of PPADS (P < 0.05 vs. 0.4 mM αβ-me ATP).

**P2X Blockade Attenuated Contraction-induced Glu Elevation**

A prior report has shown that blocking P2X receptors by dialyzing PPADS into the dorsal horn significantly attenuates the pressor response induced by static muscle contraction (12). The effect of blockade of P2X receptors is likely mediated via altering Glu. To test this possibility, 5-min contraction was first performed during ECF dialysis (Fig. 2). The contraction significantly increased [Glu] from baseline. After ECF control was obtained, 2.5, 5.0, and 10 mM of PPADS were individually dialyzed into the dorsal horn. At the end of each concentration, 5-min contractions were repeated. Figure 2 demonstrates that PPADS attenuated the contraction-induced Glu elevation. Finally, the Glu response was examined by dialyzing ECF into the dorsal horn after discontinuing PPADS. Contraction increased [Glu] from 551 ± 72 to 996 ± 123 nM (P < 0.05 vs. baseline). This result shows that blocking P2X receptors in the dorsal horn decreased the release of Glu evoked by muscle contraction.

**Cardiovascular Responses to Muscle Contraction**

**Dialysis of αβ-me ATP (n = 6).** The basal MAP and HR before dialysis of ECF were 97 ± 6 mmHg and 140 ± 6 beats/min, respectively. The averaged values of MAP and HR were not significantly altered by the dialysis of αβ-me ATP. They were 98 ± 7 mmHg and 135 ± 7 beats/min; 97 ± 7 mmHg and 134 ± 6 beats/min; and 96 ± 7 mmHg and 136 ± 7 beats/min during dialyzing of 0.1, 0.2, and 0.4 mM of αβ-me ATP, respectively.

**Dialysis of PPADS (n = 8).** The ECF was dialyzed continuously into the dorsal horn to obtain the control MAP and HR responses to muscle contraction before 2.5, 5, and 10 mM of PPADS. Table 1 shows that no significant differences were seen in baseline MAP and HR before each bout of contraction. In the control condition, muscle contraction significantly increased MAP and HR compared with basal values before contraction. However, MAP and HR increases were attenuated after dialyzing PPADS (Fig. 3). Also, Fig. 3 shows that there were no significant differences in developed tension among the interventions.
of PPADS significantly attenuated the effects of dorsal horn. An additional result shows that the prior dialysis stimulation of P2X receptors increases the release of Glu in the dorsal horn when P2X receptors were activated (Fig. 1). This result indicates that the release of Glu in the dorsal horn was increased when the pressor response to contraction. It is reasoned that blocking presynaptic P2X receptor inhibited Glu release, and thereby the muscle pressor reflex was attenuated.

In this experiment, dialyzing α,β-me ATP into the dorsal horn increased the Glu but not evoked cardiovascular changes. Thus there is a possibility that stimulation of P2X receptors can

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<th>MAP, mmHg</th>
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<td>Baseline</td>
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<tr>
<td>Control</td>
<td>113±8</td>
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<td>PPADS</td>
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Values are means ± SE; n = 8 animals. MAP, mean arterial pressure; HR, heart rate; PPADS, pyridoxal phosphate-6-azophenyl-2,4′-disulfonic acid. There is no significant difference among basal values. *P < 0.05 vs. baseline.

DISCUSSION

The main purpose of this study was to determine whether P2X receptors modulated the muscle pressor reflex by affecting the release of Glu in the dorsal horn. The first experiment demonstrates that [Glu] in the dorsal horn was increased when P2X receptors were activated (Fig. 1). This result indicates that stimulation of P2X receptors increases the release of Glu in the dorsal horn. An additional result shows that the prior dialysis of PPADS significantly attenuated the effects of α,β-me ATP, indicating that the effects of α,β-me ATP were mediated via P2X receptors. In the second experiment, we further examined the Glu and reflex cardiovascular responses to muscle contraction after blocking P2X receptors with PPADS. Our results show that blockade of P2X receptors within the dorsal horn attenuated contraction-induced [Glu] elevation, as well as the pressor response (Figs. 2 and 3). This result suggests that inhibition of Glu release within the dorsal horn of the spinal cord by blocking P2X can attenuate the reflex pressor response to muscle contraction. Spinal ATP and P2X are likely to play a role in the processing of muscle afferent inputs in mediating the muscle reflex.

