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Evidence for the involvement of spinal endogenous ATP and P2X receptors in nociceptive responses caused by formalin and capsaicin in mice

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1 The aim of the present study is to characterize the role of spinal endogenous ATP and P2X receptors in the generation of neurogenic and inflammatory pain. We examined the effects of intrathecal treatment with P2X receptor antagonists on the formalin- and capsaicin-induced nociceptive behaviours in mice.

2 Intrathecal pretreatment with the general P2 receptor antagonist, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), significantly suppressed both the first and second phases of the formalin-induced nociceptive behaviour. The second phase of the nociceptive response was also suppressed by intrathecal treatment with PPADS after the first phase. Furthermore, pretreatment with the selective antagonist for the $P2X_1$, $P2X_3$ and $P2X_{2+3}$ receptors, 2′,3′-O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP), significantly reduced the first phase, but not the second phase. The second phase was also not suppressed by intrathecal TNP-ATP after the first phase.

3 Capsaicin-induced nociceptive behaviour that has been shown to be a model for neurogenic pain, was also significantly suppressed by intrathecal pretreatment with PPADS or TNP-ATP.

4 Nociceptive behaviour in the first phase of the formalin test and in the capsaicin test were significantly inhibited by intrathecal pretreatment with α,β-methylene ATP (α,βmeATP: 5 μg $mouse^{-1}$) 15 min prior to injection of formalin or capsaicin. This treatment has been previously shown to desensitize spinal $P2X_3$ receptor subtypes *in vivo*.

5 These findings suggest that spinal endogenous ATP may play a role in (1) the formalin- and capsaicin-induced neurogenic pain *via* the PPADS- and TNP-ATP-sensitive P2X receptors which are also desensitized by α,βmeATP (perhaps the $P2X_3$ receptor subtype) and (2) formalin-induced inflammatory pain *via* PPADS-sensitive, TNP-ATP- and α,βmeATP-insensitive P2X (and/or P2Y) receptors.

Keywords: Formalin test; capsaicin test; ATP; P2X receptors; spinal cord; mouse

Abbreviations: α,βmeATP, α,β-methylene ATP; DRG, dorsal root ganglia; 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) has been reported to excite neuronal preparations in the nociceptive signalling pathway. In electrophysiological experiments, ATP or its analogue α,β-methylene ATP (α,βmeATP) evoke an inward current in dorsal root, trigeminal sensory neurons (Krishtal *et al*., 1983; 1988a,b; Bean, 1990; Robertson *et al*., 1996; Cook *et al*., 1997; Rae *et al*., 1998; Ueno *et al*., 1999) and dorsal horn neurons (Li & Perl, 1995; Bardoni *et al*., 1997). Intra-arterial injection of ATP or α,βmeATP stimulates the Aδ and C nociceptive afferent nerves innervating the knee joint in rats (Dowd *et al*., 1998). These excitatory functions of ATP have been thought to result from stimulation of ligand-gated ion channels sensitive to ATP, namely P2X receptors. The existence of P2X receptors in the nociceptive pathway was first found by Chen *et al*. (1995) and Lewis *et al*. (1995). mRNA for $P2X_3$ which is one of the seven P2X receptor subtypes ($P2X_{1-7}$) (reviewed in Ralevic & Burnstock, 1998), is selectively expressed in capsaicin-sensitive, small-sized sensory neurons in the trigeminal and dorsal root ganglion (DRG) (Chen *et al*., 1995). More recent experiments have shown that mRNA for the other P2X subunits ($P2X_{1,2,4-6}$) also exists in sensory ganglia and spinal dorsal horn neurons (Collo *et al*., 1996; Lê *et al*., 1998; Ueno *et al*., 1999; Vulchanova *et al*., 1997; Xiang *et al*., 1998). These findings have raised the possibility that these P2X receptors may play a role in the signal for nociception in the spinal cord *in vivo*.

