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Distinct Roles of P2X Receptors in Modulating Glutamate Release at Different Primary Sensory Synapses in Rat Spinal Cord

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Nakatsuka, Terumasa, Kenzo Tsuzuki, Jennifer X. Ling, Hideki Sonobe, and Jianguo G. Gu. Distinct roles of P2X receptors in modulating glutamate release at different primary sensory synapses in rat spinal cord. *J Neurophysiol* 89: 3243–3252, 2003; 10.1152/jn.01172.2002. Using spinal cord slice preparations and patch-clamp recordings in lamina II and lamina V regions, we tested a hypothesis that P2X receptor subtypes differentially modulate glutamate release from primary afferent terminals innervating different sensory regions. We found that activation of P2X receptors by α,β-methylene-ATP increased glutamate release onto >80% of DH neurons in both lamina regions. However, two distinct types of modulation, a transient and a long-lasting enhancement of glutamate release were observed. In lamina II recordings, >70% of the modulation was transient. In contrast, P2X receptor-mediated modulation was always long-lasting in lamina V. Pharmacologically, both transient and long-lasting types of modulation were blocked by 10 μM pyridxal-phospahte-6-azophenyl-2′,4′-disulphonic acid tetrasodium, a broad-spectrum P2X receptor antagonist. Transient modulation was not observed in the presence of 1 μM trinitrophenyl-ATP (TNP-ATP), a subtype-selective P2X receptor antagonist, suggesting that homomeric $P2X_3$ receptors may be involved in the transient modulation in lamina II. The long-lasting modulation remained in the presence of 1 μM TNP-ATP. Selective removal of $P2X_3$-expressing afferent terminals by the targeting toxin saporin-conjugated isolectin B4 or surgical removal of superficial DH did not affect P2X receptor-mediated long-lasting modulation in lamina V. Taken together, these results suggest that P2X receptor subtypes play distinct roles in sensory processing in functionally different sensory regions.

INTRODUCTION

ATP P2X receptors (P2X) belong to a family of nonselective cation channels gated by extracellular ATP (Jahr and Jessell 1983; Krishtal et al. 1983; North and Surprenant 2000). P2X receptors can mediate fast excitatory synaptic transmission (Bardoni et al. 1997; Edwards et al. 1992; Evans et al. 1992). They also play roles in modulating glutamatergic, GABAergic, and glycinergic synaptic transmission in the CNS (Deuchars et al. 2001; Gu and MacDermott 1997; Hugel and Schlichter 2000; Jang et al. 2001; Khakh and Henderson 1998; Li et al. 1998; Rhee et al. 2000). The roles of P2X receptors in sensory signaling in the periphery have been established (Bland-Ward and Humphery 1997; Cockayne et al. 2000; Hamilton et al. 1999; Souslova et al. 2000; Stanfa et al. 2000; Tsuda et al. 1999a,b, 2000). P2X activation also facilitates glutamate release from primary afferent central terminals in the spinal cord (Nakatsuka and Gu 2001), which implicates a central role of P2X and ATP in sensory processing.

Seven P2X subunits ($P2X_1$ to $P2X_7$) have been identified and cloned (North and Surprenant 2000). Six homomeric P2X subtypes ($P2X_1$ to $P2X_5$, $P2X_7$) and at least four heteromeric P2X subtypes ($P2X_{2+3}$, $P2X_{4+6}$, $P2X_{1+5}$, and $P2X_{2+6}$) can be formed by the seven subunits in heterologous expression systems (Khakh et al. 2001; also see Brown et al. 2002). These P2X subtypes can be classified by the ATP analogue, α,β-methylene-ATP ($\alpha\beta$meATP), into $\alpha\beta$meATP-sensitive and -insensitive subgroups. Homomeric $P2X_1$ and $P2X_3$ and heteromeric $P2X_{2+3}$, $P2X_{1+5}$ and $P2X_{4+6}$ are sensitive to $\alpha\beta$meATP, and other subtypes have very low sensitivity to $\alpha\beta$meATP (Khakh et al. 2001). $\alpha\beta$meATP-sensitive P2X receptors can be further divided into rapidly desensitizing group, including homomeric $P2X_1$ and $P2X_3$ (Chen et al. 1995; Valera et al. 1994), and weakly desensitizing group including heteromeric $P2X_{2+3}$, $P2X_{1+5}$, and $P2X_{4+6}$ (Haines et al. 1999; Le et al. 1998; Lewis et al. 1995; Torres et al. 1998). Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium (PPADS) blocks most P2X receptors with little selectivity (Khakh et al. 2001). TNP-ATP selectively blocks P2X1, $P2X_3$, and $P2X_{2+3}$ at nanomolar concentration range (Virginio et al. 1998).

Many dorsal root ganglion (DRG) neurons express P2X receptors (Jahr and Jessell 1983; Krishtal et al. 1983). Two types of currents, rapidly and weakly desensitizing currents, can be elicited by ATP or $\alpha\beta$meATP (Grubb and Evans 1999; Li et al. 1999; Ueno et al. 1999). This indicates that at least two subtypes of functional P2X receptors are expressed on DRG neurons. Rapidly and weakly desensitizing P2X receptors may have distinct sensory functions in the periphery (Cook et al. 1997; Dowd et al. 1998; Sawynok and Reid 1997; Tsuda et al. 2000). Rapidly desensitizing currents are generally thought to be mediated by homomeric $P2X_3$; weakly desensitizing P2X currents are believed to be mainly mediated by $P2X_{2+3}$ (Burgard et al. 1999; Lewis et al. 1995; Tsuda et al. 2000; Xu and Huang 2002). However, other P2X subtypes may also mediate the weakly desensitizing currents in DRG neurons because

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most P2X subunits are found to be expressed in sensory neurons (Collo et al. 1996; Xiang et al. 1998).

Spinal cord DH, the first central site for sensory processing, can be divided into structurally and functionally distinct lamina regions (Willis and Coggeshall 1991). Lamina II is a major receiving center for the inputs from fine afferent fibers that mainly carry nociceptive signals. In contrast, lamina V receives many convergent sensory inputs from both nociceptive and nonnociceptive afferent fibers (Willis and Coggeshall 1991). This region plays an important role in sensory hypersensitivity associated with allodynia and hyperalgesia. We have previously shown that P2X receptors are expressed at the central terminals of primary afferent fibers innervating lamina V neurons and that activation of these receptors modulates sensory transmission to lamina V neurons (Nakatsuka and Gu 2001). However, the properties of these P2X receptors on afferent central terminals have not been examined in detail. In lamina II, little is known about the involvement of P2X receptors in regulating glutamate release from the central terminal of primary afferent fibers. For these reasons, in the present study we have characterized P2X receptor-mediated modulation of glutamate release in both lamina II and lamina V. Further, the present study demonstrates that the modulation in these two regions is distinct due to the differential expression of P2X receptor subtypes at the central terminals of primary afferent fibers.

METHODS

Spinal-cord slice preparation

Principles of laboratory animal care (National Institutes of Health publication No. 86-23, revised 1985) were followed in all the experiments described in this study. Transverse spinal cord slices were prepared from L_5 spinal cords of rats at the postnatal age of 14–21 days as described previously (Nakatsuka and Gu 2001). Briefly, in each experiment, a rat was anesthetized with isoflurane, and then a laminectomy was performed. The lumbosacral spinal cord was taken out and placed in the preoxygenated Krebs solution at 2–4°C. After cutting all ventral and dorsal roots near the root entry zone, the pia-arachnoid membrane was removed, and the spinal cord was mounted on a Vibratome. Unless otherwise indicated, the spinal cord was cut into transverse slices each in the thickness of 500 μm. A spinal cord slice was transferred to a recording chamber (~0.5 ml) and placed on the stage of an upright IR-DIC microscope (BX50WI; Olympus, Tokyo). The slice was superfused with Krebs solution at flow rate of 10 ml/min at room temperature (22°C). The Krebs solution contained (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; the solution was saturated with 95% O_2-5% CO_2 and had pH of 7.3.