Iontophoretic ATP administration in vivo has been reported to excite the neuronal cells of the dorsal horn (11, 36, 37), suggesting postsynaptic P2X receptors appear on dorsal horn neurons. However, studies have shown that only a small proportion of the dorsal horn have purinergic synaptic input. For example, <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 blocking the glutamatergic component of excitatory postsynaptic current evoked by afferent fiber stimulation (29). Overall, previous findings support the concept that released ATP at synapses facilitates glutamatergic transmission via presynaptic P2X receptors (14, 29, 32). In addition, static muscle contraction is likely to increase ATP in the dorsal horn, because blocking ATP-sensitive P2X receptors within the dorsal horn attenuates the pressor response to contraction (12). Thus we speculate that the action of ATP at presynaptic P2X receptors to enhance Glu release plays a role in mediating the muscle pressor reflex. However, it is noted that the data provided in the present experiment cannot rule out a postsynaptic action.

Hand and colleagues previously reported that static muscle contraction increased [Glu] in the dorsal horn (15). Also, microdialysis of antagonists to N-methyl-D-aspartate/non-N-methyl-D-aspartate Glu receptors into the dorsal horn significantly attenuated the reflex cardiovascular responses to muscle contraction (16, 17, 41). These findings suggest that released Glu and its receptors play an important role in processing the muscle reflex within the dorsal horn. In the present study, our data show dialyzing PPADS into the dorsal horn decreased contraction-induced [Glu] release and blunted the reflex pressor response to contraction. It is reasoned that blocking presynaptic P2X receptor inhibited Glu release, and thereby the muscle pressor reflex was attenuated.

In this experiment, dialyzing α,β-me ATP into the dorsal horn increased the Glu but not evoked cardiovascular changes. Thus there is a possibility that stimulation of P2X receptors can...
modulate the releases of other neurotransmitters/modulators. A prior study suggests that presynaptic P2X receptors facilitate inhibitory GABAergic transmission of interneurons in rat spinal cord dorsal horn (19). It is known that GABA plays an inhibitory role in the processing of the muscle pressor reflex because activation of GABA receptors (GABAA subtype) by applying agonist muscimol to the dorsal surface of the spinal cord can abolish the muscle pressor reflex (45). The GABA engagement might attenuate effect of P2X stimulation when αβ-me ATP was dialyzed into the dorsal horn. Thus no cardiovascular changes were seen in our present experiment. In contrast, static muscle contraction stimulates thin-fiber muscle afferent nerves and then induces the releases of neurotransmitters/modulators, including Glu and substance P, etc. (43). In addition to P2X, other receptors within the dorsal horn are also engaged in the processing of the reflex responses (12, 27, 42–45). Nevertheless, it is postulated that ATP release in response to activation of primary afferent fibers, enhancing Glu release from the same afferents, is effectively a positive feedback. Also, activation of P2X receptors is likely to exert a negative action by facilitating GABAergic transmission. Clearly, additional experiments are necessary to clarify precise mechanisms.

There are seven P2X receptor subtypes (P2X1-7) with different tissue distributions (33). Among these subunits, P2X3 receptors are selectively expressed in small- and medium-diameter sensory afferent neurons in rats (34, 39). Also, P2X3 receptors have been localized at the central and peripheral terminals of these sensory neurons (3, 30). Previous studies have shown that purinergic P2X3 receptors appear in the dorsal horn (30), and activation of presynaptic P2X3 receptors at this site plays a role in the release of Glu (4). Thus it is very likely that spinal P2X3 purinoceptors play a role in regulating the reflex cardiovascular responses to muscle contraction.

However, it should be noted that we examined the effects of αβ-me ATP and PPADS on the Glu in this study. αβ-me ATP stimulates mainly P2X2, P2X3, and P2X1 receptor subtypes, and that PPADS is a nonselective blocker to P2X (4, 20, 35). Thus additional investigation is still necessary to study precise mechanism of P2X subtypes in modulating the muscle pressor reflex when specific P2X subtype agonists/blockers become available.

In conclusion, P2X modulates the reflex cardiovascular responses to activation of muscle afferent fibers by affecting the release of Glu in the dorsal horn of the spinal cord. The results further support the idea that ATP and purinergic P2X receptors in the dorsal horn are involved in the processing of the neural signal to evoke and modulate the muscle pressor reflex.

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GRANTS

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REFERENCES


