



# *In vivo* pathway of thermal hyperalgesia by intrathecal administration of $\alpha,\beta$ -methylene ATP in mouse spinal cord: Involvement of the glutamate-NMDA receptor system

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**1** The aim of the present study is to characterize the role of the P2X receptor in spinal nociceptive processing *in vivo*. We investigated the mechanisms of the P2X receptor agonist  $\alpha,\beta$ -methylene ATP ( $\alpha,\beta$ meATP)-induced modulation of acute nociceptive signalling in mouse spinal cord.

**2** Intrathecal administration of  $\alpha,\beta$ meATP produced a significant and dose-dependent thermal hyperalgesic response. This response was completely blocked by intrathecal pretreatment with the non-selective P2 receptor antagonist, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonate (PPADS) and the selective P2X<sub>1</sub>, P2X<sub>3</sub> and P2X<sub>2+3</sub> receptor antagonist, 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP). Pretreatment with  $\alpha,\beta$ meATP 15, 30 and 60 min prior to administration of a second dose of  $\alpha,\beta$ meATP diminished the  $\alpha,\beta$ meATP-induced thermal hyperalgesia.

**3** A potent agonist for the P2X<sub>1</sub> receptor,  $\beta,\gamma$ -methylene-L-ATP, did not show the hyperalgesic response, indicating that the P2X<sub>1</sub> receptor is not involved in the spinal nociceptive pathway.

**4** In fura-2 experiments using mouse dorsal root ganglion (DRG) neurons,  $\alpha,\beta$ meATP (100  $\mu$ M) increased intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>). This was not produced by a second application of  $\alpha,\beta$ meATP. The same DRG neurons also showed a marked [Ca<sup>2+</sup>]<sub>i</sub> increase in response to capsaicin (3  $\mu$ M).

**5** Intrathecal pretreatment with the Ca<sup>2+</sup>-dependent exocytosis inhibitor, botulinum neurotoxin B, abolished the thermal hyperalgesia by  $\alpha,\beta$ meATP. Furthermore, thermal hyperalgesia was significantly inhibited by the N-methyl-D-aspartate (NMDA) receptor antagonists, 2-amino-5-phosphonopentanoate (APV), dizocilpine and ifenprodil.

**6** These findings suggest that  $\alpha,\beta$ meATP-induced thermal hyperalgesia may be mediated by the spinal P2X<sub>3</sub> receptor subtype that causes unresponsiveness by repetitive agonist applications, and that  $\alpha,\beta$ meATP (perhaps through P2X<sub>3</sub> receptors) may evoke spinal glutamate release which, in turn, leads to the generation of thermal hyperalgesia *via* activation of NMDA receptors.

**Keywords:**  $\alpha,\beta$ meATP; P2X<sub>3</sub> receptor subtype; thermal hyperalgesia; glutamate; NMDA receptor; mouse spinal cord

**Abbreviations:**  $\alpha,\beta$ meATP,  $\alpha,\beta$ -methylene ATP; APV, 2-amino-5-phosphonopentanoate;  $\beta,\gamma$ me-L-ATP,  $\beta,\gamma$ -methylene-L-ATP; BoTNT/B, botulinum neurotoxin B; DRG, dorsal root ganglia; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonate; TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate

## Introduction

Extracellular adenosine 5'-triphosphate (ATP) excites many neuronal preparations by activating ATP-gated cation channels called P2X receptors (Burnstock & Wood, 1996; Inoue *et al.*, 1996; Ralevic & Burnstock, 1998). In sensory neurons, it is well known that ATP or its analogue evoke the inward current (Krishtal *et al.*, 1983; 1988a,b; Bean, 1990; Lewis *et al.*, 1995; Robertson *et al.*, 1996; Cook *et al.*, 1997; Rae *et al.*, 1998; Ueno *et al.*, 1999), proposing the role of ATP and its receptors in the generation or modulation of pain (Burnstock & Wood, 1996; Inoue *et al.*, 1996; Ralevic & Burnstock, 1998). This idea is strongly supported by the interesting finding that mRNA of the P2X<sub>3</sub> receptor subtype, which is one of seven cloned P2X receptor subtypes (P2X<sub>1</sub>–P2X<sub>7</sub>) (reviewed in Ralevic & Burnstock, 1998), is selectively expressed in capsaicin-sensitive small-diameter cell bodies of the trigeminal and dorsal root ganglion (DRG) (Chen *et al.*, 1995). Using immunological methods, P2X<sub>3</sub> receptors have

been found in the nociceptive, but not non-nociceptive, sensory nerve endings and cell bodies (Cook *et al.*, 1997). Recently, in addition to P2X<sub>3</sub>, the presence of both mRNA and protein of the other P2X receptor subtypes (P2X<sub>1,2,4–6</sub>) in sensory neurons has been shown (Collo *et al.*, 1996; Vulchanova *et al.*, 1997; Ueno *et al.*, 1999; Xiang *et al.*, 1998). Furthermore, the distribution of some of these P2X receptor subtypes in sensory neurons show different patterns (Ueno *et al.*, 1999; Xiang *et al.*, 1998), suggesting that there are several types of cell which express different P2X receptor subtypes. In fact, recent electrophysiological studies have revealed two types of ATP- and its analogue  $\alpha,\beta$ -methylene ATP ( $\alpha,\beta$ meATP)-evoked inward currents in sensory neurons (Cook *et al.*, 1997). We have further characterized the profiles of the ATP- and  $\alpha,\beta$ meATP-activated responses in DRG neurons based on current kinetic and capsaicin-sensitivity: capsaicin-sensitive DRG neurons have a rapidly desensitizing current, and capsaicin-insensitive medium-sized DRG neurons have a slowly desensitizing current (Ueno *et al.*, 1999). Together with previous evidence (Ueno *et al.*, 1998), it has

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been considered that the activation of the homomeric P2X<sub>3</sub> receptor is responsible for a rapidly desensitizing current in capsaicin-sensitive DRG neurons and that the heteromultimeric P2X<sub>2+3</sub> receptor is responsible for the slowly desensitizing current in capsaicin-insensitive medium-sized DRG neurons (Ueno *et al.*, 1999). Therefore, these findings suggest the possibility that these P2X receptors may play a role in the signal for nociceptive processing in peripheral and/or central sites such as the spinal cord *in vivo*.