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 (Olympus BX50). The microscope was coupled with a CCD camera (CCD100; DAGE-MTI, Michigan City, IN) and a video monitor screen. Whole cell patch-clamp recordings were made from DH neurons with microelectrodes filled with a solution containing (in mM) 135 K^+-gluconate, 5 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 EGTA, and 5 HEPES, pH 7.3; the electrode resistance was ~5 MΩ after filling the electrode solution. Signals were amplified with Axopatch 200B (Axon Instruments, Union City, CA), filtered at 2 kHz, sampled at 5 kHz using pCLAMP 7 (Axon Instruments), and stored in a personal computer. Stable recordings could be obtained from spinal cord slices and maintained for several hours. Spontaneous excitatory postsynaptic currents (sEPSCs) and miniature EPSCs (mEPSCs) were recorded at holding potential of −60 mV where GABAergic and glycinergic synaptic currents were negligible (Nakatsuka and Gu 2001). In some experiments, s- and mEPSCs were recorded in the presence of bicuculline (20 μM) and strychnine (2 μM). sEPSCs were recorded in the absence of TTX and mEPSCs in the presence of 0.5 μM TTX. Effects of $\alpha\beta$meATP on sEPSCs and mEPSCs were examined in lamina II and lamina V neurons. $\alpha\beta$meATP was puff-applied as well as bath-applied in lamina II recordings; $\alpha\beta$meATP was bath-applied in lamina V recordings. For puff application, pressure microinjection of 1 mM $\alpha\beta$meATP was applied at 69 kPa for 5 s by a Picrospitzer (General Valve, Fairfield, NJ). Puff electrodes were positioned with their tips being located at a distance of ~150 μm to the soma of the recorded neurons in lamina II. Puff pipettes were fabricated in the identical way as the recording micropipettes. All antagonists tested were applied through bath solution for ≥10 min before the applications of $\alpha\beta$meATP. Synaptic events including mEPSCs and sEPSCs were analyzed using Mini Analysis Program (Jaejin Software, Anderson Place, GA) with criteria being the same as previously described (Gu and MacDermott 1997). In analyzing the change of sEPSC and mEPSC frequency after bath application of $\alpha\beta$meATP, the time course of sEPSC and mEPSC frequency before and after $\alpha\beta$meATP was first constructed with time bin of 10 s. Then the average response in three bins (30 s) around the peak was used to calculate the changes in reference to the control.

Chemical removal of IB4-positive sensory terminals

In one set of experiments, electrophysiological experiments were performed on the spinal cord slice preparations obtained from rats that were preinjected with saporin-conjugated isolectin B4 (IB4-saporin) to the sciatic nerves. IB4-saporin (Advanced Targeting System, San Diego, CA) injection was performed on rats 5–7 days prior to electrophysiological recordings and immunostaining. In brief, rats were deeply anesthetized with isoflurane, and the left sciatic nerve was isolated. IB4-saporin (2 μl, 1 mg/ml) was injected into the sciatic nerve via a microelectrode (~0.5 μm in diameter) under a dissecting microscope. The wound was washed with 5 ml saline and sutured with 4-0 monofilament nylons. Transverse spinal cord slices (250 μm in thickness) were prepared from L_5 spinal cords 5–7 days after the injection. Whole cell patch-clamp recordings were then made from DH neurons of the injected side. After the electrophysiological recordings, spinal cord slices were fixed, and immunostaining was performed on these spinal cord slices. In another group of experiment, recordings were conducted on lamina V neurons in the spinal cord slices whose superficial laminae were surgically removed.

Immunohistochemistry

IB4 staining and $P2X_3$ subunit immunostaining were performed to determine the effectiveness of IB4-saporin in removing IB4-positive/$P2X_3$-expressing sensory terminals in the spinal cord. VR1-ir was also examined in some slices after recordings. In brief, the transverse spinal cord slices (250 μm in thickness) were put in a 35-mm petri dish and fixed with 4% paraformaldehyde (PFA, in PBS buffer solution) for 12 h at 4°C after the electrophysiological recordings. The slices were transferred into a 4% PFA solution containing 0.4% Triton X-100 and incubated at 4°C for 3 h. They were washed three times with PBS and then mounted onto glass slides and allow to air-dry. They were then encircled with hydrophobic resin (PAP Pen). For IB4 staining, slices were incubated with IB4-biotin (Sigma, St. Louis, MO) at room temperature for 1 h. After a rinse with 1% goat serum PBS solution three times, the slices were further incubated with Streptavidin-Alexa 350 (Molecular Probes, Eugene, OR). For $P2X_3$ immunostaining, slices were incubated with a polyclonal guinea pig anti-$P2X_3$ receptor antibody (1:3,000; Neuromics, Minneapolis, MN)

over night at 4°C. After a rinse with 1% goat serum PBS solution three times, the slices were further incubated with a secondary antibody for 3 h at room temperature. The secondary antibody (1:100 in goat serum PBS solution) was a goat anti-guinea pig IgG-conjugated with Alexa-488 (Molecular Probes). The slices were washed three times with 1% goat serum PBS solution. For VR1 immunostaining, slices were incubated with a polyclonal rabbit anti-VR1 receptor antibody (1: 3,000; Neuromics) overnight at 4°C. After a rinse with 1% goat serum PBS solution three times for 20 min, the slices were further incubated with a secondary antibody for 3 h at room temperature. The secondary antibody (1:100 in goat serum PBS solution) was a goat anti-rabbit IgG conjugated with Alexa-594 (Molecular Probes). Slices were washed three times with 1% goat serum PBS solution and then coverslip was applied with a glycerol-based anti-photobleach medium. Slices were viewed under an inverted fluorescent microscope (IX-70, Olympus, Tokyo, Japan) or a confocal microscope (1024 ES, Bio-Rad, Hercules, CA) equipped with a krypton-argon laser and T1 and T2A filter blocks, attached with an inverted fluorescent microscope (IX-70, Olympus). To provide a quantitative measure of the changes of $P2X_3$-ir in spinal cord slice sections after IB4-injection, the images were first converted to a gray scale for which the darker gray represents heavier immunostaining and the lighter gray represents less immunostaining. The immunostaining intensity was then quantitatively measured by the Scion-Imaging Program (Scion, Frederick, MD). The immunostaining intensity in lamina VII was taken as a reference of negative staining. The ratio between the immunostaining intensity in lamina IIi and lamina VII was calculated and used for the comparison of $P2X_3$-ir.

$\alpha\beta$meATP, PPADS, capsaicin, bicuculline, and strychnine were purchased from Sigma. 6-cyano-7-nitroquinoxaline-2,3-dion (CNQX) and tetrodotoxin (TTX) were purchased from Tocris (Ballowin, MO). Trinitrophenyl-ATP (TNP-ATP) was purchased from Molecular Probe. Data represent means ± SE. Paired Student's *t*-test were used for statistical comparison, and significance was considered at the $P < 0.05$ level.

