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ATP P2X Receptor-Mediated Enhancement of Glutamate Release and Evoked EPSCs in Dorsal Horn Neurons of the Rat Spinal Cord

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Presynaptic ATP P2X receptors have been proposed to play a role in modulating glutamate release from the first sensory synapse in the spinal cord. Using spinal cord slice preparations and patch-clamp recordings from dorsal horn neurons in lamina V of the rat spinal cord, we showed that the activation of P2X receptors by α,β-methylene-ATP ($\alpha\beta$m-ATP) resulted in a large increase in the frequency of spontaneous EPSCs (sEPSCs) and miniature EPSCs (mEPSCs). The increases in mEPSC frequency by $\alpha\beta$m-ATP were not blocked by the Ca^{2+} channel blocker, 30 μM La^{3+}, but were abolished in a bath solution when Ca^{2+} was omitted. The increases in mEPSC frequency by $\alpha\beta$m-ATP were blocked completely by the P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) at 10 μM. Furthermore, the EPSCs evoked by dorsal root stimulation were potentiated by $\alpha\beta$m-ATP as well as by the ecto-ATPase inhibitor ARL67156 and were depressed in the presence of P2 receptor antagonists PPADS (10 μM) and suramin (5 μM). The effects of these compounds on the evoked EPSCs were associated with the changes in glutamate release probability of primary afferent central terminals. Our results indicate that $\alpha\beta$m-ATP-sensitive P2X receptors play a significant role in modulating excitatory sensory synaptic transmission in the spinal cord, and the potential role of endogenous ATP is suggested.

Key words: ATP; purinergic receptors; EPSCs; glutamate release; primary afferent fibers; patch-clamp technique; spinal cord slice preparation

ATP P2X receptors, a family of nonselective cation channels gated by extracellular ATP (Jahr and Jessell, 1983; Krishtal et al., 1983; Khakh et al., 2001), may play an important role in somatic sensory transmission. P2X receptors are highly expressed on different functional types of primary sensory neurons (Vulchanova et al., 1997; Xiang et al., 1998; Guo et al., 1999; Li et al., 1999; Petruska et al., 2000). Immunocytochemical evidence shows the presence of different types of P2X receptors in both peripheral and central terminals of primary afferent fibers (Vulchanova et al., 1996, 1998; Guo et al., 1999; Novakovic et al., 1999). Activation of P2X receptors at peripheral sensory nerve endings may initiate sensory impulses and may be associated with nociceptive and non-nociceptive sensory signals (Cook et al., 1997; Sawynok and Reid, 1997; Dowd et al., 1998; Tsuda et al., 2000). At central terminals P2X receptors have been proposed to play a role in modulating glutamate release from sensory synapses, based on a previous study that used a coculture system of dorsal root ganglion (DRG) and dorsal horn (DH) neurons (Gu and MacDermott, 1997). Consistent with this hypothesis, ATP has been shown to have presynaptic effects in lamina II of the DH (Li and Perl, 1995), and activating presynaptic P2X receptors could have facilitative effects on sensory spinal transmission to lamina II of the DH (Li et al., 1998). Khakh and Henderson (1998) also have demonstrated a presynaptic P2X receptor-mediated facilitation of glutamate release at synapses in the brainstem. The role of endogenous ATP in modulating glutamate release from sensory afferent terminals was indicated (Khakh and Henderson, 1998; Li et al., 1998). However, it is currently not clear whether the central terminals of primary afferent fibers connecting to deep lamina DH neurons also express P2X receptors at presynaptic sites and, if so, whether the activation of these receptors may facilitate glutamate release.

To date, at least seven P2X subunits ($P2X_1$ to $P2X_7$) have been cloned (for review, see North and Surprenant, 2000). Except for $P2X_6$, all P2X subunits can form functional homomeric receptors in different heterologous expression systems (Khakh et al., 2001). Among those homomers, $P2X_1$ and $P2X_3$ receptors mediate a rapidly desensitizing response to both ATP and the agonist α,β-methylene-ATP ($\alpha\beta$m-ATP). The other five homomers mediate a nondesensitizing response to ATP and are essentially insensitive to $\alpha\beta$m-ATP (Khakh et al., 2001). Coexpression of different P2X receptor subunits results in several functional heteromers, including $P2X_{1+5}$, $P2X_{2+3}$, $P2X_{2+6}$, and $P2X_{4+6}$ (Lewis et al., 1995; Le et al., 1998, 1999; Torres et al., 1998; Haines et al., 1999; King et al., 2000). Of those heteromers, $P2X_{2+6}$ receptors are insensitive to $\alpha\beta$m-ATP (King et al., 2000). Thus, $\alpha\beta$m-ATP makes some functional P2X receptors pharmacologically distinguishable from others.

Of the seven cloned P2X receptor subunits, six ($P2X_1$ to $P2X_6$) are expressed in primary sensory neurons (Collo et al., 1996; Vulchanova et al., 1996, 1997, 1998; Xiang et al., 1998). In the spinal cord the mRNAs for $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$, and $P2X_6$ subunits have been found; mRNAs for $P2X_2$, $P2X_4$, and $P2X_6$ are the most abundant (Collo et al., 1996). Immunochemical studies on spinal cord sections with antibodies against $P2X_1$, $P2X_2$, and

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$P2X_3$ subunits have shown the expression of these subunits on the primary afferent central terminals (Vulchanova et al., 1996, 1997, 1998; Guo et al., 1999). Other P2X receptor subunits also may be expressed at the primary afferent central terminals in the spinal cord. In the present study we have explored the functions of P2X receptors at some sensory synapses in deep laminas (lamina V) of the spinal cord.

MATERIALS AND METHODS

Spinal cord slice preparation. Transverse spinal cord slices (500 μm in thickness) were prepared from L5 spinal cords of rats at postnatal age 11–21 d, as described previously (Nakatsuka et al., 2000). Unless otherwise indicated, slices without dorsal roots attached were used for recordings of spontaneous EPSCs (sEPSCs) and miniature EPSCs (mEPSCs); slices with attached L5 dorsal roots were used for evoked EPSCs (eEPSCs). A spinal cord slice was transferred to a recording chamber (~0.5 ml) and placed on the stage of an upright infrared-differential interference contrast (IR-DIC) microscope. The slice was superfused (10 ml/min) with Krebs' solution containing (in mM) 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose in 95% O_2/5% CO_2, pH 7.3, at 22°C.

Preparation of acutely dissociated DRG neurons. Dorsal root ganglia were removed from rats (age of 4–7 weeks) and placed in a 35°C bath solution containing dispase (neutral protease, 5 mg/ml; Boehringer Mannheim, Indianapolis, IN) and collagenase (2 mg/ml; Sigma type 1, St. Louis, MO). After 1 hr of incubation the DRGs were triturated to dissociate the neurons. The dissociated cells were plated on coverslips precoated with poly-L-lysine and allowed to adhere for 1 hr before recording.

Preparation of cultured DRG neurons. Dorsal root ganglia were isolated from rat embryos aged 16 d (E16) *in utero*, exposed to 0.25% trypsin for 20 min, and dissociated. The dissociated primary sensory neurons were plated on glass coverslips previously prepared with a monolayer of rat cortical astrocytes. At the time of plating, 2.5S NGF (10 ng/ml) and 5-fluoro-2′-deoxyuridine (10 μM) were added, and 2.5S NGF was added once every week when the cells were fed with fresh media. The cultures of 2–3 weeks were used for the experiments. *Principles of Laboratory Animal Care* (National Institutes of Health publication 86-23; revised, 1985) was followed in all of the tissue preparation procedures described above.

