ATP P_2x Receptors and Sensory Synaptic Transmission Between Primary Afferent Fibers and Spinal Dorsal Horn Neurons in Rats

PING LI, AMELITA A. CALEJESAN, AND MIN ZHUO
Department of Anesthesiology, Washington University, School of Medicine, St. Louis, Missouri 63110-1093

INTRODUCTION

The superficial dorsal horn of the spinal cord is important for sensory transmission, including information coding nociceptive information (Christensen and Perl 1970; Light et al. 1979; Light and Perl 1979; Kumazawa and Perl 1978; Pearson 1952; Rethelyi et al. 1989; Sugiuera et al. 1986). Glutamate is a major neurotransmitter, and fast synaptic transmission in the dorsal horn is mainly mediated by alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate and N-methyl-D-aspartate (NMDA) receptors (Glaum et al. 1994; Horii et al. 1996; Nastrom et al. 1994; Randic et al. 1993; Schneider and Perl 1988, 1994; Yoshimura and Jessell 1990; see Yaksh and Malmberg 1994 for a review). ATP may serve as a neuromodulator to enhance glutamate induced responses by a postsynaptic mechanism (Li and Perl 1998). Although a synaptic action of ATP P_2x receptors on glutamate release was recently reported (Gu and MacDermott 1997), the involvement of postsynaptic P_2x receptors in synaptic transmission between primary afferent fibers and dorsal horn neurons is still unclear. ATP may serve as a neuromodulator to enhance glutamate induced responses by a postsynaptic mechanism (Li and Perl 1995) or induce postsynaptic currents in a subpopulation of dorsal horn neurons (Bardoni et al. 1997). In this study, we used in vitro whole cell patch-clamp recording techniques and in vivo behavioral tests to examine the role of P_2x receptors in sensory synaptic transmission and behavioral nociception.

METHODS

Slice preparation

Transverse spinal cord slices from postnatal (P4–P17) rats (Sprague-Dawley; Harlan) were prepared with the method of Taka-hashi (1990) with some modifications. Rats were deeply anesthetized with halothane and then cooled on ice for 10 min. The lumbar spinal cord was exposed, and ice-cold physiological saline solution (all in mM: 113 NaCl, 3 KCl, 25 NaHCO_3, 1 NaH_2PO_4, 2 CaCl_2, 1 MgCl_2, and 25 d-glucose, equilibrated with 95% O_2-5% CO_2, pH 7.3) was poured over it to cool the spinal cord. A segment of lumbar spinal cord was quickly removed and immersed in oxygenated, ice-cold saline for 3 min. The dura was removed and cross-section slices (150–200 μm in thickness) were cut with a Vibratome. The slices were kept in a filtering funnel filled with oxygenated saline at room temperature for 2 h.

Whole cell patch-clamp recording

Whole cell voltage-clamp experiments were performed with an Axopatch 200B patch-clamp amplifier. The slice was continuously perfused with oxygenated saline. Visually guided whole cell patch-clamp recordings were performed with the aid of an Axioskop microscope. The exact sites of recorded neurons were determined by biocytin labeling. Biocytin (0.5%) was added into the recording solution to label the cell. Whole cell patch-clamp recordings were made with 5–10 MΩ electrodes without fire polishing. The recording pipette solution contained (in mM) 110 CsMeSO_4, 5 MgCl_2, 1 ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid (EGTA), 40 N-2-hydroxyethylpiperazine-N′-2-eth-
FIG. 1. Excitatory monosynaptic responses in the superficial dorsal horn of the lumbar spinal cord are mediated by glutamatergic by \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors and \(N\)-methyl-D-aspartate (NMDA) receptors. A: evoked excitatory postsynaptic currents (EPSCs) induced electrical stimulation of the dorsal root nerves. Five responses induced by stimulation at a low intensity were displayed (with a 20-s interval). The time of stimulation is indicated by an arrow. B: evoked EPSCs induced electrical stimulation of the dorsal root nerves. In the same experiment as A, electrical stimulation at a high intensity elicited polysynaptic responses. Two responses induced at a 20-s interval were displayed. C: EPSCs induced by electrical stimulation of dorsal root nerves were completely blocked by application of both a AMPA/kainate receptor antagonist CNQX (10 \(\mu\)M) and a NMDA receptor antagonist AP-5 (50 \(\mu\)M). D: evoked monosynaptic EPSCs induced by placing a stimulating electrode at the dorsal root entry zone (DREZ) in the dorsal horn. The time of stimulation is indicated by an arrow. Five responses induced at a 20-s interval were displayed. One response of reduced magnitude was induced by stimulation at a low intensity. No polysynaptic response was observed. E: EPSCs induced by stimulation at the DREZ were completely blocked by application of both an AMPA/kainate receptor antagonist CNQX (10 \(\mu\)M) and a NMDA receptor antagonist AP-5 (50 \(\mu\)M). F: distribution of cells, which were labeled and from which no residual current was recorded after the blockade of glutamatergic receptors, illustrated on a representative coronal spinal cord slice (Paxinos and Watson 1997). Twenty-eight of 56 cells were successfully labeled.