*In vivo* evidence of the role of P2X receptors at peripheral sites have been shown previously in several animal models for nociception (Bland-Ward & Humphrey, 1997; Sawynok & Reid, 1997; Dowd *et al.*, 1998) and in humans (Bleehan & Keele, 1977; Bleehan, 1978; Coutts *et al.*, 1981), and have suggested the involvement of P2X receptors on peripheral nerve endings of primary afferent neurons in acute nociceptive behaviour (Burnstock & Wood, 1996). As for the central site, in spite of recent studies concerning the localization of P2X receptors in the spinal cord (Collo *et al.*, 1996; Vulchanova *et al.*, 1997; 1998; Lê *et al.*, 1998; Llewellyn-Smith & Burnstock, 1998), there has been no further investigation since Driessen *et al.* (1994) demonstrated that intrathecal injection of  $\alpha,\beta$ meATP and 2-methylthio ATP decreased tail-flick latency. Thus, the nociceptive pathway of the P2X receptor-mediated pain-modulating effect in the spinal cord is still unknown. It seems worthy to investigate the *in vivo* mechanisms of P2X receptor-mediated modulation of nociceptive processing in the spinal cord because neuronal function in the spinal cord has been considered to play a pivotal role in pathological pain in the clinic (Coderre *et al.*, 1993).

In the present study, to characterize the functional consequence of P2X receptor activation in the spinal nociceptive pathway *in vivo*, we studied (1) whether the intrathecal administration of  $\alpha,\beta$ meATP produces the increase in the thermal nociceptive response (thermal hyperalgesia); (2) which P2X receptor subtypes are involved in the  $\alpha,\beta$ meATP-induced thermal hyperalgesia and (3) whether  $\alpha,\beta$ meATP-induced thermal hyperalgesia is mediated by a signalling pathway through the activation of N-methyl-D-aspartate (NMDA) receptors in the spinal cord.

## Methods

### Animals

Male ddY mice (20–23 g) were obtained from the Shizuoka Laboratory Center (Shizuoka, Japan). The animals were housed at a temperature of  $22 \pm 1^\circ\text{C}$  with a 12 h light–dark cycle (light on 08 30 to 20 30). Food and water were available *ad libitum*.

### Assessment of nociception

To assess the thermally evoked paw withdrawal response, we measured the paw withdrawal latency to a radiant heat stimulus. Mice were gently held by hand with their right hindpaw positioned in an apparatus (Ugo Basile, Italy) for radiant heat stimulation on the plantar surface. Intensity of heat stimulus was adjusted for paw withdrawal latency of 9–10 s in normal animals. The latency of paw withdrawal response in control mice averaged 9.3 s over the course of the experiments. The thermal hyperalgesia was expressed as change in paw withdrawal latency (latency of paw withdrawal response after  $\alpha,\beta$ meATP injection minus latency before that). A cut-off time was set at 20 s to avoid injury to the hindpaw.

### Intrathecal injection

An intrathecal administration was performed according to the procedure described by Hylden & Wilcox (1980) using a 25- $\mu\text{l}$  Hamilton syringe with 28-gauge needle. The paw withdrawal response was measured 5 min after intrathecal injection of  $\alpha,\beta$ meATP. Pyridoxal-phosphate-6-azophenyl-2',4'-disulphonate (PPADS: 1.0–5.0  $\mu\text{g}$  per mouse) and 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP: 0.25–1.0  $\mu\text{g}$  per mouse) were intrathecally injected 10 min before  $\alpha,\beta$ meATP injection. Botulinum neurotoxin type B (BoNT/B: 0.1–10 ng per mouse) was intrathecally injected 12 h before  $\alpha,\beta$ meATP injection. The schedule for treatment with BoNT/B was as reported previously (Pierce & Kalivas, 1997). Mice were injected intrathecally with 2-amino-5-phosphonopentanoate (APV: 0.1–1.0  $\mu\text{g}$  per mouse), dizocilpine (0.1–0.4  $\mu\text{g}$  per mouse) or ifenprodil (5–20  $\mu\text{g}$  per mouse) 10 min before  $\alpha,\beta$ meATP injection.