Patch-clamp recordings from spinal cord slices. Lamina regions were identified under a 10× objective, and individual neurons were identified with a 40× objective under IR-DIC microscope. Whole-cell patch-clamp recordings were made from DH neurons with electrodes (~5 MΩ) filled with a solution containing (in mM) 135 K^+-gluconate, 5 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 EGTA, and 5 HEPES. Signals were amplified and filtered at 2 kHz (Axopatch 200B, Axon Instruments, Foster City, CA) and sampled at 5 kHz. Cells were held at −60 mV, which was close to the reversal potential for $GABA_A$ and glycine receptors under the experimental conditions. At this holding potential the outward IPSCs were minimized and usually were undetectable. sEPSCs were recorded in the absence of TTX. mEPSCs were recorded in the presence of 0.5 μM TTX (the term mEPSCs is used for simplicity; "sEPSCs in the presence of TTX" may be a more suitable term). In some experiments mEPSCs were recorded in the presence of 20 μM bicuculline and 2 μM strychnine in bath solution. αβm-ATP, capsaicin, and other testing compounds described in Results were applied via bath solution. In some experiments the effects of 100 μM ATP on sEPSC frequency were tested in the presence of 50 μM ARL67156 plus 2 mM caffeine. The application intervals for testing compounds were 20 min. At this interval the agonist responses were reproducible. Analyses of sEPSCs and mEPSCs were performed as described previously (Gu and MacDermott, 1997).

To record eEPSCs from lamina V, we applied a stimulus (~120 μA, 0.1 msec) to a dorsal root with a suction electrode. Monosynaptic connection was judged by constant latency of the eEPSCs when multiple stimuli were applied (Nakatsuka et al., 2000). Most lamina V neurons that we recorded had monosynaptic connections with primary afferent fibers (93% from 28 recordings) in our slice preparations. The stimulation condition usually yielded few synaptic failures. In the experiments to determine synaptic potentiation of eEPSCs by P2X receptor activation, we first identified monosynaptic eEPSCs and then gradually reduced the intensity (Malinow and Tsien, 1990) to a level at which some synaptic failures (30–70%) occurred. For higher sensitivity the cells with higher synaptic failure rates were assigned for tests with αβm-ATP or ARL67156, and the cells with lower failure rates were tested with P2X receptor antagonists. Synaptic failure was judged by current levels in the range of current noise baseline of Gaussian distribution (±3 pA). One to three cells were recorded from a slice of each rat.

Patch-clamp recordings from acutely dissociated or cultured DRG neurons. The coverslips with dissociated DRG neurons were mounted in a 0.5 ml recording chamber and placed on the stage of an Olympus IX70 microscope. Cells were perfused continuously with bath solution (22°C) flowing at 1 ml/min. The bath solution contained (in mM) 150 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, pH-adjusted to 7.4 with NaOH; osmolarity was adjusted to 320 mOsm with sucrose. Cells were voltage clamped at −70 mV in the whole-cell configuration. Signals were amplified and filtered at 2 kHz (Axopatch 200B) and sampled at 5 kHz. The recording electrode internal solution contained (in mM) 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, and 20 HEPES, pH-adjusted to 7.4 by KOH, with an osmolarity of 315–325 mOsm. Recording electrode resistance was between 2.0 and 5.0 MΩ. αβm-ATP (10 μM) or capsaicin (1 μM) was applied rapidly to neurons through a glass tube (inner diameter, ~500 μm) positioned 1.0 mm away from the cell, and each was applied for 2 sec. The gravity-driven solution flow was controlled electronically by solenoid valves and triggered from a computer.

The potential nonspecific effects of pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) on action potentials and Na^+ and Ca^{2+} channel activity were examined in cultured DRG neurons. Conditions, including the bath solution and electrode internal solutions for patch-clamp recordings from cultured DRG neurons, were similar to the recordings from acutely dissociated DRG neurons. Under current clamp the action potentials were evoked by current steps of 600 pA for 4 msec. The tests were performed both in normal bath solution and after a 10 min continuous perfusion of 50 μM PPADS in the bath solution. To examine the potential nonspecific effects of PPADS on Na^+ and Ca^{2+} channel activity, we held the cells at −80 mV under perforated voltage-clamp configuration, and the electrode internal solution contained Cs^+ (Gu et al., 1996). Inward currents with Na^+ and Ca^{2+} channel components (Gu and MacDermott, 1997) were evoked by voltage steps to +10 mV for 200 msec. The tests were performed both in normal bath solution and after a 10 min continuous perfusion of 50 μM PPADS in the bath solution.

ATP, αβm-ATP, ARL67156, PPADS, suramin, capsaicin, caffeine, bicuculline, strychnine, and $LaCl_3$ were purchased from Sigma. CNQX and TTX were purchased from Tocris (St. Louis, MO). Unless otherwise indicated, data represent mean ± SEM; paired Student's *t* tests were used for statistical comparison, and significance was considered at the $p < 0.05$ level.

RESULTS

P2X receptor-mediated enhancement of spontaneous glutamate release

We performed patch-clamp recordings from DH neurons in lamina V of spinal cord slices. Under IR-DIC microscopy lamina regions in the dorsal horn were identified first with a 10× objective (Fig. 1*A*), and then individual neurons in lamina V were visualized under a 40× objective (Fig. 1*B*). To test whether the activation of P2X receptors may release glutamate from afferent central terminals and/or DH interneuron terminals onto lamina V DH neurons, we bath-applied αβm-ATP (100 μM), a metabolic stable ATP analog and selective P2X receptor agonist, to determine its effects on the frequency of sEPSCs. Application of αβm-ATP (100 μM) produced a large increase in sEPSC frequency (Fig. 1*C,D*) in most lamina V neurons (Fig. 1*E*). Of 23 cells that were recorded, 21 cells showed substantial increases in sEPSC frequency, and only two cells showed no increase after αβm-ATP (Fig. 1*E*). The overall changes of sEPSC frequency were 355 ± 58% of control ($p < 0.05$, paired *t* test; $n = 23$). The effects lasted for ~200 sec (202 ± 12 sec; $n = 21$) after a 60 sec αβm-ATP application (Fig. 1*D*). Figure 1*E* shows the changes of sEPSC frequency in all of the 23 lamina V neurons that were tested after αβm-ATP application. When αβm-ATP was applied repeatedly at 10 min intervals, it produced similar increases in