In studies using behavioural pharmacology, intrathecal administration of α,βmeATP produces potentiation of nociception evaluated by the tail-flick test (Driessen *et al*., 1994). We have recently demonstrated that α,βmeATP produces a thermal hyperalgesia *via* $P2X_3$ receptors in mouse spinal cord (Tsuda *et al*., 1999). These studies have shown that ATP receptor activation by exogenous application of agonists can evoke a hyperalgesic response. However, the role of spinal endogenous ATP in nociception *in vivo* remains unclear. Although there is only one report describing the antinociceptive effects of P2 receptor antagonists in tail-flick test and formalin test (Driessen *et al*., 1994), suramin and reactive blue 2 are not selective for P2 receptors and potently inhibit the response to glutamate through N-methyl-D-aspartate (NMDA) and non-NMDA receptors (Nakazawa *et al*., 1995;

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Peoples & Li, 1998). Many studies have shown that glutamate and its receptors play a major role in spinal nociceptive transmission (Coderre & Melzack, 1992; Li *et al*., 1998; Lutfy *et al*., 1997; Murray *et al*., 1991; Yamamoto & Yaksh, 1992), suggesting the irrelevance of this blockade of spinal P2X receptors against antinociceptive effects. Indeed, the selective P2 receptor antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) which has no effect on glutamate receptors (Dale & Gilday, 1996; Peoples & Li, 1998), fails to produce the antinociceptive effect in the tail-flick test (Driessen *et al*., 1994; Li *et al*., 1998).

The formalin test, which evaluates nociception caused by injection of dilute formalin solution into the hindpaw, has been widely used and is considered one of the standard animal models of nociception (Tjølsen *et al*., 1992). Injection of formalin into the mouse hindpaw produces two distinct phases of nociceptive behaviours: an early transient phase (first phase; 5 min after injection) and a late persistent phase (second phase; 15–30 min after injection). The first phase has been considered to reflect direct stimulation of primary afferent fibres (neurogenic pain) while the second phase is dependent on peripheral inflammation (inflammatory pain) (Dubuisson & Dennis, 1977; Shibata *et al*., 1989; Tjølsen *et al*., 1992). Since formalin evokes a continuous nociceptive response generated by injured tissue, the activation of various endogenous pain regulatory substrates has been proposed. Thus, the formalin test is considered to be of greater relevance for clinical situations than tests with more phasic stimulus such as the tail-flick test (Dubuisson & Dennis, 1977; Tjølsen *et al*., 1992). Therefore, we investigated the effects of intrathecal treatment with the general P2 receptor antagonist, PPADS, and the selective $P2X_1$, $P2X_3$ and $P2X_{2+3}$ receptors antagonist, 2′,3′-O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP), (Virginio *et al*., 1998) on formalin-induced neurogenic and inflammatory pain. Furthermore, the effects of these antagonists on capsaicin-induced nociceptive behaviour (reported as a model for neurogenic pain; Sakurada *et al*., 1992) were also investigated.

Methods

Animals

Male ddY mice (20–23 g) were obtained from Shizuoka Laboratory Center (Shizuoka, Japan). The animals were housed at a temperature of $22 \pm 1^{\circ}C$ with a 12 h light-dark cycle (light on 08.30–20.30 h). Food and water were available *ad libitum*.

Formalin test

The procedure used for the formalin test was according to a previously described method (Sakurada *et al*., 1993) with minor modifications. Each mouse was placed in an observation cylinder 10 min before the injection of diluted formalin to allow acclimatization to the new environment. Twenty μl of a 2% or 0.0625% solution of formalin in 0.9% saline was injected s.c. into the plantar surface of the left hindpaw. Each mouse was then returned to the observation cylinder and the nociceptive response was recorded for a period of 30 min. The summation of time (s) spent in licking and biting of the formalin-injected hindpaw during each 5 min block was measured as an indicator of the nociceptive response. The duration of the response in the first 5 min and then from 10–30 min represent the first and second phases, respectively. An intrathecal administration was performed according to the procedure described by Hylden & Wilcox (1980) using a 25-μl Hamilton syringe with 28-gauge needle. Mice were injected intrathecally with PPADS (1.0–5.0 μg mouse^{-1}) or TNP-ATP (1.0 μg mouse^{-1}) 10 min before the formalin injection. In addition, to investigate the effect on the second phase of the formalin test, mice were treated intrathecally with PPADS (5.0 μg mouse^{-1}) or TNP-ATP (1.0 μg mouse^{-1}) 5 min after the formalin injection. The doses of PPADS and TNP-ATP used here were based on our recent study (Tsuda *et al*., 1999). In addition, to assess the effects of these P2X antagonists on formalin-induced paw inflammation, the thickness of the hindpaw was measured by vernier calipers 30 min after injection of 2% formalin. To desensitize P2X receptors in the spinal cord which are sensitive to α,βmeATP, mice were injected intrathecally with α,βmeATP (5.0 μg mouse^{-1}) 15 min before the formalin injection (Tsuda *et al*., 1999).