### Measurement of $[\text{Ca}^{2+}]_i$ in acutely dissociated mouse DRG neuron

Male ddY mice were decapitated under ether anaesthesia and the DRG were removed from the L4–6 segments. The DRG were treated first with 20 unit  $\text{ml}^{-1}$  papain (Worthington Biochemical Co., NJ, U.S.A.) dissolved in Tyrode's solution for 10 min at  $37^\circ\text{C}$ . Tissue was then treated with 4 mg  $\text{ml}^{-1}$  collagenase type II (CLS2; Worthington Biochemical Co.) and 2.5 unit  $\text{ml}^{-1}$  Dispase (Calbiochem, CA, U.S.A.) dissolved in Tyrode's solution for 30 min at  $37^\circ\text{C}$ . At the end of this treatment, the enzyme solution was removed and the cells were then mechanically dissociated by trituration through a pasteur pipette. Cells were plated on poly-L-lysine (Sigma, MO, U.S.A.)-coated glass coverslips with silicon rubber walls (Flexiperm, W.C. GmbH, Germany). The increase in  $[\text{Ca}^{2+}]_i$  in single cells was measured by the fura-2 technique (Grynkiewicz *et al.*, 1985) with minor modifications (Koizumi *et al.*, 1994). The cells were incubated with 5  $\mu\text{M}$  fura-2 acetoxymethylester (fura-2 AM; Dojindo, Kumamoto, Japan) for 30 min in balanced salt solution (BSS; composition in mM: NaCl 150, KCl 5,  $\text{CaCl}_2$  1.2,  $\text{MgCl}_2$  1.2, D-glucose 10 and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 25; pH 7.4). Then, the cells were washed with BSS and mounted on an inverted fluorescence microscope (TMD-300, Nikon, Tokyo, Japan) equipped with a Xenon-lamp and band-pass filters of 340 nm wavelength and 360 nm wavelength. The emission fluorescence was measured at 510 nm. Image data, recorded by a high-sensitivity silicon intensifier target camera (C-2741-08, Hamamatsu Photonics, Hamamatsu, Japan), were processed by a  $\text{Ca}^{2+}$ -analysing system (Furusawa Lab. Appliance Co., Kawagoe, Japan).  $\alpha,\beta$ meATP (100  $\mu\text{M}$ ) and capsaicin (3  $\mu\text{M}$ ) were applied to the cells for 15 s with each application.

### Drugs

$\alpha,\beta$ -Methylene ATP ( $\alpha,\beta$ meATP) and  $\beta,\gamma$ -methylene-L-ATP ( $\beta,\gamma$  me-L-ATP) (Sigma, MO, U.S.A.) were dissolved in phosphate-buffered saline (PBS; composition in mM: NaCl 137, KCl 2.7,  $\text{KH}_2\text{PO}_4$  1.5,  $\text{NaH}_2\text{PO}_4$  8.1; pH 7.4). Pyridoxal-phosphate-6-azophenyl-2',4'-disulphonate tetrasodium (PPADS: RBI, MA, U.S.A.), 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP: Molecular Probes, OR, U.S.A.), 2-amino-5-phosphonopentanoic acid (APV: RBI, MA, U.S.A.), dizocilpine (RBI, MA, U.S.A.) and botulinum neurotoxin type B (BoNT/B: Calbiochem-Nova-

biochem, CA, U.S.A.) were dissolved and diluted in saline. Ifenprodil (RBI, MA, U.S.A.) was dissolved in 1% dimethyl sulphoxide (DMSO) in saline.

### Statistical analysis

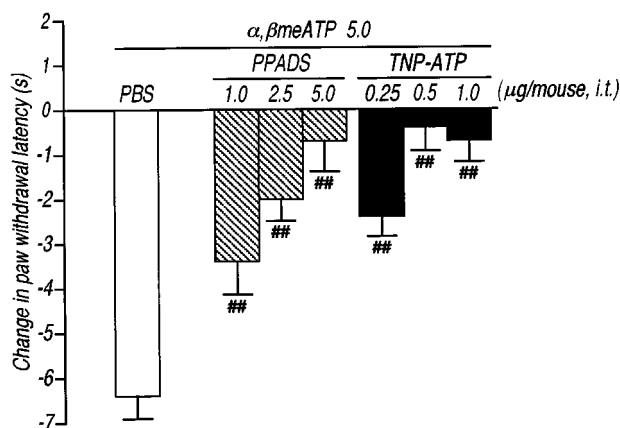
The latency of paw withdrawal responses was evaluated statistically using the Student's *t*-test or Wilcoxon test.

## Results

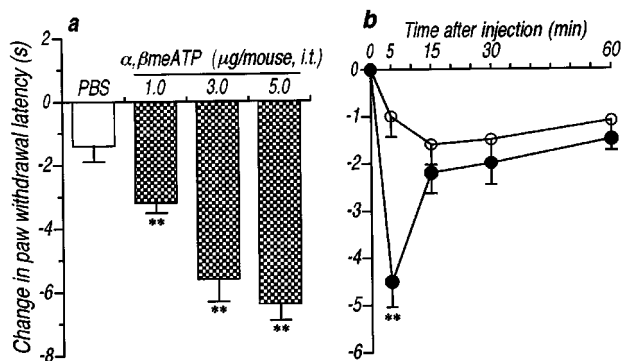
Intrathecal administration of  $\alpha,\beta$ meATP (1.0–5.0  $\mu$ g per mouse) produced a significant and dose-dependent thermal hyperalgesic response (the decrease in the paw withdrawal latency to noxious heat stimulus) (Figure 1a). The thermal hyperalgesic response was relatively short acting: at 5.0  $\mu$ g per mouse of  $\alpha,\beta$ meATP, hyperalgesia peaked at 5 min after intrathecal injection of  $\alpha,\beta$ meATP ( $P < 0.01$ ) and recovered after 15 min (Figure 1b). In Figure 2, antagonistic effects of non-selective and selective antagonists of P2 receptors were examined. The  $\alpha,\beta$ meATP-induced thermal hyperalgesia was blocked in a dose dependent fashion by intrathecal pretreatment with the non-selective P2 receptor antagonist, PPADS (1.0–5.0  $\mu$ g per mouse;  $P < 0.01$ ). Furthermore, pretreatment with the selective P2X<sub>1</sub>, P2X<sub>3</sub> and P2X<sub>2+3</sub> receptor antagonist, TNP-ATP (Virginio *et al.*, 1998) at doses of 0.25, 0.5 and 1.0  $\mu$ g per mouse inhibited the  $\alpha,\beta$ meATP-induced thermal hyperalgesia in a dose-dependent manner ( $P < 0.01$ ). The two largest doses of TNP-ATP completely blocked the  $\alpha,\beta$ meATP-induced thermal hyperalgesic response, suggesting that this response is mediated by P2X<sub>1</sub>, P2X<sub>3</sub> or P2X<sub>2+3</sub> receptors in the spinal cord. Paw withdrawal latency was not changed by each antagonist alone (control:  $9.5 \pm 0.5$ , PPADS 5.0  $\mu$ g per mouse:  $9.3 \pm 0.5$  s, TNP-ATP 1.0  $\mu$ g per mouse:  $9.8 \pm 0.5$  s). Intrathecal administration of  $\beta,\gamma$  me-L-ATP (5.0 and 10.0  $\mu$ g per mouse), which is a potent agonist for P2X<sub>1</sub> receptors, did not affect the paw withdrawal latency (Figure 3). Therefore, it is not likely that the P2X<sub>1</sub> receptor subtype is involved in the  $\alpha,\beta$ meATP-induced thermal hyperalgesia. As shown in Figure