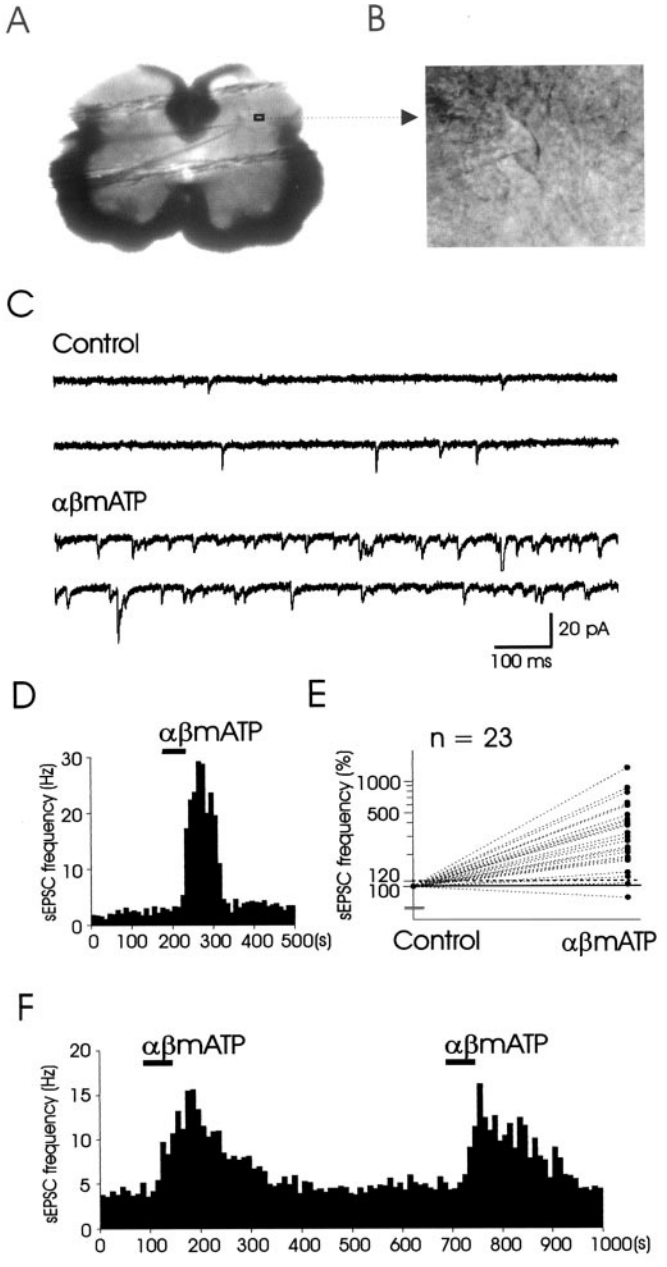

Figure 1. Effects of αβm-ATP on sEPSC frequency recorded from lamina V dorsal horn neurons. *A, B*, Spinal cord slice preparation viewed under an IR-DIC microscope. Lamina regions were identified under 10× objective (*A*). A part of a patch electrode is seen in *A* also. The electrode tip is inside the tissue ~70 μm from the surface, and its lamina location is indicated by a *box*. A neuron in the *boxed region* can be seen under 40× objective (*B*, *center* of the field). The patch electrode is to the *left*. *C*, Sample traces of sEPSCs recorded from a lamina V neuron in normal bath solution (*Control*, *top two traces*) and after bath application of 100 μM αβm-ATP (*bottom two traces*). *D*, Time course of the increases in sEPSC frequency after bath application of αβm-ATP. The time of αβm-ATP application is indicated by a *horizontal bar*. Time bin is 10 sec. *E*, Results from 23 lamina V neurons. The change in sEPSC frequency after αβm-ATP is expressed as the percentage of the control EPSC frequency in logarithmic scale. Each *filled circle* represents the response recorded from a cell. *F*, Effects on sEPSC frequency by two repeated applications of 100 μM αβm-ATP. Similar results were obtained in the other nine cells.

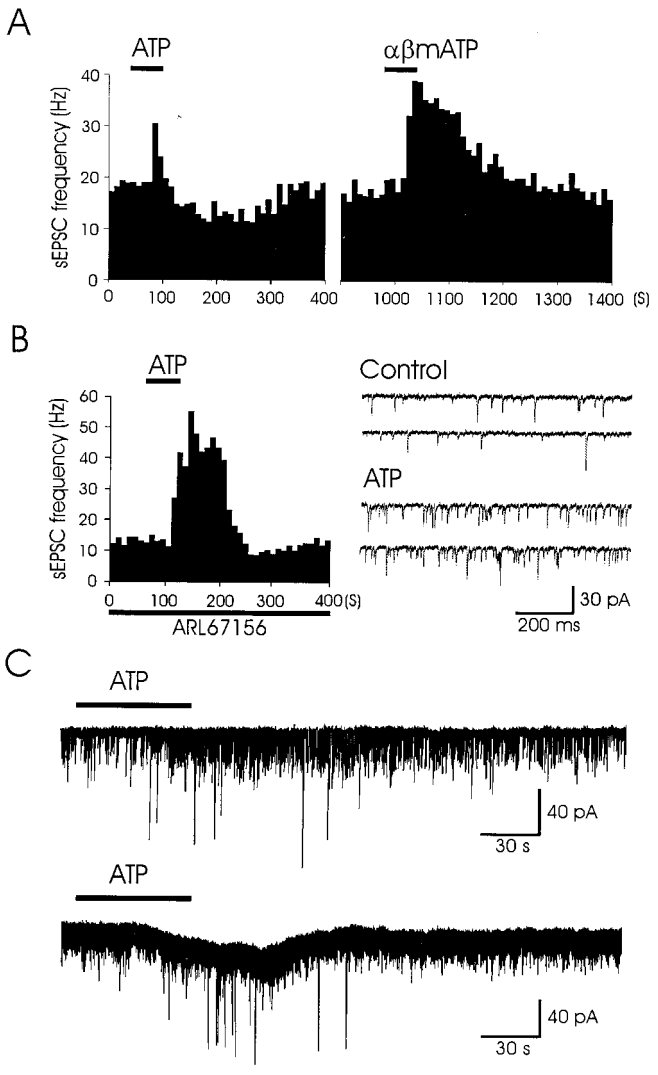

Figure 2. Inhibition of ATP metabolism by ARL67156 and the effects of ATP on sEPSC frequency. *A*, Bath application of 100 μM ATP produced a biphasic change in sEPSC frequency, with a transient increase followed by a depression phase in a lamina V neuron. In the same cell 100 μM αβm-ATP produced prolonged increases in sEPSC frequency. Of eight cells that were tested as shown in *A*, three showed biphasic responses and five cells had only the depression phase. *B*, ATP (100 μM) produced a prolonged increase of sEPSC frequency in the presence of the ecto-ATPase inhibitor ARL67156 (50 μM). Traces on the *right* are sample traces of sEPSCs in normal bath solution (*Control*) and after the application of 100 μM ATP in the presence of 50 μM ARL67156. Similar results were obtained in the other nine cells. *C*, ATP (100 μM) also induced inward whole-cell currents in some DH neurons in the presence of 50 μM ARL67156. Of 10 cells that were recorded, inward whole-cell currents were undetectable (*top trace*) in eight cells but were detected (*bottom trace*) in two cells. In the experiments shown in *B* and *C*, the bath solution also contained 2 mM caffeine.

sEPSC frequency ($n = 10$) (Fig. 1*F*). However, when 100 μM ATP was applied for 60 sec, sEPSC frequency showed either a biphasic change with initially a transient increase (<20 sec) followed by a depression phase ($n = 3$) (Fig. 2*A*) or had only a depression phase ($n = 5$). This was most likely attributable to the rapid metabolism of ATP and the effects of its metabolite adenosine. To test this possibility, we next performed experiments in the presence of the ecto-ATPase inhibitor ARL67156 (50 μM) and the adenosine receptor antagonist caffeine (2 mM) (Salter et al., 1993; Westfall

et al., 1996). Under this condition a 60 sec application of ATP (100 μM) produced a nearly threefold increase of sEPSC frequency (270 ± 30%; $p < 0.05$; $n = 10$) (Fig. 2*B*) that lasted for ~120 sec (123 ± 6 sec), a result similar to the effects of αβm-ATP on sEPSC frequency. In addition to the increases of sEPSC frequency, ATP also produced small inward currents (11 ± 7 pA) in 2 of 10 recorded neurons (Fig. 2*C*). Because the use of ATP may have potentially more complications than the use of αβm-ATP (see Discussion), we used αβm-ATP as the P2X receptor agonist in the remaining study.

We tested whether αβm-ATP-sensitive terminals were connected monosynaptically with most lamina V neurons. This was done by determining the effects of αβm-ATP on sEPSCs in the presence of 500 nM TTX (mEPSCs will be used for simplicity in the following parts). The presence of TTX blocks active interneuronal transmission between DH neurons. Under this condition the bath application of 100 μM αβm-ATP for 60 sec still significantly increased mEPSC frequency (Fig. 3*A,B*) in most neurons that were recorded (Fig. 3*C*). Of 23 cells that were recorded, 22 of them showed large increases in mEPSC frequency, and one cell had little change (Fig. 3*C*). The overall changes of mEPSC frequency were 382 ± 49% of control ($p < 0.05$, paired *t* test; $n = 23$). The effects lasted for ~170 sec (174 ± 13 sec; $n = 22$). Of those cells showing the increases in mEPSC frequency by αβm-ATP, 19 of them also were tested with capsaicin (2 μM). mEPSC frequency was not affected by a 60 sec capsaicin application ($n = 19$) (Fig. 3*B,D*).