RESULTS

P2X receptor-mediated modulation of glutamate release onto lamina II neurons

The metabolically stable ATP analogue $\alpha\beta$meATP was used in this study because its action in spinal cord slice preparations was found to be mainly on primary afferent central terminals, and it had little effect on DH neurons (Nakatsuka and Gu 2001). Puff application (5 s) was used to rapidly deliver $\alpha\beta$meATP (~0.1 μl) to lamina II near the places where the recorded lamina II neurons were located. Although the concentration of $\alpha\beta$meATP inside the puff electrode was 1 mM, its actual concentration that reached afferent central terminals should be much lower because of instant dilution by surrounding buffer solution. sEPSC frequency was increased in 9 of 11 lamina II neurons recorded (82%, Fig. 1, *A*, *B*, and *E*) after a 5-s puff application of $\alpha\beta$meATP. The responses had very rapid onset. The effects were not due to mechanical force by puff pressure because puff application of bath solution did not produce any change in sEPSC frequency. The overall changes in sEPSC frequency were 359 ± 119% of controls ($n = 11$). Of the nine cells showing the increased sEPSC frequency, seven of them showed transient responses that were terminated 1–2 s before the end of 5-s $\alpha\beta$meATP application (Fig. 1*A*). The remaining two neurons showed responses that lasted for >10 s with the 5-s $\alpha\beta$meATP application (Fig. 1*B*). Similar to its effects on sEPSC frequency, mEPSC frequency in seven of the nine lamina II neurons recorded also was increased after puff application of $\alpha\beta$meATP (Fig. 1, *C*, *D*, and *F*). The overall changes in mEPSC frequency were 437 ± 147% ($n = 9$). Of the seven cells showing the increased mEPSC frequency, five of them showed transient responses that were terminated 1–2 s

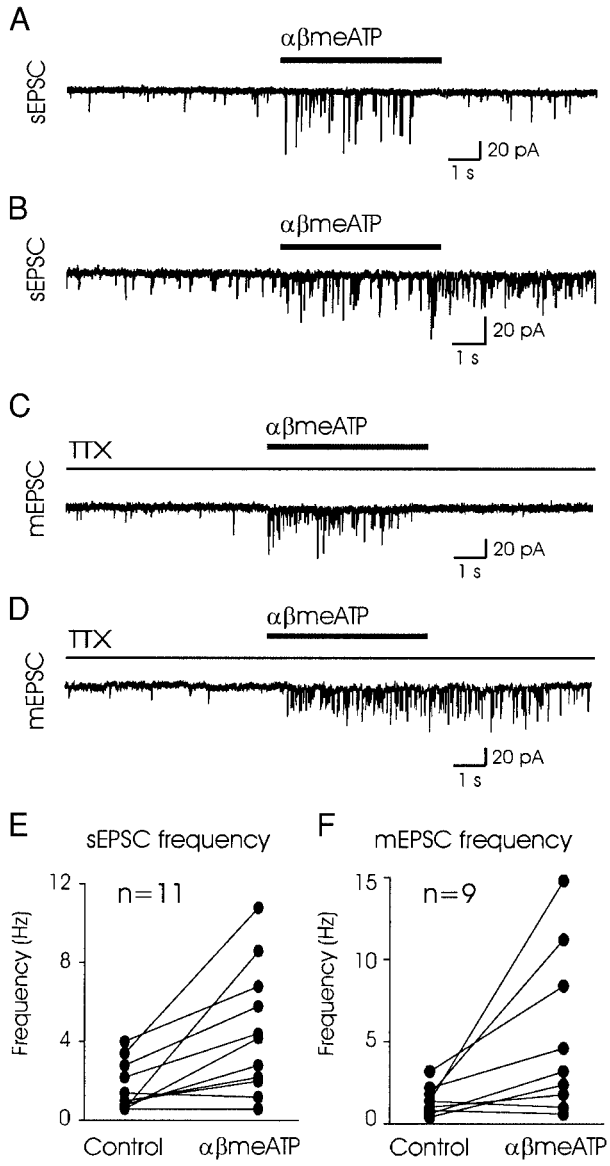

FIG. 1. Increases of spontaneous and miniature excitatory postsynaptic current (sEPSC and mEPSC) frequency in lamina II neurons following puff application of α,β-methylene-ATP ($\alpha\beta$meATP). *A*: an example shows a transient increase of sEPSC frequency in a lamina II neuron after puff application of $\alpha\beta$meATP. The response was desensitized before the end of a 5-s $\alpha\beta$meATP application. $\alpha\beta$meATP concentration inside puff electrode was 1 mM. The line above the trace indicates the duration of $\alpha\beta$meATP application. *B*: an example shows a prolonged increase of sEPSC frequency in a different lamina II neuron after puff application of 1 mM $\alpha\beta$meATP. The response was sustained during a 5-s $\alpha\beta$meATP application and lasted for several seconds after the application. *C* and *D*: puff applications of $\alpha\beta$meATP (1 mM) produced a transient increase (*C*) and a prolonged increase (*D*) of mEPSC frequency in the presence of 500 nM TTX in 2 lamina II neurons. TTX was continuously present in bath solution and was also included in puff solution. *E* and *F*: pooled results show $\alpha\beta$meATP-induced increases of sEPSC (*E*) and mEPSC (*F*) frequency in lamina II neurons. sEPSC frequency was increased in 9 of 11 lamina II neurons recorded (*E*). Of these 9 cells, 7 cells had transient and 2 cells had the prolonged responses. mEPSC frequency was increased in 7 out of 9 lamina II cells recorded (*F*). Of these 7 cells, 5 cells had transient and 2 cells had the prolonged responses.

before the end of 5-s αβmeATP application (Fig. 1*C*). The remaining two neurons showed responses that lasted for >10 s with 5-s αβmeATP application (Fig. 1*D*). In the preceding experiments, αβmeATP did not directly evoke P2X receptor-mediated whole cell currents from the recorded DH neurons because there was little change in baseline current levels during αβmeATP applications (Fig. 1, *A–D*). The sEPSCs and mEPSCs in the preceding experiments were mediated by the release of glutamate from terminals connecting to the recorded neurons because they were abolished completely in the presence of 10 μM 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX, not shown). It was noted that when a cell had a transient response to αβmeATP, a second application of αβmeATP in the same cell usually could not induce an increase in the frequency of sEPSCs and mEPSCs even when the time interval between two applications was >10 min. αβmeATP-induced transient responses rarely recovered completely, even with very long time wash in normal bath solution (not shown). This phenomenon is consistent with the previous findings that some P2X receptors such as homomeric $P2X_3$ receptors recover very slowly from desensitization (Khakh et al. 2001).

The effects of two P2X receptor antagonists, PPADS and TNP-ATP, on αβmeATP-induced responses were tested in lamina II recordings. Due to the very slow and incomplete recovery from the desensitization of αβmeATP-induced responses in most recordings, αβmeATP was applied only one time in the presence of an antagonist in each recording. Therefore the effects of antagonists were analyzed by group comparison. This approach is valid because ~80% of cells responded to αβmeATP in the absence of P2X antagonists. In the presence of 10 μM PPADS in bath perfusion solution, puff application of αβmeATP (1 mM αβmeATP mixed with 10 μM PPADS inside puff electrode) did not significantly increase sEPSC frequency (102 ± 4% of control) in any of nine neurons recorded in lamina II (Fig. 2, *A* and *D*). Similar experiments were performed to test the effects of TNP-ATP. In 10 neurons recorded in the presence of 1 μM TNP-ATP, puff application of 1 mM αβmeATP (1 mM αβmeATP mixed with 1 μM TNP-ATP inside puff electrode) did not significantly increase sEPSC frequency in 8 neurons (Fig. 2*B*). Two neurons clearly showed an increase of sEPSC frequency (Fig. 2*C*), but the responses in these two cells were different from the transient responses observed in most lamina II neurons. They had longer-lasting responses that continue for several seconds after the termination of the 5-s αβmeATP application. The average response induced by αβmeATP in the presence of 1 μM TNP-ATP is presented in Fig. 2*D*. The average response includes data from eight nonresponsive and two responsive neurons.