4, the thermal hyperalgesic response was significantly reduced by intrathecal pretreatment with  $\alpha,\beta$ meATP (5.0  $\mu$ g per mouse). The prior injection of  $\alpha,\beta$ meATP at 15 and 30 min extended the paw withdrawal latency as compared with non-treatment value, indicating that the P2X receptor involved in thermal hyperalgesic response is inactivated by pretreatment with  $\alpha,\beta$ meATP. The inactivation by pretreatment with  $\alpha,\beta$ meATP recovered slowly and lasted for 120 min.

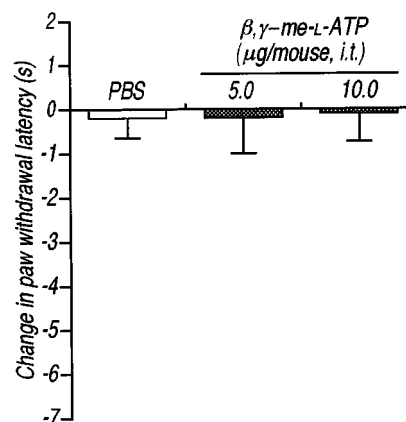
Since ATP and related compounds have been previously shown to stimulate P2X receptors located on sensory neurons, causing intense pain, the present study explored the possibility that  $\alpha,\beta$ meATP-induced thermal hyperalgesia is produced by induction of neurotransmitter release in the spinal cord. First, we examined whether  $\alpha,\beta$ meATP could increase the Ca<sup>2+</sup> influx that is necessary to evoke the neurotransmitter release. Using the fura-2 technique,  $\alpha,\beta$ meATP (100  $\mu$ M) induced a



**Figure 2** Effect of the antagonists for the non-selective P2 receptor PPADS and the selective P2X<sub>1</sub>, P2X<sub>3</sub> and P2X<sub>2+3</sub> receptor TNP-ATP on the thermal hyperalgesic response by intrathecal injection of  $\alpha,\beta$ meATP in mice. Mice were injected intrathecal with PPADS (1.0–5.0  $\mu$ g per mouse; hatched columns) or TNP-ATP (0.25–1.0  $\mu$ g per mouse; closed columns) 10 min prior to intrathecal injection of  $\alpha,\beta$ meATP (5.0  $\mu$ g per mouse; open column). Paw withdrawal response was measured 5 min after injection with  $\alpha,\beta$ meATP. Ordinate: change in paw withdrawal latency(s). Each column represents the mean  $\pm$  s.e.mean of 7–11 mice. ## $P < 0.01$  vs  $\alpha,\beta$ meATP (5.0  $\mu$ g per mouse)-injected control group.

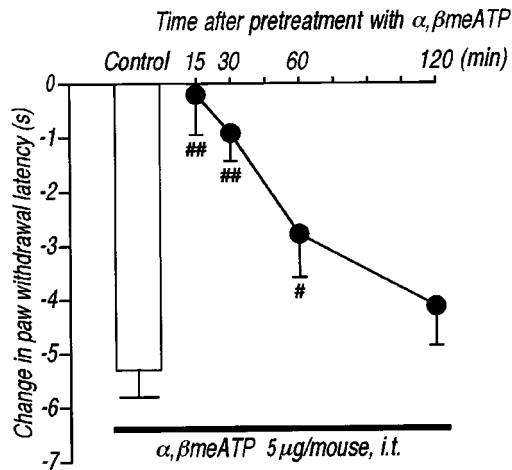


**Figure 1** An intrathecal administration of  $\alpha,\beta$ meATP caused the thermal hyperalgesic response in mice. (a) Dose-response and (b) time-course of the thermal hyperalgesic response by intrathecal  $\alpha,\beta$ meATP injection. (a) Paw withdrawal response was measured 5 min after intrathecal injection of  $\alpha,\beta$ meATP (1.0–5.0  $\mu$ g per mouse; dotted columns) or PBS (5  $\mu$ l; open column). (b) Paw withdrawal response was measured 0, 5, 15, 30 and 60 min after intrathecal injection of  $\alpha,\beta$ meATP (5.0  $\mu$ g per mouse; closed circle) or PBS (5  $\mu$ l; open circle). Ordinate: change in paw withdrawal latency (s); latency of paw withdrawal response after  $\alpha,\beta$ meATP or PBS injection minus latency before that). Each point and column represent the mean  $\pm$  s.e.mean of 10 mice. \*\* $P < 0.01$  vs PBS-treated group.