Biocytin staining

Slices were fixed in cold 10 mM phosphate-buffered saline (PBS; pH 7.2) containing 4% paraformaldehyde (1–7 days at 4°C) and were then rinsed several times for 10 min each in PBS. To block endogenous peroxidases, slices were transferred into 1% anesulfonic acid sodium, 2 MgATP, 0.1 Na,GTP (pH 7.2, HCl), and the osmolarity was adjusted to 295–300 mosmol. After touching the soma with the pipette tip, gentle suction was applied to improve the seal until a high resistance (>10 GΩ) was reached. Membrane potential was clamped at −70 mV (liquid junction potential not corrected). Typical series resistance was 14–39 MΩ and monitored throughout the experiments.

Postsynaptic excitatory postsynaptic potentials (EPSCs) were evoked at 0.02–0.05 Hz with a bipolar tungsten electrode placed at the dorsal root entry zone (DREZ) or dorsal root afferent fibers. EPSCs were filtered at 1 kHz and digitized at 5 kHz. Monosynaptic EPSCs were identified with two criteria (Hori et al. 1996); 1) the response latency did not change with increasing intensities of electrical stimulation, and 2) EPSCs followed high-frequency stimulation (50 Hz) with reduced amplitude and without changing response latency. Inhibitory influences were removed by including bicuculline methiodide (10 \(\mu\)M) and strychnine hydrochloride (1 \(\mu\)M) in the perfusion solution.

Behavioral nociceptive tests

Adult, male rats (Sprague-Dawley; Harlan) were used. The spinal nociceptive tail-flick reflex was evoked by noxious heat focused on the underside of the tail. The latency to reflexive removal of the tail from the heat was measured. A cutoff time of 12 s was employed to minimize damage to the skin of the tail. The hot-plate reflex was measured with a controlled metal plate at 50°C. A cutoff time of 65 s was used. The nociceptive responses were licking, lifting hindpaw, or jumping off the hot-plate, and the latency for response was recorded.

Intrathecal drug administration

The intrathecal injection was carried out as described previously (Hylden and Wilcox 1980) with some modifications. To avoid
FIG. 2. Presynaptic ATP $P_2^\times$ receptors regulate the release of glutamate. A: example of EPSCs to a paired-pulse stimulation delivered at a 50-ms interval before (left) or 10 min after (right) application of 10 $\mu$M pyridoxal-phosphate-6-azophenyl-2'4'-disulfonic acid (PPADS). The time of stimulation is indicated by arrows. B: summary of data from 6 experiments. The paired-pulse stimulation ratio ($P$ ratio = the 1st peak current/the 2nd peak current × 100) was calculated before and 10 min after 10 $\mu$M PPADS. Data are presented as means ± SE, * $P < 0.05$. C: model explaining the effect of PPADS on fast synaptic transmission in the lumbar spinal cord. At normal condition, fast synaptic transmission is mediated by postsynaptic glutamatergic AMPA and/or kainate receptors. ATP released from primary afferent fibers and/or spinal cord interneurons binds to presynaptic P2$^\times$ receptors and enhances the release of glutamate (Glu). ATP may also act on postsynaptic P2$^\times$ receptors to modulate glutamatergic synaptic transmission (see Li and Perl 1995).