We examined the modulation of glutamate release in lamina II after the prolonged P2X receptor activation. In this experiment, 100 μM αβmeATP was applied through bath solution for a period of 60 s. Of 22 cells recorded in lamina II, only 5 of them (23%) showed long-lasting increases of sEPSC frequency after αβmeATP application (Fig. 3, *A* and *C*). The responses lasted for >150 s (Fig. 3*A*). In the remaining 17 cells (77%) recorded, neither transient nor long-lasting response was observed (Fig. 3, *B* and *C*). We further examined the effects of αβmeATP on mEPSCs in the presence of 0.5 μM TTX. Similar to the effects on sEPSC frequency, bath application of αβmeATP only produced long-lasting increases of mEPSC frequency in a small percent of neurons recorded (4 of 18 neurons, 22%, Fig. 3, *D* and *F*). The majority of lamina II neurons recorded (14 of 18 cells, 78%) showed no change in mEPSC frequency (Fig. 3, *E* and *F*).

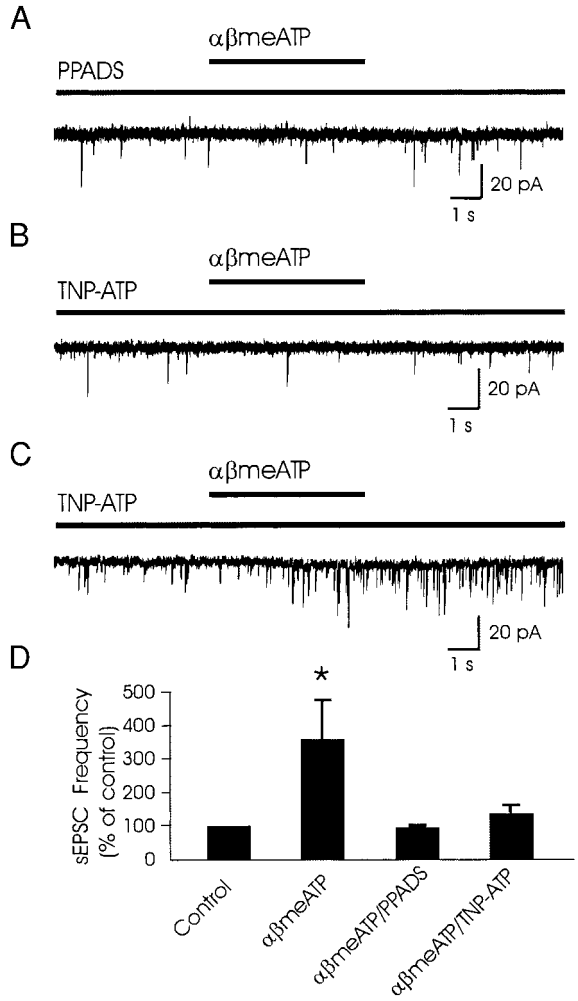

FIG. 2. Effects of pyridxal-phospahte-6-azophenyl-2′,4′-disulphonic acid tetrasodium (PPADS) and trinitrophenyl-ATP (TNP-ATP) on αβmeATP-induced changes of sEPSC frequency in lamina II. *A*: a sample trace shows the lack of response to a 5-s puff application of 1 mM αβmeATP when 10 μM PPADS was present. *B*: a sample trace shows the lack of response to a 5-s puff application of 1 mM αβmeATP in a lamina II cell when 1 μM TNP-ATP was present. *C*: in a different lamina II cell, a 5-s puff application of 1 mM αβmeATP produced a prolonged increase of sEPSC frequency in the presence of 1 μM TNP-ATP. *D*: pooled results show the overall responses to the puff applications of 1 mM αβmeATP in normal bath (αβmeATP, $n = 9$), in the presence of PPADS (αβmeATP/PPADS, $n = 9$), and in the presence of TNP-ATP (αβmeATP/TNP-ATP, $n = 10$). The pooled responses in the presence of TNP-ATP include 8 cells as illustrated in *B* and 2 cells as illustrated in C. Data represent means ± SE; $*P < 0.05$, Student's *t*-test.

P2X receptor-mediated modulation of glutamate release onto lamina V neurons

Although P2X receptor-mediated modulation of glutamate release onto lamina V neurons was reported previously (Nakatsuka and Gu 2001), the properties of this modulation have not been characterized in detail. The following experiments were designed to compare P2X receptor-mediated synaptic modulation in lamina V with that in lamina II.

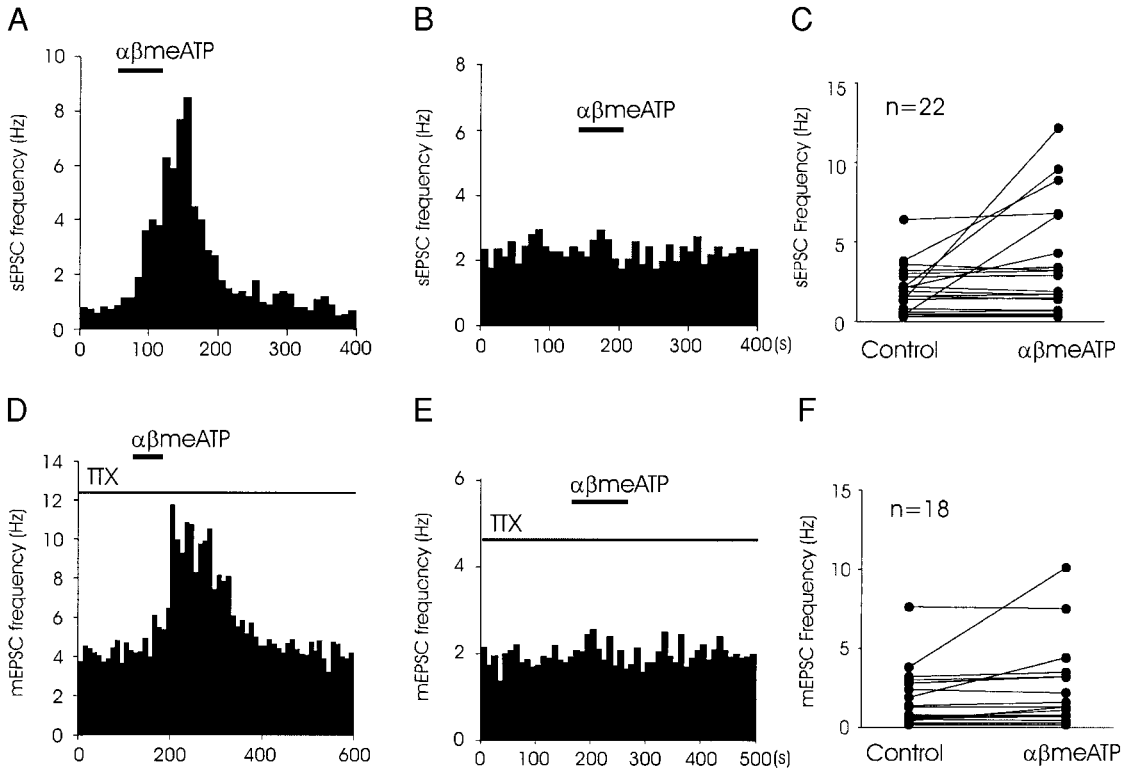

FIG. 3. Changes in the frequency of sEPSCs and mEPSCs in lamina II neurons after the prolonged bath application of $\alpha\beta$meATP. *A*: a histogram shows the time course of the long-lasting increase of sEPSC frequency in a lamina II neuron after bath application of 100 μM $\alpha\beta$meATP for 60 s. The line above the histogram indicates the duration of $\alpha\beta$meATP application. *B*: an example shows that bath application of 100 μM $\alpha\beta$meATP for 60 s did not produce any significant increase of sEPSC frequency in a lamina II neuron. *C*: pooled results from 22 lamina II cells. Five cells had long-lasting responses to $\alpha\beta$meATP as illustrated in *A* and the remaining 17 cells had little response. Each line connecting 2 solid circles represents a recording from a lamina II neuron before and after the application of $\alpha\beta$meATP. *D–F*: the experiments were the same as that shown in *A–C* except mEPSCs were recorded in the presence of 500 nM TTX ($n = 18$). $\alpha\beta$meATP-induced long-lasting increases of mEPSC frequency were only observed in 4 lamina II neurons (*F*). In all the recordings for sEPSCs and mEPSCs, no transient response was observed after bath applications of $\alpha\beta$meATP.