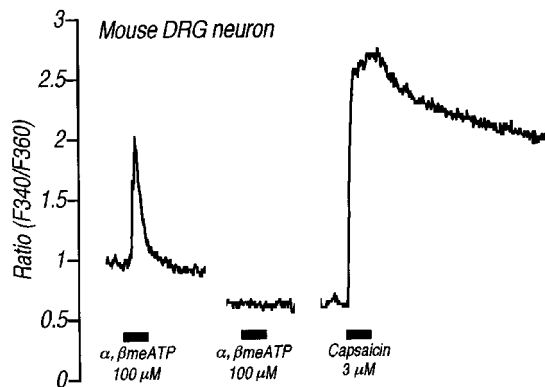


**Figure 3** Effect of the intrathecal injection of  $\beta,\gamma$ me-L-ATP on the paw withdrawal latency in mice. The paw withdrawal response was measured 5 min after intrathecal injection of  $\beta,\gamma$  me-L-ATP (5.0 and 10.0  $\mu$ g per mouse; dotted columns) or PBS (5  $\mu$ l; open column). Ordinate: change in paw withdrawal latency(s). Each column represents the mean  $\pm$  s.e.mean of 5–7 mice.

transient  $[Ca^{2+}]_i$  increase in acutely dissociated mouse DRG neurons (Figure 5). The  $[Ca^{2+}]_i$  increase by  $\alpha, \beta$ meATP was not observed following a second application of  $\alpha, \beta$ meATP (100  $\mu$ M). Treatment with capsaicin (3  $\mu$ M) also caused an increase in  $[Ca^{2+}]_i$  in this neuron, indicating that activation of the P2X receptors located on capsaicin-sensitive DRG neurons triggers the increase in the intracellular  $Ca^{2+}$  concentration. Second, we examined the involvement of neurotransmitters release in  $\alpha, \beta$ meATP-induced thermal hyperalgesia using mice which had been injected with BoNT/B which inhibits the  $Ca^{2+}$ -dependent exocytosis of neurotransmitters. As shown in Figure 6, the  $\alpha, \beta$ meATP-induced thermal hyperalgesia was markedly inhibited by pretreatment with BoNT/B [0.1 ( $P < 0.05$ ), 0.5, 1, 5 and 10 ng per mouse ( $P < 0.01$ )] in a dose-dependent manner. Pretreatment with BoNT/B alone (control:  $10.7 \pm 0.7$  s, BoNT/B 10 ng per mouse:  $12.0 \pm 0.4$  s), caused a small increase in paw withdrawal latency but this was not statistically significant. These results suggest that the evoked release of certain neurotransmitters by intrathecal injection of  $\alpha, \beta$ meATP may be involved in  $\alpha, \beta$ meATP-induced

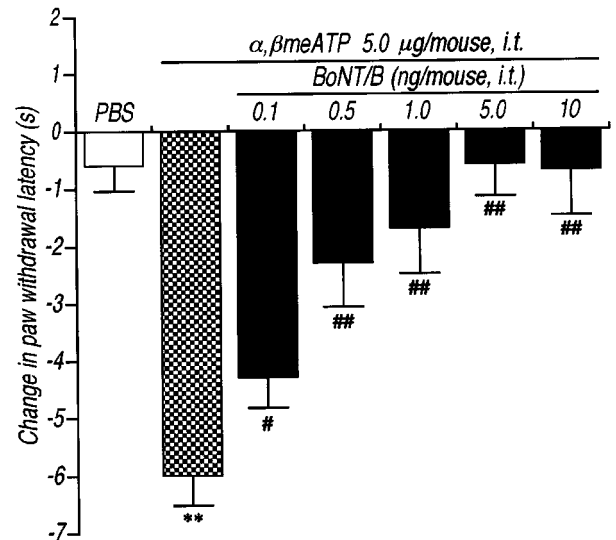


**Figure 4** Effect of the intrathecal pretreatment with  $\alpha, \beta$ meATP on the thermal hyperalgesic response by intrathecal injection of  $\alpha, \beta$ meATP in mice. Mice were injected intrathecal with  $\alpha, \beta$ meATP (5.0  $\mu$ g per mouse) 15, 30, 60 and 120 min prior to intrathecal injection of  $\alpha, \beta$ meATP (5.0  $\mu$ g per mouse) (closed circles). Paw withdrawal response was measured 5 min after injection with  $\alpha, \beta$ meATP. Ordinate: change in paw withdrawal latency(s). Each column represents the mean  $\pm$  s.e. mean of 8–12 mice. # $P < 0.05$ , ## $P < 0.01$  vs  $\alpha, \beta$ meATP (5.0  $\mu$ g per mouse)-injected control group.