To determine whether Ca^{2+} entry through P2X receptors at presynaptic terminals may contribute directly to the increases in spontaneous glutamate release, we preapplied 30 μM La^{3+} to block the potential Ca^{2+} entry through voltage-gated Ca^{2+} channels (Gu and MacDermott, 1997). Under this condition and in the presence of 500 nM TTX, 100 μM αβm-ATP still produced a large increase in the frequency of mEPSCs (273 ± 20% of control; $p < 0.05$; $n = 4$) (Fig. 4*Aa*). On the other hand, when experiments were performed in a 0 Ca^{2+} bath solution (Ca^{2+} was omitted from normal bath solution), αβm-ATP did not produce a significant increase of mEPSC frequency (117 ± 16% of control; $n = 5$) (Fig. 4*Ab*). Whereas αβm-ATP increased mEPSC frequency in almost all lamina V neurons, mEPSC amplitude remained within 99 ± 2% of control ($n = 26$) (Fig. 4*B*). We tested whether the increase of mEPSCs by αβm-ATP could be blocked by the P2X receptor antagonist PPADS (10 μM). In five cells 10 μM αβm-ATP-induced significant increases of mEPSCs (261 ± 25% of control; $p < 0.05$) in normal bath solution; in these five cells, when 10 μM PPADS was present, the effects of αβm-ATP on mEPSC frequency were abolished completely (99 ± 4% of control) (Fig. 4*C*). CNQX (10 μM), a non-NMDA receptor antagonist, was tested on those cells for which αβm-ATP induced increases in the frequency of mEPSCs or sEPSCs. CNQX completely blocked mEPSCs ($n = 5$) (Fig. 4*D*) as well as sEPSCs (data not shown; $n = 5$). These results suggest that P2X receptors are present at glutamatergic terminals monosynaptically contacting lamina V neurons.

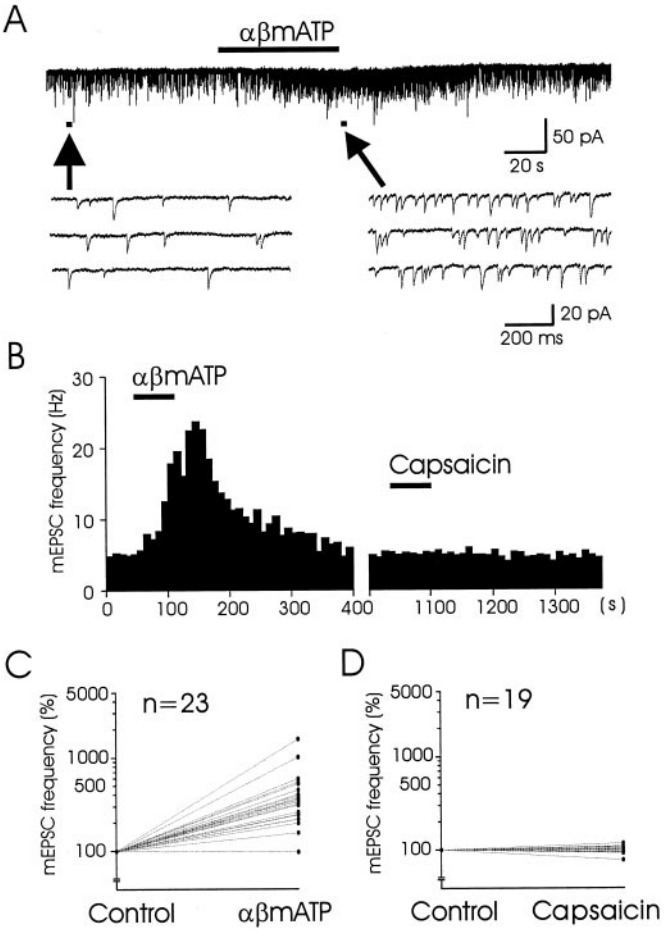

Figure 3. Effects of αβm-ATP on mEPSC frequency recorded from lamina V dorsal horn neurons. *A*, The *top trace* shows mEPSCs recorded from a lamina V neuron before and after the application of 100 μM αβm-ATP. The *bottom traces* show, at an expanded time scale, the mEPSCs before (*left three traces*) and after (*right three traces*) αβm-ATP application. *B*, Histogram shows the time course and degree of the increases in mEPSC frequency after 100 μM αβm-ATP. It also shows, in the same recording, that 2 μM capsaicin did not have an effect on mEPSCs. *C*, Results from 23 lamina V neurons show that most lamina V neurons responded to αβm-ATP with an increase in mEPSC frequency. *D*, Of the 23 cells in *C*, 19 neurons were tested with 2 μM capsaicin, and little change in mEPSC frequency was observed. In all experiments, αβm-ATP and capsaicin were applied for 60 sec. The time bin is 10 sec in the histogram.

Whole-cell currents directly evoked by αβm-ATP in sensory neurons

To explore whether αβm-ATP-sensitive terminals were derived mainly from the central terminals of primary afferent fibers that remained in the spinal sections or mainly from terminals of DH interneurons, we determined the expression of αβm-ATP-sensitive P2X receptors on DH neurons and DRG neurons. This was done by examining αβm-ATP-evoked whole-cell currents from DH or DRG neurons. In 101 spinal cord DH neurons, 55 in lamina V and 46 in lamina II, 100 μM αβm-ATP did not induce any detectable whole-cell current directly (Fig. 5*A*). We next directly examined αβm-ATP-evoked whole-cell currents in acutely dissociated DRG neurons. Of 20 cells that were recorded, six of them were large DRG neurons (diameter, >50 μm) and had no response to 10 μM αβm-ATP (data not shown). Thirteen cells were small-to-medium DRG neurons and had responses to the application of 10 μM αβm-ATP. Of the 13 αβm-ATP-sensitive DRG neurons, six (cell size 35–45 μm) showed αβm-ATP-evoked P2X currents that had nondesensitizing current components (Fig. 5*B*, *left*). The amplitude of nondesensitizing components was 560 ± 120 pA ($n = 6$). Interestingly, all of those six cells did not have a measurable response to 1 μM capsaicin (Fig. 5*B*, *right*). For the other seven αβm-ATP-sensitive DRG neurons (cell