Different from most recordings in lamina II, >90% of lamina V neurons showed long-lasting increases of sEPSC and mEPSC frequency (Fig. 4) after bath application of 100 μM $\alpha\beta$meATP for 60 s. Of 22 cells recorded for sEPSCs (Fig. 4, *A* and *B*), 20 cells showed the increased sEPSC frequency by $\alpha\beta$meATP. The effects lasted for 210 ± 14 s ($n = 20$), about three times longer than the period of $\alpha\beta$meATP application. The remaining two cells did not show a change in sEPSC frequency. The overall changes of sEPSC frequency were 371 ± 48% of control ($n = 22$) after $\alpha\beta$meATP application. The effects of 100 μM $\alpha\beta$meATP on mEPSCs were tested in 20 cells (Fig. 4, *C* and *D*), 19 of them (95%) showed the increased mEPSC frequency. The effects lasted for 181 ± 15 s. Only one cell showed no response. The overall changes in mEPSC frequency were 342 ± 41% of control ($n = 20$) after bath application of 100 μM $\alpha\beta$meATP. The increases of sEPSC and mEPSC frequency could be reproduced by multiple applications of 100 μM $\alpha\beta$meATP at a time interval of 10 min (not shown, but see Fig. 5*B*). $\alpha\beta$meATP at the concentration of 10 μM was tested in eight lamina V neurons; these neurons were also tested with 100 μM $\alpha\beta$meATP (Fig. 4*E*). mEPSC frequency was increased to 168 ± 27% of control ($P < 0.05$, $n = 8$) with 10 μM $\alpha\beta$meATP and to 376 ± 54% of control with 100 μM $\alpha\beta$meATP ($P < 0.05$, $n = 8$).

The two P2X receptor antagonists, PPADS and TNP-ATP, were examined to determine antagonist profiles of $\alpha\beta$meATP-induced responses in lamina V neurons. Because $\alpha\beta$meATP-induced increases of mEPSCs and sEPSCs showed essentially identical responses (Fig. 4), TTX was omitted from bath solution, and sEPSCs were measured in all of the following experiments. The $\alpha\beta$meATP-induced increases of sEPSC frequency were sensitive to the block by PPADS. As shown in Fig. 5, *A* and *C*, the increases of sEPSC frequency by 100 μM $\alpha\beta$meATP (403 ± 89% of control, $n = 6$) were completely abolished in the presence of 10 μM PPADS (104 ± 5% of control, $n = 6$). In contrast to PPADS, TNP-ATP did not significantly inhibit $\alpha\beta$meATP-induced increases of sEPSCs (Fig. 5*B*,*C*). Of six lamina V neurons recorded, $\alpha\beta$meATP (100 μM) facilitated sEPSC frequency to 472 ± 89% of control in the absence of TNP-ATP; $\alpha\beta$meATP still increased sEPSC frequency to 448 ± 99% of control in the same neurons when 1 μM TNP-ATP was present (Fig. 5, *B* and *C*).

$\alpha\beta$meATP-induced long-lasting increases of glutamate release onto lamina V neurons after the removal of $P2X_3$-expressing afferent terminals

$P2X_3$-ir in the spinal cord section was restricted in inner lamina II (Fig. 6*B*), and overlapped with IB4-positive primary afferent central terminals (Fig. 6*A*). However, many lamina V

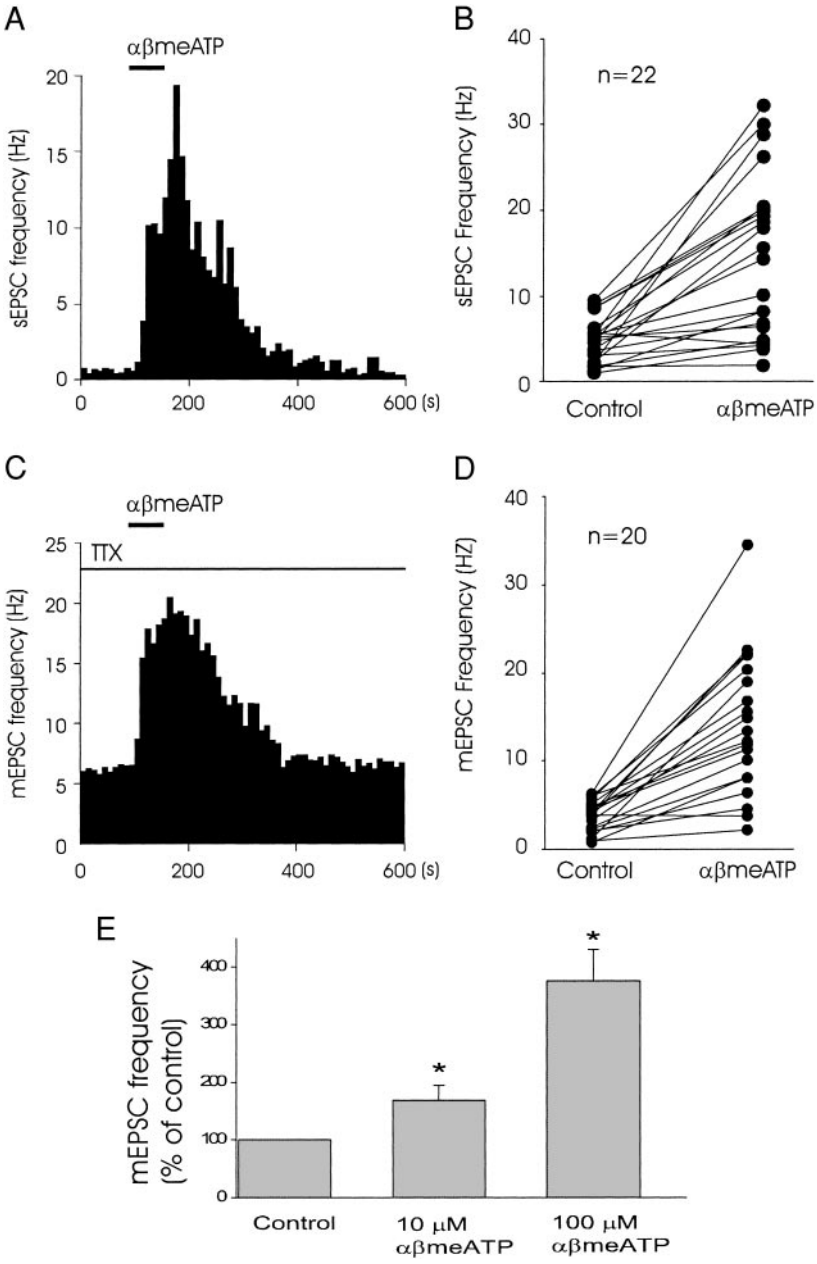

FIG. 4. Changes in the frequency of sEPSCs and mEPSCs in lamina V neurons after the prolonged bath application of $\alpha\beta$meATP. *A*: a histogram shows a long-lasting increase of sEPSC frequency in a lamina V neuron after bath application of 100 μM $\alpha\beta$meATP for 60 s. *B*: pooled results show that the long-lasting increases of sEPSC frequency occurred in almost all neurons recorded in lamina V ($n = 22$). *C* and *D*: similar to *A* and *B* except the experiments were conducted in the presence of 500 nM TTX. $\alpha\beta$meATP induced long-lasting increases of mEPSC frequency in almost all lamina V neurons recorded ($n = 20$). *E*: $\alpha\beta$meATP-induced increases of mEPSC frequency at the concentrations of 10 μM and 100 μM of $\alpha\beta$meATP in lamina V neurons ($n = 8$).