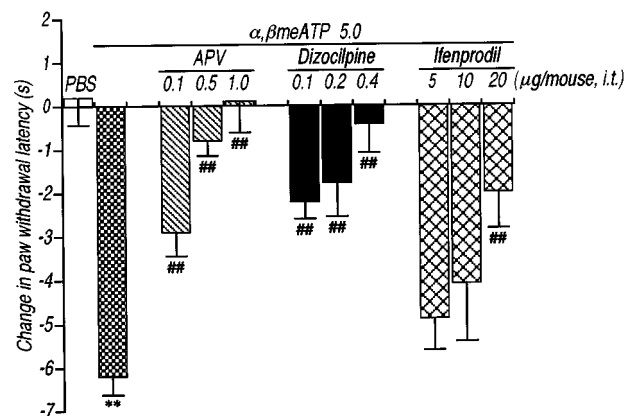


**Figure 5** Effects of  $\alpha, \beta$ meATP on  $[Ca^{2+}]_i$  in acutely dissociated DRG neuron from adult mouse. Horizontal solid bars show the applications of  $\alpha, \beta$ meATP (100  $\mu$ M) and capsaicin (3  $\mu$ M) for 15 s. A second application of  $\alpha, \beta$ meATP (100  $\mu$ M) was applied 5 min after the first application.

thermal hyperalgesia. Thus, we investigated the role of NMDA receptors in the spinal cord. The  $\alpha, \beta$ meATP-induced thermal hyperalgesia was abolished by pretreatment with the competitive NMDA receptor antagonist APV (0.1–1.0  $\mu$ g per mouse:  $P < 0.01$ ) (Figure 7). Furthermore, the non-competitive NMDA receptor antagonists dizocilpine and ifenprodil significantly inhibited the thermal hyperalgesic response (dizocilpine 0.1–0.4  $\mu$ g per mouse:  $P < 0.01$ , ifenprodil 20  $\mu$ g per mouse:  $P < 0.01$ ), indicating the involvement of spinal



**Figure 6** Effects of pretreatment with botulinum neurotoxin type B (BoNT/B) on the thermal hyperalgesic response by intrathecal injection of  $\alpha, \beta$ meATP in mice. Mice were injected intrathecal with BoNT/B (0.1–10.0 ng per mouse; closed columns) 12 h prior to intrathecal injection of  $\alpha, \beta$ meATP (5.0  $\mu$ g per mouse). Paw withdrawal response was measured 5 min after the injection of  $\alpha, \beta$ meATP or PBS. Ordinate: change in paw withdrawal latency(s). Each column represents the mean  $\pm$  s.e. mean of 7–12 mice. \*\* $P < 0.01$  vs PBS-injected group; # $P < 0.05$ , ## $P < 0.01$  vs  $\alpha, \beta$ meATP (5.0  $\mu$ g per mouse)-injected control group.



**Figure 7** Effects of the pretreatment with NMDA receptor antagonists on the thermal hyperalgesic response by intrathecal injection of  $\alpha, \beta$ meATP in mice. Mice were injected intrathecal with APV (0.1–1.0  $\mu$ g per mouse; hatched columns), dizocilpine (0.1–0.4  $\mu$ g per mouse; closed columns) and ifenprodil (5–20  $\mu$ g per mouse; cross hatched columns) 10 min prior to the intrathecal injection of  $\alpha, \beta$ meATP (5.0  $\mu$ g per mouse). Paw withdrawal response was measured 5 min after the injection of  $\alpha, \beta$ meATP or PBS. Ordinate: change in paw withdrawal latency(s). Each column represents the mean  $\pm$  s.e. mean of 6–18 mice. \*\* $P < 0.01$  vs PBS-injected group; ## $P < 0.01$  vs  $\alpha, \beta$ meATP (5.0  $\mu$ g per mouse)-injected control group.

NMDA receptors. In contrast to the effect of APV and dizocilpine which completely inhibited the  $\alpha,\beta$ meATP-induced thermal hyperalgesia, the inhibitory effect of ifenprodil was weaker than that of APV and dizocilpine. In addition, the latency of paw withdrawal response was not changed by each NMDA receptor antagonist alone [control:  $9.3 \pm 0.6$  s ( $n=8$ ), APV  $1.0 \mu\text{g}$  per mouse:  $10.5 \pm 0.5$  s ( $n=10$ ), dizocilpine  $0.4 \mu\text{g}$  per mouse:  $10.5 \pm 0.6$  s ( $n=10$ ), ifenprodil  $20 \mu\text{g}$  per mouse:  $9.6 \pm 0.7$  s ( $n=11$ )].

## Discussion

Recent *in vitro* studies have provided evidence that strongly supports the proposal that P2X receptors could play a role in the signal for nociceptive processing in the spinal cord (see Introduction). However, the *in vivo* mechanisms of P2X-mediated modulation of nociceptive processing in the spinal cord are unclear. The present study first demonstrated that intrathecal administration of  $\alpha,\beta$ meATP significantly and dose-dependently evokes thermal hyperalgesia in mice. This is consistent with a previous finding that intrathecal injection of  $\alpha,\beta$ meATP produces hyperalgesia using the tail-flick test in rats (Driessen *et al.*, 1994), confirming the presence of a pain-modulating effect by  $\alpha,\beta$ meATP in the spinal cord. The  $\alpha,\beta$ meATP-induced thermal hyperalgesic response appears to be mediated by the activation of P2X receptors because the thermal hyperalgesic response was completely blocked by the non-selective P2 receptor antagonist PPADS and by the selective P2X<sub>1</sub>, P2X<sub>3</sub>, and P2X<sub>2+3</sub> receptor antagonist TNP-ATP (Figure 2). Furthermore, intrathecal injection of  $\beta,\gamma$ me-L-ATP, which is a potent agonist for P2X<sub>1</sub> receptors (Evans *et al.*, 1995; Trezise *et al.*, 1995; Chen *et al.*, 1995), failed to produce a thermal hyperalgesic response (Figure 3). These data indicate that the P2X<sub>3</sub> and/or P2X<sub>2+3</sub> receptor subtypes in the spinal cord may be involved in the signalling system of nociception. In agreement, our previous electrophysiological study has shown that the kinetics of  $\alpha,\beta$ meATP-evoked inward current in rat DRG neurons (Ueno *et al.*, 1998b) are not the same as those in cells transfected with P2X<sub>1</sub> receptors (Werner *et al.*, 1996; Parker, 1998). Furthermore, ATP and  $\beta,\gamma$ me-D-ATP (which is a potent agonist of P2X<sub>1</sub> and P2X<sub>3</sub> receptors) evoke a concentration-dependent inward current in rat DRG neurons while  $\beta,\gamma$ me-L-ATP is less active (Rae *et al.*, 1998). Therefore, it is concluded that the  $\alpha,\beta$ meATP-induced thermal hyperalgesia is mediated by the activation of P2X<sub>3</sub> or P2X<sub>2+3</sub> receptor subtypes in the spinal cord.