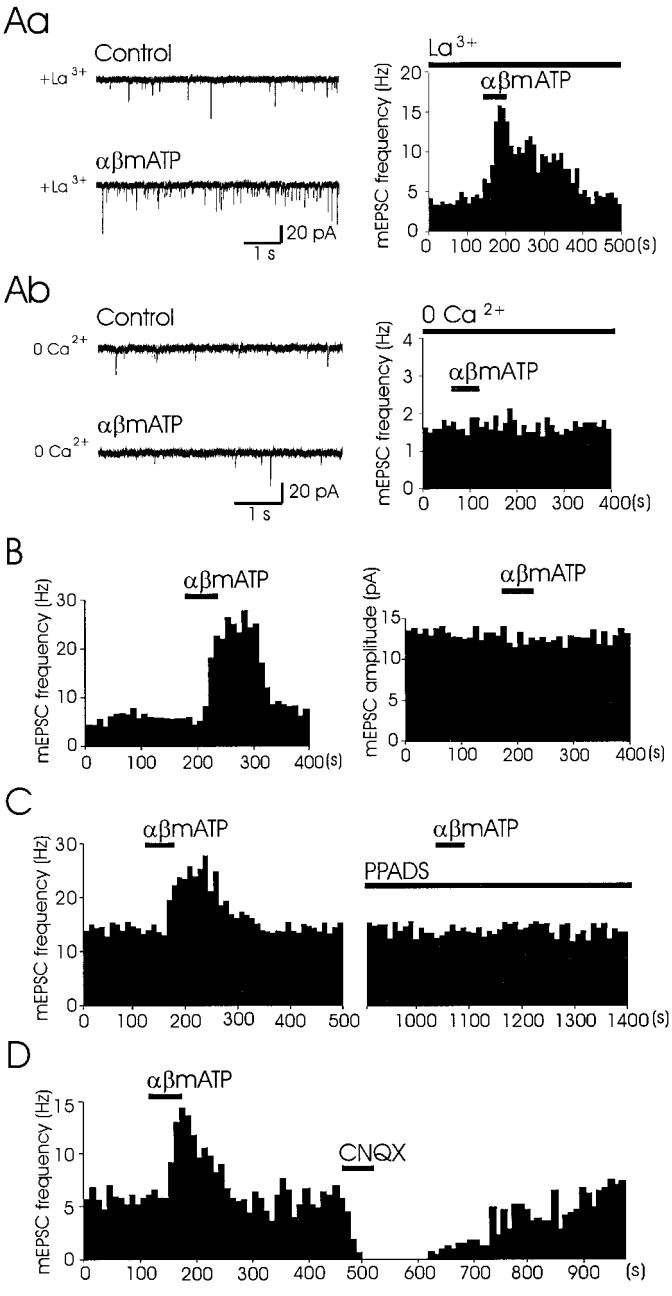

Figure 4. Characterization of αβm-ATP-induced increases of mEPSC frequency. *Aa*, All experiments were performed in the continuous presence of 30 μM La^{3+} to block voltage-gated Ca^{2+} channels. Two sample traces on the *left* represent mEPSCs from a lamina V neuron before (*Control*, *top trace*) and after the bath application of 100 μM αβm-ATP (*bottom*). Histogram on the *right* shows the time course and degree of the increases in mEPSC frequency in the presence of 30 μM La^{3+}. Similar results were obtained in three other cells. *Ab*, Sample traces (*left*) and frequency histogram (*right*) show little change of mEPSC frequency after the application of 100 μM αβm-ATP in the 0 Ca^{2+} both solution. Similar results were obtained in the other four cells. *B*, Histograms of mEPSC frequency (*left*) and amplitude (*right*) indicate that αβm-ATP (100 μM) increased the frequency, but not the amplitude, of mEPSCs. *C*, The increase of mEPSC frequency by αβm-ATP was blocked by 10 μM PPADS ($n = 5$). *D*, mEPSCs were inhibited completely by 10 μM CNQX ($n = 5$).

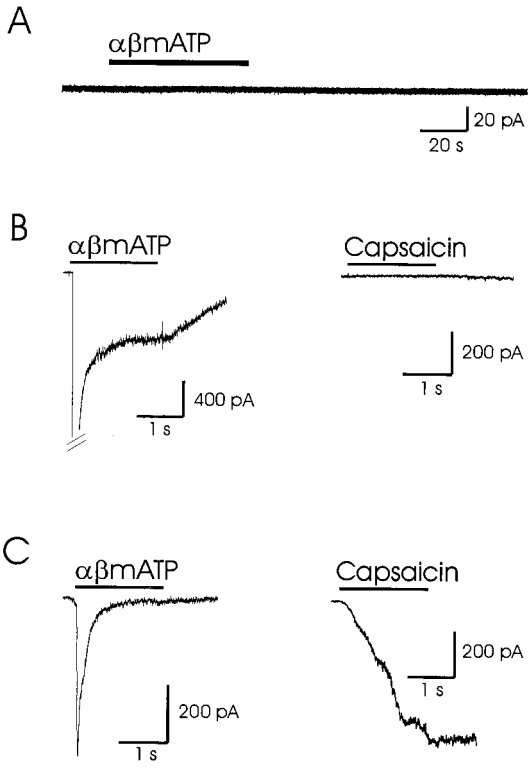

Figure 5. Examination of αβm-ATP-evoked, P2X receptor-mediated, whole-cell currents on DH and DRG neurons. *A*, The recording is from a DH neuron in lamina V in a slice preparation. The αβm-ATP application is indicated with a *horizontal bar* above the recording trace. Holding current is −20 pA, with the cell being held at −70 mV. Bath solution contained (in μM) 20 CNQX, 50 APV, 20 bicuculline, and 2 strychnine. αβm-ATP (100 μM) did not evoke any detectable inward current directly. Similar results were obtained from the other 100 DH neurons in slices. Among those recordings, 55 cells were from lamina V, and the remaining cells were from lamina II. *B*, An inward current with a large nondesensitizing component was evoked by 10 μM αβm-ATP in an acutely dissociated DRG neuron (*left trace*). The initial desensitizing phase is truncated. The steady-state current component was sustained during the 2 sec αβm-ATP application. The *right trace* shows that in the same neuron 1 μM capsaicin did not induce any detectable current. Similar results were obtained in five other DRG neurons. *C*, A rapidly desensitizing current (*left*) was evoked by 10 μM αβm-ATP in a DRG neuron. In the same neuron, 1 μM capsaicin evoked an inward current (*right*). The desensitization to αβm-ATP was complete and reached baseline in ~500 msec during the 2 sec αβm-ATP application. Similar results were obtained in six other DRG neurons.

size 20–30 μm), the αβm-ATP-evoked currents (830 ± 230 pA; $n = 7$; peak currents) showed rapid and complete desensitization during a 2 sec αβm-ATP application (Fig. 5*C*, *left*). In this type of αβm-ATP-sensitive neurons, 1 μM capsaicin also evoked inward currents (Fig. 5*C*, *right*).

αβm-ATP-sensitive synapses and A-afferent fiber central terminals

We recorded eEPSCs to determine the properties of primary afferent terminals that synapsed with lamina V neurons, using spinal cord slices with attached dorsal roots (Fig. 6*A*). In the same neurons we also examined the effects of αβm-ATP on the sEPSC frequency. In 12 lamina V cells that showed increases in sEPSC frequency by 100 μM αβm-ATP (306 ± 39% of control) (Fig. 6*B*), stimulation of the dorsal root always evoked EPSCs with monosynaptic A-afferent properties (Fig. 6*C*). Conduction velocities of afferent fibers for those recordings were 2.3 ± 0.2 m/sec ($n = 12$),

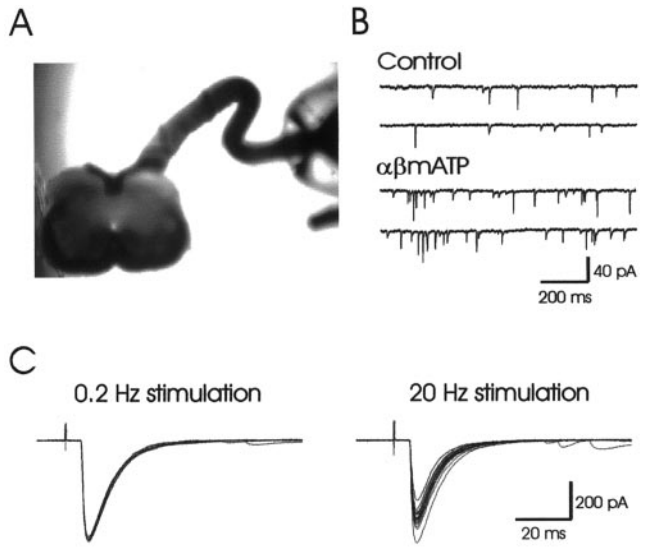

Figure 6. αβm-ATP-sensitive terminals and monosynaptic A-afferent EPSCs recorded in lamina V neurons. *A*, The image shows a spinal cord slice with an attached dorsal root. A suction electrode for root stimulation is shown on the *right*. A part of the root was sucked into the stimulation electrode. *B*, Sample traces show the increase of sEPSC frequency by 100 μM αβm-ATP in a lamina V neuron. *C*, In the same neuron as in *B*, dorsal root stimulation at 0.2 Hz (*left*) and 20 Hz (*right*) elicited monosynaptic A-afferent eEPSCs. Repeated stimulation (20 times) at either frequency did not change the latency of eEPSCs. The same results were obtained in 11 other neurons in lamina V. Conduction velocity is 2.3 ± 0.2 m/sec (n = 12), within Aδ-afferent fiber range.

within the range of Aδ-afferent fiber conduction velocities for the same age group of rats (Nakatsuka et al., 2000). Latency of eEPSCs after repeated stimulation at different stimulation frequencies was constant in each recording (Fig. 6*C*). One cell that showed monosynaptic A-afferent eEPSCs had no response to 100 μM αβm-ATP. Of those 12 cells as shown in Figure 6, eight of them were tested with the bath application of capsaicin (2 μM). Little change in mEPSC frequency was observed in those cells (100 ± 2% of control). The association of αβm-ATP-sensitive terminals and Aδ-afferent terminals was also evident in the experiments below (see Fig. 8).