neurons are known to extend their dendrites dorsally into superficial laminae, making it possible that $P2X_{2+3}$-expressing afferent terminals were involved (North 2002). To address whether or not $P2X_{2+3}$ receptors were responsible for the long-lasting modulation in lamina V recordings, we examined effects of $\alpha\beta$meATP on sEPSC frequency after chemically destroying $P2X_3$-expression afferent neurons using IB4-saporin (Fig. 6). Five days after the injection of 2 μl IB4-saporin (1 mg/ml) into the sciatic nerve, IB4 staining in the ipsilateral side disappeared (Fig. 6*A*) from the spinal cord slice preparations of L_5 section. We used the ratio of $P2X_3$-ir intensity between lamina II and lamina VII as a quantitative measure for the change of $P2X_3$-ir after IB4-saporin injection. The ratio of $P2X_3$-ir intensity was 3.00 ± 0.43 ($n = 6$) in the control sides, and it became 1.01 ± 0.11 ($n = 6$, $P < 0.05$) in the IB4-injected side. Thus $P2X_3$-ir was almost completely lost in the ipsilateral side (Fig. 6*B*).

Electrophysiological experiments were performed in such slices prior to immunostaining of $P2X_3$ receptors. The removal of $P2X_3$-expression terminals in the recorded slice sections was confirmed in each experiment after recordings (Fig. 6*B*). $\alpha\beta$meATP-induced responses in lamina V on the ipsilateral site showed similar responses to those recordings from slices of un-injected rats. As shown in Fig. 6, *C* and *D*, bath application of 100 μM $\alpha\beta$meATP still largely enhanced sEPSC frequency to $305 \pm 42\%$ of control ($n = 20$). The effects were observed in almost all lamina V neurons (90%, 18 of 20 neurons from 6 slices) in the IB4-saporin injected sides (Figs. 6, *C* and *D*, and 7*D*).

To further confirm that a subtype of P2X receptors other than $P2X_{2+3}$ receptors was responsible for the long-lasting modulation in lamina V recordings, we examined $\alpha\beta$meATP-induced responses in lamina V neurons after the removal of superficial DH (lamina I–III; Fig. 7). A second purpose of this

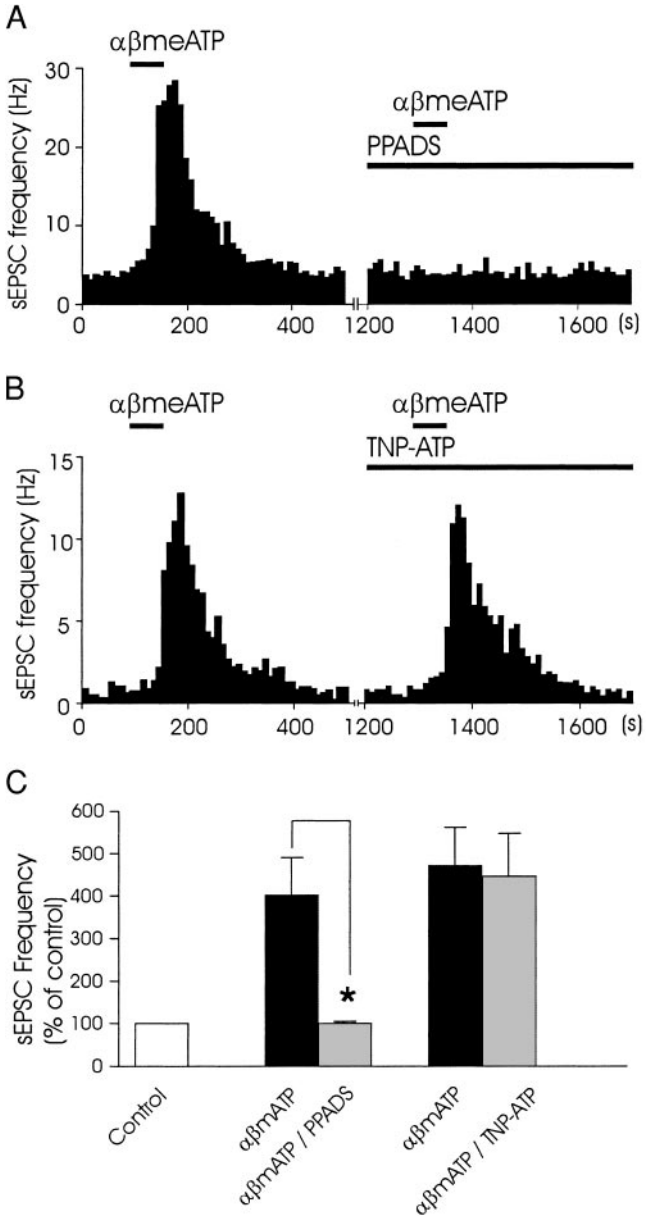

FIG. 5. Effects of PPADS and TNP-ATP on $\alpha\beta$meATP-induced changes of sEPSC frequency in lamina V. *A*: a histogram shows the changes of sEPSC frequency in a lamina V neuron after 60-s bath applications of 100 μM $\alpha\beta$meATP in the absence and presence of 10 μM PPADS. *B*: a histogram shows the changes of sEPSC frequency in a lamina V neuron after 60-s bath applications of 100 μM $\alpha\beta$meATP in the absence and presence of 1 μM TNP-ATP. *C*: pooled results show the overall responses to bath applications of 100 μM $\alpha\beta$meATP in the absence of antagonists ($\alpha\beta$meATP, $n = 6$), in the presence of 10 μM PPADS ($\alpha\beta$meATP/PPADS, $n = 6$), or in the presence of 1 μM TNP-ATP ($\alpha\beta$meATP/TNP-ATP, $n = 6$). Control was sEPSC frequency before $\alpha\beta$meATP application and was scaled as 100%. Data represent means ± SE; *$P < 0.05$, paired Student's *t*-test.

experiment was to determine the location (superficial vs. deep laminae) of these $\alpha\beta$meATP-sensitive synapses. Figure 7, *A* and *B*, illustrates the position of $P2X_3$-ir and its relative position to the portion of the dorsal horn that was surgically removed. In addition, Fig. 7*A* also showed that $P2X_3$-ir largely overlapped with VR1-ir in the inner lamina II (Fig. 7*A*). The removal of superficial lamina abolished capsaicin-induced increases of sEPSC frequency in lamina V neurons ($n = 10$, not shown) (also see Nakatsuka et al. 2002), indicating the effectiveness of removing VR1/$P2X_3$-expressing terminals. When $\alpha\beta$meATP (100 μM) was tested in such preparation, bath