Possible stress induced during intrathecal injection, rats were anesthetized with halothane (2%) during injection. After the injection, it took 2–3 min for rats to recover. The volume of the injection was 10 $\mu$l. Although baseline nociceptive thresholds were not affected by intrathecal administration of saline, we cannot completely exclude the potential damage introduced by intrathecal administration.

Data and analysis

Data are presented as means ± SE. Statistical comparisons were made with the use of analyses of variance (Newman-Keuls tests for post hoc comparison) or Student’s $t$-test. $P < 0.05$ was considered significant.

Results

To induce synaptic responses in the superficial dorsal horn, stimulating electrodes were placed at the DREZ ($n = 120$) or attached dorsal root nerves ($n = 10$). EPSCs were recorded from dorsal horn neurons in both cases. Direct stimulation of the dorsal root nerves induced monosynaptic EPSCs at low intensities (Fig. 1A); however, polysynaptic responses were often recorded when the intensity was increased ($n = 10$; see Fig. 1B). To stimulate a smaller population of afferent fibers, we placed stimulating electrodes near the DREZ. Short and constant latency fast EPSCs were induced. These EPSCs were monosynaptic because the response latency did not change with increasing intensities of electrical stimulation. Furthermore, they followed high-frequency stimulation (50 Hz) with reduced amplitude and without changing in latency. The amplitudes of EPSCs were stable when single stimulation pulses were delivered at $\leq 0.02$ Hz.

To test if ATP may also be involved in fast synaptic transmission, we carried out experiments with two types of glutamate receptor antagonists, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 $\mu$M) for AMPA/kainate receptors and AP-5 (50 or 100 $\mu$M) for NMDA receptors, to block glutamate receptor-mediated synaptic responses. If there are other fast transmitters involved, we should have been able to detect some residual currents. In a total of 56 neurons tested, fast EPSCs were completely blocked by co-application of CNQX and AP-5 (Fig. 1E); 28 neurons were successfully labeled. They were located in lamina I ($n = 5$), II ($n = 14$), and III ($n = 5$). Similar results were obtained with direct stimulation of dorsal root nerves ($n = 10$; Fig. 1C). The intensity of electrical stimulation used was strong enough to activate high-threshold nociceptive unmyelinated C fibers because at the same intensity slow, substance P/neurokinin A-mediated synaptic currents were induced when six pulses of stimuli were delivered at 25 Hz (Li and Zhuo, unpublished data).

$P_2^\times$ receptors are highly expressed in dorsal root ganglia (Brake et al. 1994; Chen et al. 1995; Cook et al. 1997), and activation of presynaptic P2$^\times$ receptors elicited glutamate
release (Gu and MacDermott 1997). Several drugs were used to block P2X receptors. Among them, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) is a selective antagonist for P2X receptors (see Dale and Gilday 1996). We thus investigated possible presynaptic effects of PPADS. EPSCs to a paired-pulse stimulation at a 50-ms interval were recorded before and after application of 10 μM PPADS (PPADS at this dose did not affect other receptors) (see Dale and Gilday 1996). Interestingly, EPSCs were significantly decreased (n = 6, from 80.7 ± 19.8 pA to 38.9 ± 9.7 pA; P < 0.05). This inhibition was accompanied by significant changes in the ratio of paired-pulse responses (Fig. 2). In an example shown in Fig. 2, paired-pulse depression was observed before the application. At 10 min after PPADS, paired-pulse facilitation was induced (Fig. 2). Similar results were obtained from another five experiments (Fig. 2), indicating that PPADS produced its effect by blocking presynaptic P2X receptors.

A previous study showed that PPADS did not produce any antinociceptive effect in the tail-flick (TF) test (Driessen et al. 1994). We reexamined the effect of PPADS in the TF reflex and also carried out experiments with the hot-plate (HP) test. Intrathecal administration of PPADS (0.6 or 5.9 μg) into the lumbar spinal cord produced no significant change in response latencies (Fig. 3). The skin temperature of the tail was not significantly affected (mean 22.5 ± 0.5°C, n = 5). At a higher dose (59 μg), PPADS induced abnormal aggressive behavior and made measurements impossible (n = 3). Rats became hypersensitive to gentle touch and thus no more testing was carried out. Intrathecal injection of vehicle saline (10 μl) affected neither the TF nor HP latency (n = 5; Fig. 3). Morphine, however, produced significant antinociceptive effects in the TF (n = 5) and HP (n = 5) tests when administered intrathecally at a similar quantity (10 μg; Fig. 3).