P2X receptor-mediated enhancement of glutamate release in responding to the stimulation of Aδ-primary afferent fibers

ATP could be released in DH regions (Salter et al., 1993; Bardoni et al., 1997; Li et al., 1998). If released, will endogenous ATP increase glutamate release from αβm-ATP-sensitive terminals onto lamina V neurons? Repetitive stimulation (20 times at 20 Hz, 1 sec duration) was applied to a dorsal root at Aδ-fiber stimulation intensity (~120 μA, 0.1 msec). At the end of this repetitive stimulation there was a brief increase in sEPSC frequency that lasted for ~5 sec. The sEPSC frequency after the stimulation (post-stim) was 260 ± 23% (n = 11) (Fig. 7*A,B*) of sEPSC frequency before the stimulation (pre-stim). When the same repetitive stimulation was conducted in the presence of 10 μM PPADS, post-stim sEPSC frequency was 171 ± 23% (n = 7) of pre-stim sEPSC frequency, significantly lower than the post-stim sEPSC frequency in normal bath solution (Fig. 7*A,B*). Similar experiments also were performed in the presence of 5 μM suramin. The post-stim sEPSC frequency was 187 ± 33% (n = 4; not illustrated as a figure) of pre-stim sEPSC frequency in the presence of suramin, significantly lower than the post-stim sEPSC frequency in normal bath solution. The effects of PPADS or suramin at the concentrations that were used were unlikely to be nonspecific actions on glutamatergic transmission because pre-stim sEPSC frequency was not affected in the presence of 10 μM PPADS (n = 4) (Fig. 7*A,B*) or 5 μM suramin (data not shown). There was also no change in the basal frequency and amplitude of sEPSCs by 10 μM PPADS (frequency, 101 ± 3% of control; amplitude, 99 ± 1% of control; n = 8) (Fig. 7*C*). We performed an analysis of the amplitude of individual electrically evoked EPSCs during the train of dorsal root stimulation under control conditions and in the presence of PPADS. Similar to the finding by Li et al. (1998), we found that there were changes in the paired pulse ratio in the presence of 10 μM PPADS. The paired pulse ratio was 78 ± 16% under control conditions and 192 ± 38% in the presence of PPADS (n = 4; not illustrated as a figure). These results suggested that the effects of PPADS were on the presynaptic sites (Li et al., 1998).

Because some sEPSCs might be generated by action potentials and involved in Ca^{2+} entry through voltage-gated Ca^{2+} channels, we determined whether PPADS had effects on the size and shape of action potentials as well as Na^+ and Ca^{2+} channel activity in cultured DRG neurons (Fig. 7*D,E*). The size and shape of action potentials elicited by a current step showed identically in normal bath solution and after a 10 min perfusion of cells with bath solution containing 50 μM PPADS (n = 6) (Fig. 7*D*). Furthermore, the inward currents of both the Na^+ channel component and Ca^{2+} component (Gu and MacDermott, 1997) were also identical in normal bath solution and in the bath solution containing 50 μM PPADS (n = 6) (Fig. 7*E*).

Activation of other presynaptic ligand-gated cation channels has been shown to result in either potentiation or depression of the evoked EPSCs at glutamatergic synapses in the brain (MacDermott et al., 1999). Will activation of αβm-ATP-sensitive P2X receptors result in synaptic potentiation of Aδ-afferent glutamatergic transmission to lamina V neurons? Stimuli were applied at a reduced intensity (see Materials and Methods) to a dorsal root, and monosynaptic Aδ-eEPSCs and synaptic failures occurred randomly (Fig. 8*Aa*, *bottom*). Glutamate release likely was occurring at a few afferent central terminals during this stimulation protocol, as was evident by a much smaller amplitude of eEPSC (compare with eEPSC size in Fig. 6*C*). After the application of 1 μM αβm-ATP for 5 min, the synaptic failure rates that followed the same stimulation protocol were decreased significantly (Fig. 8*Ab*, *bottom*), and the mean amplitude of eEPSCs was increased significantly (Fig. 8*Ab*, *top*). When the ecto-ATPase inhibitor ARL67156 (10 μM for 5 min) was used to prevent the breakdown of endogenous ATP, we also observed effects similar to αβm-ATP (Fig. 8*B*). Figure 8*C* summarizes the effects of αβm-ATP (n = 4) and ARL67156 (n = 4) on synaptic failure rates and mean amplitudes and shows significant decreases of failure rates (Fig. 8*Ca*) and increases in mean eEPSC amplitude (Fig. 8*Cb*). On the other hand, in the presence of 10 μM PPADS or 5 μM suramin the failure rates increased significantly (Fig. 8*D*).

DISCUSSION

In the present study we have provided the electrophysiological evidence for the presence of presynaptic P2X receptors at the central terminals of primary afferent fibers connecting to lamina V neurons of the spinal cord dorsal horn. Our results indicate that the activation of these receptors can result in a robust increase of spontaneous glutamate release, as evidenced by a large increase