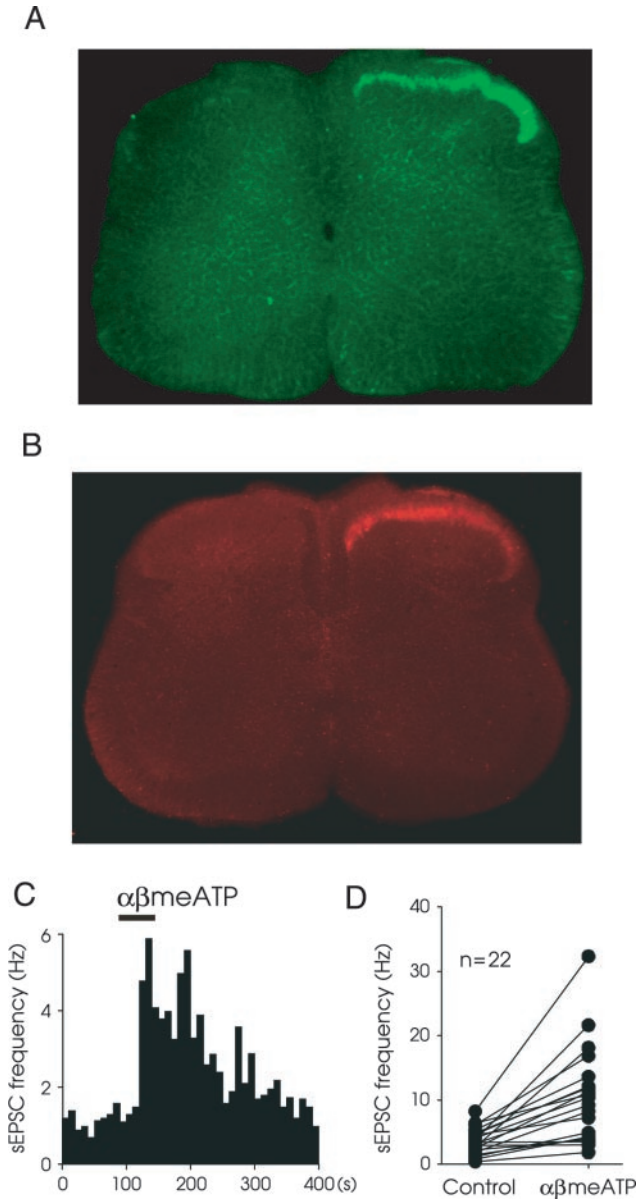

FIG. 6. $\alpha\beta$meATP-induced long-lasting increases of sEPSC frequency in lamina V neurons after the removal of $P2X_3$-expressing afferent terminals by saporin-conjugated isolectin B4 (IB4-saporin). *A*: the micrograph shows IB4 staining in lamina II of control side (right side) and the lack of IB4 staining in the left side in a L_5 spinal cord slice section. The slice was obtained from a rat for which IB4-saporin (IB4-SAP) was injected into the left sciatic nerve. The L_5 spinal cord slice section was obtained and IB4 staining performed 5 days after the injection of IB4-SAP. *B*: the experiment was the same as *A* except immunostaining was performed for $P2X_3$ subunit. $P2X_3$-ir was shown in lamina II of right side (control side) and disappeared from left side (IB4-SAP injected side). *C*: an example shows a recording from a lamina V neuron in the IB4-SAP injected side. $\alpha\beta$meATP still induced long-lasting increases of sEPSC frequency in the lamina V neuron when $P2X_3$-expressing terminals were removed by IB4-SAP. *D*: pooled results from 22 lamina V neurons of IB4-SAP injected sides. The removal of IB4/$P2X_3$-expressing terminals had little effect on $\alpha\beta$meATP-induced responses in lamina V. In all experiments, the removal of $P2X_3$-expressing terminals in the IB4-SAP-injected sides was confirmed by $P2X_3$ immunostaining after each electrophysiological experiment.

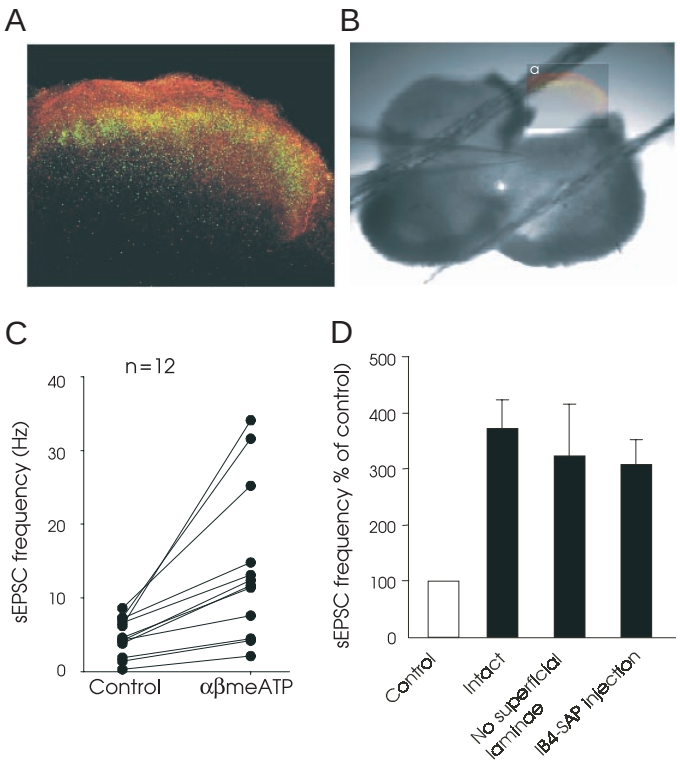

FIG. 7. αβmeATP-induced long-lasting increases of sEPSC frequency in lamina V after surgical removal of superficial laminae. *A*: a confocal image of immunoreactivity for $P2X_3$ subunit and VR1 receptor in a spinal cord slice. $P2X_3$-ir (green) was restricted in lamina II and VR1-ir (red) across both lamina I and lamina II regions. In lamina II, $P2X_3$-ir and VR1-ir overlapped (yellow) substantially. *B*: the micrograph shows a spinal cord slice whose superficial laminae on the right side were removed surgically. A recording electrode is shown in the field, and the tip of the electrode is located in lamina V region on the right side. An *inset* in *B* is a resized image of *A* to show the relative position of $P2X_3$-ir to the surgically removed portion of the dorsal horn. *C*: αβmeATP-induced increases of sEPSC frequency in the preparation as illustrated in *B* ($n = 12$). *D*: a summary shows αβmeATP-induced changes of sEPSC frequency in intact spinal cord slice ($n = 22$), in the slices whose superficial laminae were surgically removed ($n = 12$), and in the slice obtained from IB4-SAP injected rats ($n = 20$). Data represent means ± SE; Student's *t*-test were used for comparison, and no significant differences were found between the intact slice group and 2 other groups.

application of αβmeATP still largely increased sEPSC frequency in all 12 lamina V neurons recorded (Fig. 7, *C* and *D*). The overall changes of sEPSC frequency were 320 ± 89% of control ($n = 12$), similar to the responses in intact slices. Thus the removal of $P2X_3$-expressing terminals either chemically or surgically did not significantly change the αβmeATP-induced responses in lamina V neurons (Fig. 7*D*).

DISCUSSION

We have demonstrated that P2X receptors play distinct roles in modulating glutamate release at different primary sensory synapses in the dorsal horn of the spinal cord. A transient type of modulation, mediated by rapidly desensitizing subtype of P2X receptors, is predominant in the primary sensory synapses of lamina II. Whereas in lamina V, a long-lasting type of modulation is engaged in almost all the primary sensory synapses and is mediated by weakly desensitizing subtype of P2X receptors. These results implicate that P2X receptor subtypes play distinct roles in central processing of different primary sensory inputs. Because both lamina II and V are involved in nociceptive transmission, the facilitation of glutamate release by presynaptic P2X receptors may have implications in the changes of synaptic plasticity during physiological and pathological pain states.