**DISCUSSION**

At the superficial dorsal horn of the lumbar spinal cord, we confirmed results of previous studies that glutamate is a major fast excitatory transmitter. No evidence was found for the involvement of P2X receptors in synaptic transmission between primary afferent fibers and dorsal horn neurons. Consistent with previous reports (Li and Perl 1995; Bardoni et al. 1997), our results suggest that P2X receptors do not play a major role in sensory transmission between primary afferent fibers and dorsal horn neurons at the lumbar spinal cord. In support of studies using cultured neurons (Gu and MacDermott 1997), presynaptic P2X receptors regulate the presynaptic release of glutamate. Behavioral tests showed no antinociceptive effect produced after the blockade of spinal P2X receptors by PPADS; if anything, there is a hyperalgesic effect at a high dose.

ATP has been proposed as an excitatory transmitter for primary afferent fibers (Jahr and Jessell 1983; Salter and Henry 1985; Salter and Hicks 1994). P2X receptors are ligand-gated ion channels and were identified in dorsal root ganglion cells as well as the superficial dorsal horn of the spinal cord (Brake et al. 1994; Buell et al. 1996; Collo et al. 1996; Cook et al. 1997; Vulchanova et al. 1996; see Burnstock and Wood 1996 for a review). At the cervical spinal cord, EPSCs induced by focal electrical stimulation were mediated by P2X receptors in a subpopulation (<5%) of dorsal horn neurons (Bardoni et al. 1996), providing evidence that ATP may contribute to sensory synaptic transmission. However, other studies failed to show any postsynaptic response mediated by P2X receptors in cultured neurons or the lumbar spinal cord (Gu and MacDermott 1997; Li and Perl 1995). We found that at the lumbar spinal cord fast EPSCs were mediated by glutamatergic AMPA/kainate receptors, consistent with other reports (Hori et al. 1996; Jahr and Jessell 1985; Randic et al. 1993; Schneider and Perl 1988, 1994; Yoshimura and Jessell 1990). However, ATP may act as a neuromodulator to enhance synaptic transmission in the superficial dorsal horn of the spinal cord (Gu and MacDermott 1997; Li and Perl 1995). We showed that PPADS significantly decreased the amplitude of fast EPSCs and altered paired-pulse responses, indicating the involvement of a presynaptic mechanism (Schulz et al. 1994; Zalutsky and Nicoll 1990). Our finding of a presynaptic action of P2X receptors, however, does not exclude a potential post-
synaptic modulatory effect of P2x as reported by Li and Perl (1995).

The involvement of P2x receptors in nociception is less clear. Behavioral studies with different antagonists find that inhibition of P2x receptors in the spinal cord is antinociceptive (Driessen et al. 1994). However, some of these drugs are not selective for P2x receptors and could affect other transmitter receptors as well (e.g., NMDA receptors) (see Dale and Gilday 1996). PPADS, a more selective antagonist than other drugs, did not produce any antinociceptive effect in this study (see also Driessen et al. 1994). This suggests that P2x receptors did not contribute to spinal nociceptive transmission. However, these studies do not completely exclude the involvement of other P2x subtype receptors (such as P2x4), which are less sensitive to PPADS (Buell et al. 1996; Collo et al. 1996) in acute nociceptive modulation as well as persistent pain.

In summary, our results suggest that ATP P2x receptors modulate excitatory synaptic transmission in the superficial dorsal horn of the lumbar spinal cord by a presynaptic mechanism, and such a mechanism does not play an important role in behavioral nociceptive responses to noxious heating to the hindpaw or tail.

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Address for reprint requests: M. Zhuo, Dept. of Anesthesiology, Campus Box 8054, Washington University, School of Medicine, St. Louis, MO 63110.

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