Under the present experimental conditions, determination of the involvement of P2X receptors (P2X<sub>3</sub> or P2X<sub>2+3</sub>) in  $\alpha,\beta$ meATP-induced thermal hyperalgesia is difficult, however, we have presumed the importance of the P2X<sub>3</sub> receptor subtype in the thermal hyperalgesia by  $\alpha,\beta$ meATP from the following observations. In capsaicin-sensitive small-sized DRG neurons that have been shown to be nociceptors (Simone *et al.*, 1989; Holzer, 1991; Szallasi & Blumberg, 1996),  $\alpha,\beta$ meATP evokes a rapidly desensitizing inward current which is dramatically decreased by a second application of the compound (Ueno *et al.*, 1999). This electrophysiological data appears to be similar to the present behavioural profile, in that  $\alpha,\beta$ meATP-induced thermal hyperalgesia was short-lived and produced a lesser response in mice that had previously received  $\alpha,\beta$ meATP intrathecally. The  $\alpha,\beta$ meATP-activated response in capsaicin-sensitive DRG neurons has been thought to be mediated by P2X<sub>3</sub> receptors (Ueno *et al.*, 1999). In fact, the P2X<sub>3</sub> receptor subtype is observed in capsaicin-sensitive small-sized DRG neurons

(Chen *et al.*, 1995; Ueno *et al.*, 1999; Vulchanova *et al.*, 1998), and is found in greater numbers than other P2X receptor subtypes (Llewellyn-Smith & Burnstock, 1998). In contrast, capsaicin-insensitive, medium-sized DRG neurons possess the  $\alpha,\beta$ meATP-evoked slowly desensitizing current that retains its responsiveness after first application, and this type of current has been thought to be mediated *via* heteromeric P2X<sub>2+3</sub> receptors (Ueno *et al.*, 1999). The specific localization of the P2X<sub>3</sub> receptor subtype in small-sized DRG neurons is of particular interest in the light of the demonstration that small-, but not large-, diameter DRG neurons evoke the inward current by application of noxious heat stimulation (Reichling & Levine, 1997). The activation of small DRG neurons by noxious heat has been proposed to mediate the heat-evoked nociceptive response *in vivo* (Cesara & McNaughton, 1996; Caterina *et al.*, 1997; Kirschstein *et al.*, 1997; Reichling & Levine, 1997; Tominaga *et al.*, 1998). On the basis of these observations, it is reasonable to suggest that the thermal hyperalgesia by intrathecal injection of  $\alpha,\beta$ meATP may be associated with the spinal P2X<sub>3</sub> receptor activation-mediated enhancement of the signalling of noxious heat stimulation in heat-sensitive small-diameter DRG neurons, although the possible involvement of other P2X subtypes can not be completely excluded. This possibility will be clarified by the development of new selective compounds for the P2X receptor subtypes.

Immunohistochemical studies have revealed that P2X<sub>3</sub> receptors in the spinal cord are localized in the central presynaptic terminal sites of primary afferent neurons, but not in the cell bodies of superficial dorsal horn neurons (Cook *et al.*, 1997; Vulchanova *et al.*, 1997; 1998; Llewellyn-Smith & Burnstock, 1998). This evidence would suggest that activation of P2X<sub>3</sub> receptors leads to the release of certain neurotransmitters in the spinal cord, and that these released neurotransmitters play an important role in  $\alpha,\beta$ meATP-induced thermal hyperalgesia. We have undertaken three experiments to clarify this possibility. Since Ca<sup>2+</sup> has been shown to be important in the release of neurotransmitters, we first examined the ability of  $\alpha,\beta$ meATP to increase intracellular Ca<sup>2+</sup> concentration in adult mouse DRG neuron using the fura-2 method. In the present study,  $\alpha,\beta$ meATP produced an increase in the [Ca<sup>2+</sup>]<sub>i</sub> in acutely dissociated adult mouse DRG neuron. This is in agreement with previous findings in neurons cultured from neonatal rat DRG (Bouvier *et al.*, 1991), confirming that  $\alpha,\beta$ meATP causes the Ca<sup>2+</sup> influx into DRG neurons. Furthermore, this  $\alpha,\beta$ meATP-induced increase [Ca<sup>2+</sup>]<sub>i</sub> was not produced by a second application of  $\alpha,\beta$ meATP and in addition, capsaicin treatment dramatically increased the [Ca<sup>2+</sup>]<sub>i</sub>. These findings lead to the suggestion that activation of P2X (perhaps P2X<sub>3</sub>) receptors located in the central terminal of capsaicin-sensitive DRG neurons may trigger the Ca<sup>2+</sup> influx. In the second experiment we used BoNT/B that specially cleaves the synaptic vesicle protein synaptobrevin. This cleavage event has been proposed to be part of the fusion machinery involved in the fusion between synaptic vesicles and the presynaptic plasma membrane (Schiavo *et al.*, 1992; Poulain *et al.*, 1993; Burgoyne & Morgan, 1995; Almeida *et al.*, 1997), which inhibits Ca<sup>2+</sup>-dependent neurotransmitter release both *in vitro* (Schiavo *et al.*, 1992; McMahan *et al.*, 1992; Poulain *et al.*, 1993) and *in vivo* (Pierce & Kalivas, 1997). We found in the present behavioural study that intrathecal pretreatment with BoNT/B caused a dose-dependent blockade of the  $\alpha,\beta$ meATP-induced thermal hyperalgesia. This behavioural finding using BoNT/B strongly supports our theory and suggests that the Ca<sup>2+</sup>-dependent exocytosis of synaptic vesicular storing