P2X receptor-mediated modulation of glutamate release onto lamina II neurons

Immunochemical evidence has shown the expression of P2X subunits at the central terminals of primary afferent fibers (Guo et al. 1999; Vulchanova et al. 1996–1998). Although activation of P2X receptors produced glutamate release from sensory synapses in a DGR-DH co-culture system (Gu and MacDermott 1997; Labrakakis et al. 2000), only one study suggests that P2X receptors may play a role in modulating glutamate release in lamina II (Li et al. 1998). Using puff application to rapidly deliver αβmeATP, we found that the frequency of sEPSCs and mEPSCs, i.e., synaptic release of glutamate, was increased in most recordings in lamina II. Most responses were shown to be transient and rapidly desensitized. In contrast, most recordings did not reveal any significant increase in the frequency of sEPSCs and mEPSCs when αβmeATP was bath applied. The lack of transient responses during bath application of αβmeATP was most likely due to the slow penetration of αβmeATP to the tissue, which inactivated the rapidly desensitizing P2X receptors before it could be activated. Thus most afferent terminals connecting to lamina II neurons expressed rapidly desensitizing P2X receptors, and activation of these receptors produces a transient increase of glutamate release. Pharmacologically, we showed that the transient responses in lamina II were sensitive to the block by both PPADS and TNP-ATP. Because $P2X_3$ subunits are high expressed at the afferent terminals innervating lamina II regions (Vulchanova et al. 1997, 1998) and homomeric $P2X_3$ receptors are highly sensitive to the block by TNP-ATP (Virginio et al. 1998; also see Burgard et al. 1999; Grubb and Evans 1999), the transient responses induced by αβmeATP in lamina II were most likely mediated by homomeric $P2X_3$ receptors. However, the potential contribution of $P2X_1$ receptors cannot be excluded.

We have shown that in a small population of lamina II neurons, focal and bath application of αβmeATP induced a prolonged increase of glutamate release. This suggests that the weakly desensitizing P2X receptors are expressed on a small number of afferent terminals innervating lamina II. In DRG neurons, weakly desensitizing P2X receptors that could be activated by αβmeATP were highly speculated to be $P2X_{2+3}$ receptors (Burgard et al. 1999; Lewis et al. 1995; Xu and Huang 2002). $P2X_{2+3}$ receptors are sensitive to the block by TNP-ATP in nanomolar concentration range (Virginio et al. 1998). However, we found that during continuous perfusion of 1 μM TNP-ATP, αβmeATP still could produce a long-lasting increase of glutamate release in a small number of lamina II neurons. This raises a possibility that some afferent central terminals innervating lamina II regions may express a P2X subtype different from $P2X_{2+3}$. It should be noted that our experimental protocol with TNP-ATP in this study could not allow us to determine the potential role of $P2X_{2+3}$ heteromeric receptors (Xu and Huang 2002) in αβmeATP-induced long-lasting responses because TNP-ATP were present through the experiments in lamina II recordings. $P2X_{2+3}$-like currents have

recently been demonstrated in the soma of DRG neurons (Burgard et al. 1999; Grubb and Evans 1999; Rae et al. 1998; Tsuda et al. 2000; Ueno et al. 1999) although co-expression of $P2X_2$ and $P2X_3$ subunits at the same central terminals of primary afferent fibers was not clearly evident immunocytochemically with confocal microscopic study (Vulchanova et al. 1997).

P2X receptor-mediated modulation of glutamate release onto lamina V neurons

$\alpha\beta$meATP largely increased sEPSC and mEPSC frequency in almost all lamina V neurons for a prolonged period. This effect was distinct from $\alpha\beta$meATP-induced transient responses recorded in lamina II cells. These results suggest that weakly desensitizing P2X receptors are expressed at the central terminals of primary afferents innervating lamina V neurons. $\alpha\beta$meATP-induced increases of mEPSCs and sEPSCs in lamina V were unlikely due to its action on DH neurons because $\alpha\beta$meATP-sensitive P2X receptors were found on DH inhibitory interneurons not on glutamatergic DH neurons (Hugel and Schlichter 2000; Jang et al. 2001; Rhee et al. 2000). The expression of $\alpha\beta$meATP-sensitive P2X receptors on Aδ-afferent terminals in lamina V has been more directly demonstrated recently by the effects of $\alpha\beta$meATP on Aδ-afferent-evoked EPSCs recorded in lamina V neurons; $\alpha\beta$meATP at low concentrations potentiated Aδ-afferent-evoked EPSCs (Nakatsuka and Gu 2001) and failed the evoked EPSCs in lamina V neurons at high concentrations (Tsuzuki et al. 2003).

It has been widely thought that P2X receptors on the central terminals of Aδ-afferent fibers are $P2X_{2+3}$ receptors (North 2002). However, $\alpha\beta$meATP-induced responses in lamina V recordings were remained in the presence of 1 μM TNP-ATP, raising a possibility that these weakly desensitizing P2X receptors were not $P2X_{2+3}$ receptors. One possibility for the lack of TNP-ATP effects could be due to its metabolic instability in tissue preparations (Lewis et al. 1998). However, the abolishment of transient responses in lamina II suggested that TNP-ATP reached targets at a concentration high enough to completely block homomeric $P2X_3$ receptors. Because $P2X_{2+3}$ receptors were shown to be slightly less sensitive to TNP-ATP antagonism than $P2X_3$ receptors (Virginio et al. 1998), we used two TNP-ATP-independent approaches to test the idea that those $\alpha\beta$meATP-activated P2X receptors were not $P2X_{2+3}$. One approach was to chemically remove $P2X_3$-expressing afferent fibers, and the other approach was to surgically remove superficial laminae. $P2X_3$ subunits have been shown to be predominantly, if not exclusively, expressed on IB4-positive DRG neurons (Vulchanova et al. 1998). IB4-positive DRG neurons could be selectively destroyed by IB4-saporin (IB4-SAP) (Vulchanova et al. 2001). We found that IB4-staining was negative and $P2X_3$-ir was lost in L_5 spinal slice sections after sciatic nerve injections of IB4-SAP. In such a spinal cord slice preparation, we found that $\alpha\beta$meATP still produced long-lasting increases of glutamate release onto lamina V neurons. Furthermore, $\alpha\beta$meATP-induced responses were not altered in lamina V recordings when $P2X_3$-ir regions (superficial laminae) in the spinal cord were surgically removed. The results with surgical removal of superficial laminae also indicate that afferent terminals expressing the weakly desensitizing P2X receptors make synaptic contacts with lamina V neurons in deep laminae. Taken together, the P2X receptors that mediated long-lasting enhancement of glutamate release onto lamina V neurons were unlikely to be $P2X_{2+3}$ receptors. The pharmacological profiles of $\alpha\beta$meATP-mediated synaptic modulation in lamina V appear to be consistent with the involvement of $P2X_{1+5}$ and/or $P2X_{4+6}$. We have recently shown the similar pharmacological profiles of $\alpha\beta$meATP-evoked currents directly recorded from dissociated DRG neurons (see Tsuzuki et al. 2003). However, in view that unidentified P2X heteromeric receptors (North 2002) may be present in native cells, it remains to be possible that P2X receptors on Aδ-afferent terminals innervating lamina V region are new P2X receptor subtypes.

In conclusion, differential modulation of sensory inputs into different sensory regions by P2X receptor subtypes represents an important mechanism of sensory processing in the spinal cord dorsal horn.

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