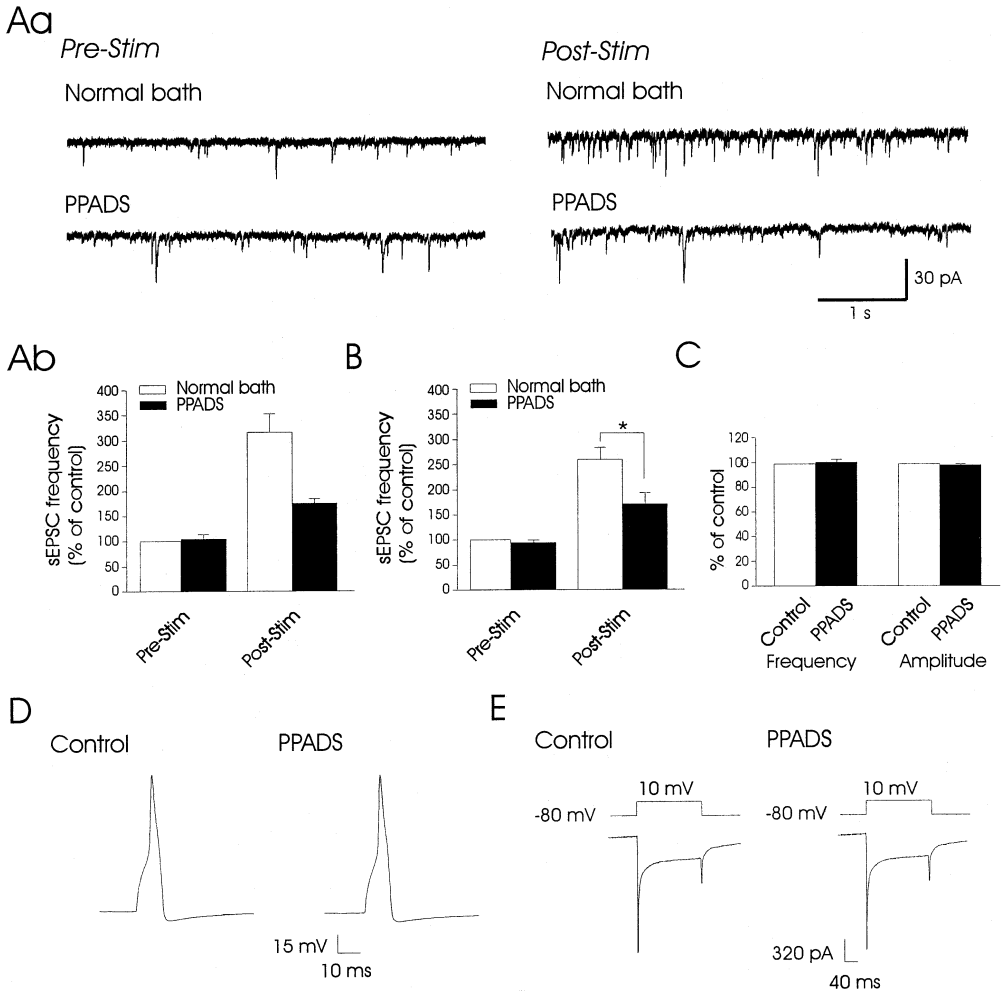

Figure 7. Effects of P2X antagonists on sEPSCs after repetitive stimulation to afferent fibers. *Aa*, *Top two traces* show sEPSCs recorded from a lamina V neuron before and after repetitive stimulation (20 times at 20 Hz, 1 sec duration) in normal bath solution. *Bottom two traces* show, in the same cell in the presence of 10 μM PPADS, the sEPSCs before and after the same repetitive stimulation. Stimulation intensity was similar to that in Figure 6*C*. *Ab*, An example shows responses of three trials in the same cell. The *Post-Stim* effects were tested three times in normal bath solution and three times in the presence of PPADS. The interval of each test was 2 min. The number of sEPSCs was counted for 5 sec before and immediately after the finish of the repetitive stimulation. Similar results were obtained in two other cells. *B*, A summary of the changes of sEPSC frequency before and after the repetitive stimulation in normal bath solution (*open bars*) and in 10 μM PPADS (*filled bars*; $n = 7$ cells). PPADS significantly reduced the stimulation-induced enhancement of sEPSC frequency ($p < 0.05$). *C*, The frequency and amplitude of basal sEPSCs were not changed after a 10 min perfusion of 10 μM PPADS ($n = 8$). *D*, Two traces show action potentials elicited by current steps in a DRG neuron in normal bath solution (*left*) and after the perfusion of 50 μM PPADS for 10 min. Similar results were obtained in five other cells. *E*, Two traces show currents elicited by voltage steps from −80 to +10 mV in a DRG neuron in normal bath solution (*left*) and after a 10 min perfusion of 50 μM PPADS (*right*). The initial current component with rapid decay phase is attributable mainly to Na^+ channels, and the subsequent steady-state current component represents mainly voltage-gated Ca^{2+} channel activity (Gu and MacDermott, 1997). No change was observed after 50 μM PPADS; similar results were obtained in five other cells. Recordings in *E* were performed with perforated patch-clamp technique; Cs^+-internal electrode solution was used (Gu et al., 1996).

in the frequency of sEPSCs and mEPSCs. Furthermore, we have provided evidence suggesting that P2X receptor activation can modulate the evoked glutamate release after the stimulation of primary afferent fibers. Our results also suggest that endogenously released ATP, associated with primary sensory fiber stimulation, may enhance spontaneous and evoked glutamate release.

P2X receptors on primary afferent central terminals versus P2X receptors on DH neurons

We have shown that $\alpha\beta$m-ATP-sensitive terminals extensively synapse to lamina V neurons. Many of those terminals might be derived from primary afferent fibers. This is consistent with the presence of $\alpha\beta$m-ATP-sensitive P2X receptors on DRG neurons. It is supported further by our findings that the eEPSCs after stimulation of primary afferent fibers were potentiated by $\alpha\beta$m-ATP and ARL67156 and depressed by PPADS and suramin. The effects of those purine compounds were at the central terminals of primary afferent fibers, because the potentiation of eEPSCs was associated with the increase of presynaptic release probability (Fig. 8).

There is a possibility that some $\alpha\beta$m-ATP-sensitive terminals may be derived from DH interneurons. Previously, it was reported that presynaptic P2X receptors were expressed on inhibitory DH neurons; their activation resulted in the release of GABA or glycine (Hugel and Schlichter, 2000; Rhee et al., 2000). Interestingly, presynaptic P2X receptors on inhibitory DH neurons in those studies were found to be $\alpha\beta$m-ATP-insensitive. It will be very interesting to know whether P2X receptors are expressed at presynaptic terminals of excitatory DH neurons. In attempting to determine whether $\alpha\beta$m-ATP-induced increases in mEPSC frequency might be attributable partially to its direct action on DH neurons, we examined the possible expression of functional $\alpha\beta$m-ATP-sensitive P2X receptors in DH neurons. However, after we tested >100 DH neurons, 100 μM $\alpha\beta$m-ATP did not evoke any detectable whole-cell current on those neurons. This result may suggest that $\alpha\beta$m-ATP-sensitive DH neurons, if present, will be a very small population. This suggestion is also supported by a previous study with acutely dissociated DH neurons (Bardoni et al., 1997). However, we still should not rule out completely the possible presence of $\alpha\beta$m-ATP-sensitive P2X receptors at presynaptic terminals of glutamatergic DH neurons.

In this study we also have performed some experiments by using ATP. It appears that, before exogenously applied ATP reached the recorded neurons (usually inside slices ~70 μm from the surface of the tissue), there had been substantial ATP metabolism that primarily decreased actual ATP concentrations