certain neurotransmitters would be involved in the generation of thermal hyperalgesic response by  $\alpha,\beta$ meATP. For several neurotransmitters that have their release prevented by BoNT/B (Schiavo *et al.*, 1992; McMahon *et al.*, 1992; Poulain *et al.*, 1993; Pierce & Kalivas, 1997), we presumed that one potential candidate involved in thermal hyperalgesia is glutamate. Glutamate is a major excitatory neurotransmitter in the central nervous system including the spinal cord and is located in the presynaptic termini of the spinal cord (De Biasi & Rustioni, 1988). The release of glutamate from these termini results in hyperalgesia *via* activation of NMDA receptors (Aanonsen & Wilcox, 1987; Coderre & Melzack, 1992; Malmberg & Yaksh, 1995; Ren *et al.*, 1992; Dickenson *et al.*, 1997). In the third experiment, we determined whether several NMDA receptor antagonists inhibit the  $\alpha,\beta$ meATP-induced thermal hyperalgesia. The competitive antagonist APV and the non-competitive antagonists dizocilpine and ifenprodil blocked the  $\alpha,\beta$ meATP-induced thermal hyperalgesia, indicating the involvement of spinal NMDA receptors in the  $\alpha,\beta$ meATP-induced thermal hyperalgesia. However, the inhibitory effect of ifenprodil on  $\alpha,\beta$ meATP-induced thermal hyperalgesia was weaker than that of APV and dizocilpine. Ifenprodil is known to be a selective antagonist for NMDAR2B (NR2B) subunit-containing NMDA receptors (Williams, 1993; Molinoff *et al.*, 1994). In spinal lamina I–III, antibodies for NR2A and NR2B subunits stain with fair intensity (Petralia *et al.*, 1996). Thus, the inability of the complete blockade of the hyperalgesic response by ifenprodil is likely to be due to the residual action *via* NR2A-containing NMDA receptors in the spinal cord. The putative release of glutamate by  $\alpha,\beta$ meATP in the present study is strongly supported by the study of Gu & MacDermott that shows that ATP evokes the glutamate release from cultured sensory neuron synapses *via* the activation of P2X receptor that are localized at presynaptic termini of DRG neurons (Gu & MacDermott, 1997). Similar to the presynaptic localization of P2X<sub>3</sub> receptors (Llewellyn-Smith & Burnstock, 1998), immunoreactivity for glutamate is also observed in many dark scalloped terminal in substantial gelatinosa (De Biasi & Rustioni, 1988). Furthermore, glutamate is released by

capsaicin from capsaicin-sensitive primary afferent termini (Ueda *et al.*, 1993) that are known to express P2X<sub>3</sub> receptors (Vulchanova *et al.*, 1998), and this glutamate release by capsaicin has been considered to play a role in capsaicin-evoked nociception *in vivo* (Sakurada *et al.*, 1998). Moreover, P2X<sub>3</sub> receptors are presynaptically localized in the inner portion of lamina II in the dorsal horn of the spinal cord (Vulchanova *et al.*, 1998), and interestingly, dense immunoreactivity of NR1, the essential subunit for the NMDA receptor, is also found particularly in the inner lamina II (Liu *et al.*, 1994). Although there is no direct evidence concerning the localization of either P2X<sub>3</sub> receptors, glutamate or NMDA receptors using immunohistochemical studies, the close relationship of P2X<sub>3</sub> receptors with the glutamate-NMDA receptor pathway may be considered. Besides glutamate, sensory neurons contain neuropeptides such as substance P and somatostatin that play a pivotal role in the signal transduction of pain. However, recent studies have shown the very limited localization of substance P and somatostatin in P2X<sub>3</sub>-immunoreactive positive termini in the dorsal horn (Vulchanova *et al.*, 1997; 1998). From the present findings *in vivo* together with previous evidence, it is, therefore, suggested that the glutamate-NMDA receptor pathway in the spinal cord may be involved mainly in the generation of  $\alpha,\beta$ meATP-induced thermal hyperalgesia *via* the P2X<sub>3</sub> receptor subtype.

In summary, the present study using a behavioural approach has shown that thermal hyperalgesia by the intrathecal injection of  $\alpha,\beta$ meATP may be mediated by activation of the P2X<sub>3</sub> receptor subtype in the spinal cord, and that intrathecal injection of  $\alpha,\beta$ meATP may evoke spinal glutamate release which, in turn, leads to the generation of thermal hyperalgesia *via* NMDA receptors. These findings provide the first *in vivo* evidence for the involvement of glutamate-NMDA receptors pathway in the P2X receptors-mediated modulation of acute nociceptive signalling in the spinal cord. In addition to the peripheral site, this pain-modulating pathway, mediated by P2X receptors in the spinal cord, may lead to the discovery of a new class of compounds that suppress pain and help to elucidate the role of ATP and its receptors in pathological pain.

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