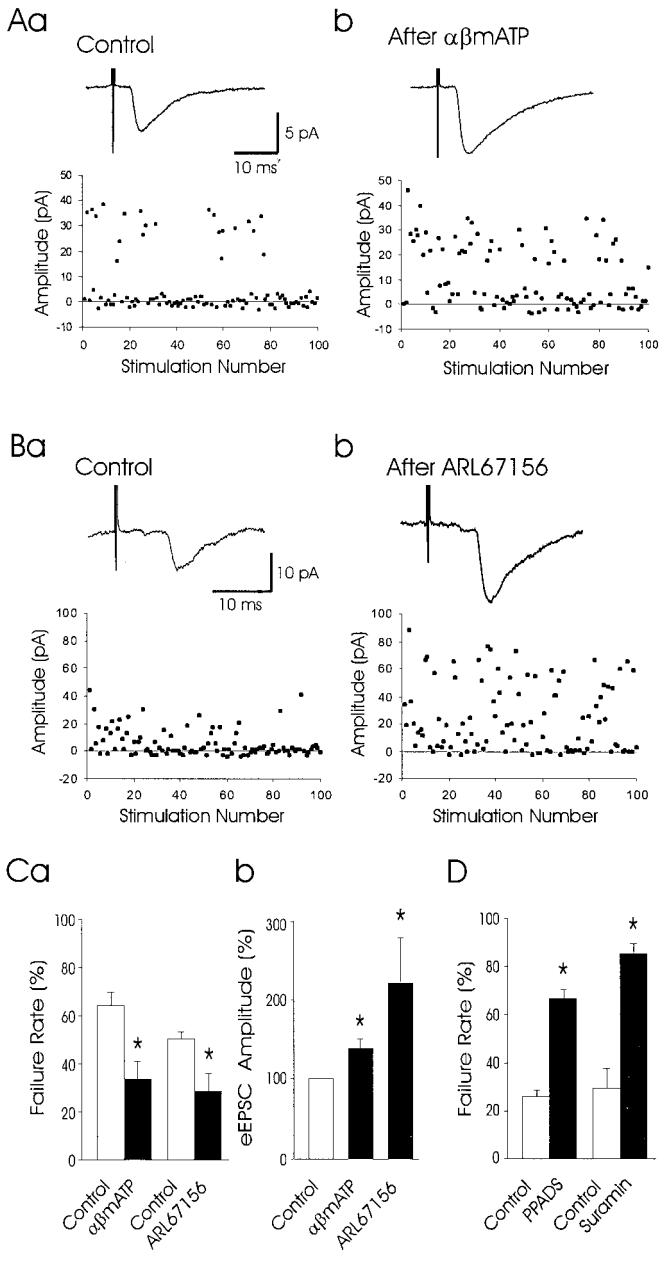

Figure 8. P2X receptor-mediated potentiation of evoked EPSCs. *Aa*, The *bottom panel* shows consecutive eEPSCs and synaptic failures recorded on a lamina V neuron in control. The mean amplitude is shown on the *top*. Basal noise level was within ± 2.5 pA around the current level of baseline. *Ab*, Synaptic failures (*bottom*) were decreased and mean amplitude (*top*) was increased after the bath application of 1 μM αβm-ATP. *B*, The experiment was the same as that in *A*, except that the effects of 10 μM ARL67156 were tested. The conduction velocity was 4 m/sec in *A* and 2 m/sec in *B*. *C*, A summary of the decrease of failure rates (*Ca*) and the increase of mean eEPSC amplitude (*Cb*) by 1 μM αβm-ATP ($n = 4$) and 10 μM ARL67156 ($n = 4$). *D*, A summary of the increase of failure rates after the application of 10 μM PPADS ($n = 4$) and 5 μM suramin ($n = 4$). In all experiments eEPSCs were induced by dorsal root stimulation (see Materials and Methods). Data represent the mean ± SEM; $^*p < 0.05$, paired Wilcoxon test.

around the recorded cells. However, the ecto-ATPase inhibitor ARL67156 effectively might prevent ATP metabolism in the spinal cord slice. ATP may have complicated effects in the slice preparations, not only because of its metabolism but also because of its direct effects on some DH neurons (see also Salter et al., 1993; Li and Perl, 1995; Bardoni et al., 1997; Li et al., 1998).

αβm-ATP-sensitive terminals and P2X receptor subtypes

Although primary afferent neurons express six P2X subunits (Collo et al., 1996; Xiang et al., 1998), the spinal cord distribution of P2X receptor-expressing afferent terminals has not been well characterized. Immunoreactivity to $P2X_1$, $P2X_2$, and $P2X_3$ subunits is present at primary afferent terminals in superficial laminas (Vulchanova et al., 1996, 1997). The αβm-ATP-sensitive synapses shown in this study could be located in deep laminas, but they also could be located in the superficial laminas because deep lamina neurons extend their dendrites to superficial laminas (Ritz and Greenspan, 1985). $P2X_3$ subunits are expressed extensively at primary afferent terminals innervating inner lamina II, raising a possibility that our αβm-ATP-sensitive terminals are derived from $P2X_3$ receptor-expressing terminals. However, this possibility may be inconsistent with the finding that our αβm-ATP-sensitive afferent terminals are capsaicin-insensitive and are not from C-afferent fibers. Nevertheless, ~20% of primary afferent neurons did not coexpress $P2X_3$ subunits and capsaicin VR1 receptors (Guo et al., 1999). Furthermore, $P2X_3$ subunits might not be expressed exclusively on unmyelinated, IB4-positive sensory neurons as previously thought (Petruska et al., 2000). Thus, we cannot rule out the possibility that $P2X_3$-expressing afferent terminals synapse to lamina V dendrites. If $P2X_3$-expressing afferent terminals were involved in our study, the subtypes of P2X receptors that mediated the augmented glutamate release would most likely be heteromeric $P2X_{2+3}$ receptors. Heteromeric $P2X_{2+3}$ receptors respond to αβm-ATP with weak desensitization and probably are expressed on some DRG neurons (Lewis et al., 1995; Burgard et al., 1999; North and Surprenant, 2000). We showed that αβm-ATP produced a sustained increase in spontaneous glutamate release with little or weak desensitization, which appears to be consistent with some properties of heteromeric $P2X_{2+3}$ receptors. However, it has been shown recently that heteromeric $P2X_{1+5}$ receptors and heteromeric $P2X_{4+6}$ receptors in heterologous expression systems also respond to αβm-ATP with a weakly desensitizing current component (Le et al., 1998, 1999; Torres et al., 1998; Haines et al., 1999). A more recent study has provided first evidence suggesting that functional heteromeric $P2X_{1+5}$ receptors are expressed in native tissues (Surprenant et al., 2000). Thus, these two heteromeric P2X receptors, if expressed on primary afferent fibers, may account for the effects of αβm-ATP shown in this study.

Future studies with subtype-selective P2X receptor antagonists such as TNP-ATP (North and Surprenant, 2000; Surprenant et al., 2000; Khakh et al., 2001) may help to reveal the subtype(s) of P2X receptors mediating the augment of glutamate release onto lamina V neurons.

Primary afferent type(s) of αβm-ATP-sensitive terminals

We have provided evidence suggesting that some αβm-ATP-sensitive terminals may be derived from Aδ-afferent fibers. The results that Aδ-eEPSCs can be potentiated after P2X receptor activation directly support this point. Few C-afferent fibers have monosynaptic connections with lamina V neurons (Willis and Coggeshall, 1991), suggesting that the αβm-ATP-sensitive terminals in our study were unlikely to be derived from C-afferent fibers. However, there is a possibility that some Aα/Aβ fibers also

may express αβm-ATP-sensitive P2X receptors, because lamina V neurons receive input from large primary afferents as well (Brown, 1982). In our study the stimulation of dorsal root with intensity sufficient to activate Aα/Aβ fibers did not evoke Aα/Aβ monosynaptic responses in lamina V neurons. Similar results have been reported (Yoshimura et al., 1992). This is thought to be attributable to the impairment of large myelinated fibers in the transverse spinal slice preparations because of their descending and ascending path before terminating in lamina V (Brown, 1982). Nevertheless, the involvement of Aα/Aβ-afferent terminals in αβm-ATP-induced glutamate release is discounted by our finding that large-diameter (>50 μm) DRG neurons had little response to αβm-ATP or ATP (see also Li et al., 1999). Aδ-afferent terminals to lamina V are known to carry nociceptive and non-nociceptive sensory information (Willis and Coggeshall, 1991). We have shown that most Aδ-afferent terminals to the lamina V region were αβm-ATP-sensitive/capsaicin-insensitive. This raises a good possibility that at least some nociceptive Aδ-afferent fibers may express αβm-ATP-sensitive P2X receptors at their central terminals. One possible origin of the αβm-ATP-sensitive central terminals could be from Aδ-mechanoreceptors of keen joints because the Aδ-mechanoreceptors were found to be αβm-ATP-sensitive/capsaicin-insensitive (Dowd et al., 1998).

The potential origin of endogenous ATP

Our results suggest that endogenous ATP may be released in the dorsal horn regions during dorsal root stimulation. The simplest interpretation of our results is that ATP was released from primary afferent terminals. The endogenously released ATP then acted on presynaptic P2X receptors on the primary afferent terminals, which in turn enhanced glutamate release probability (Figs. 7, 8). The possible release of ATP from primary afferent central terminals was proposed first by Holton and Holton (1954). Using spinal cord synaptosomal preparations, White et al. (1985) demonstrated the release of ATP by high K^+ and suggested that ATP may be released from both primary afferent terminals and dorsal horn interneurons. Consistently, with the use of electrophysiological approaches, ATP was suggested to be released in the spinal cord dorsal horn after focal electric stimulation (Bardoni et al., 1997; Li et al., 1998). It was suggested that the endogenously released ATP might function as a fast synaptic transmitter (Bardoni et al., 1997) or modulator (Li et al., 1998). Jo and Schlichter (1999) showed that ATP could be released from many dorsal horn interneurons. It also has been shown that glutamate can evoke the release of ATP from astrocytes (Queiroz et al., 1999). Thus, in addition to the potential release of endogenous ATP from primary afferent terminals, there is also a possibility that ATP is released from postsynaptic dorsal horn neurons and/or surrounding astrocytes as a consequence of glutamate receptor action on these cells. If the latter hypothesis is true, then ATP is a retrograde signaling molecule in the sensory synapses.

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