Capsaicin test

The procedure used for the capsaicin test was according to a previously described method (Sakurada *et al*., 1992). Before testing, the mice were placed individually in an observation cylinder 10 min before the injection of capsaicin to allow acclimatization to the new environment. Following this, 20 μl of capsaicin (1.6 μg paw^{-1}) was injected s.c. into the plantar surface of the left hindpaw. Each mouse was then returned to the observation cylinder and the nociceptive response was recorded for a period of 5 min. The summation of time (s) spent in licking and biting of the capsaicin-injected hindpaw was measured as an indicator of the nociceptive response. Mice were injected intrathecally with PPADS (5.0 μg mouse^{-1}) or TNP-ATP (1.0 μg mouse^{-1}) 10 min before the capsaicin injection. Mice treated with α,βmeATP (5.0 μg mouse^{-1}) were intrathecally injected 15 min before the capsaicin injection.

Rotarod performance test

The effects of PPADS and TNP-ATP on motor coordination were assessed using the rotarod performance test according to a previously described method (Tsuda *et al*., 1996). Each mouse was trained to run in a rotarod (3 cm diameter, 8 r.p.m.; Natsume Seisakusho, Tokyo, Japan) until it could remain there for 60 s without falling. The mice were then evaluated in a rotarod performance test for 60 s 10 min after the PPADS or TNP-ATP injection.

Drugs

Formalin (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) was diluted in saline. Capsaicin (Wako Pure Chemical Industries, Tokyo, Japan) was dissolved in 1% dimethyl sulphoxide (DMSO) in saline. Pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonate (PPADS: RBI, MA, U.S.A.), 2′,3′-O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP: Molecular Probes, OR, U.S.A.) and α,β-methylene ATP (α,βmeATP: Sigma, MO, U.S.A.) were dissolved in phosphate-buffered saline (PBS; composition in mM): NaCl 137, KCl 2.7, KH_2PO_4 1.5, NaH_2PO_4 8.1; pH 7.4.

Statistical analysis

The duration of biting and licking response and thickness of hindpaw were evaluated statistically using the Student's *t*-test or one factor repeated measures analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons

test (when more than one dose administered). Analysis of the time-course of the duration of biting and licking response was performed by two factors (group × times) repeated measures analysis of variance (ANOVA). Time on rotarod was evaluated using the non-parametric Wilcoxon test.

Results

Effects on the formalin-induced nociceptive behaviour in mice

S.c. injection of 2% formalin into the hindpaw produced biphasic biting and licking behaviour of the injected paw. Intrathecal administration of PPADS 10 min prior to the formalin injection did not change the pattern of nociceptive responses, but significantly suppressed the response time of the formalin-induced biting and licking behaviour (1.0 μg mouse^{-1}: $F_{1,126}=4.183$ $P<0.05$, 2.5 μg mouse^{-1}: $F_{1,126}=7.421$ $P<0.01$, 5 μg mouse^{-1}: $F_{1,126}=27.501$ $P<0.01$) (Figure 1a). The total response time of each phase is shown in Figure 1b,c. Pretreatment with intrathecal PPADS produced a dose-dependent and significant suppression of the time spent in the biting and licking responses in both the first ($F_{3,36}=5.929$: $P<0.01$; 2.5 and 5 μg mouse^{-1}: $P<0.01$) (Figure 1b) and second phases ($F_{3,36}=9.259$: $P<0.01$; 1 μg mouse^{-1}: $P<0.05$, 2.5 and 5 μg mouse^{-1}: $P<0.01$) (Figure 1c). However, none of the doses of PPADS affected formalin-induced hindpaw inflammation (Table 1). To confirm the inhibitory effect of PPADS on the first phase, mice were injected in the hindpaw with a low concentration of formalin solution (0.0625%) that exhibited only the first phase of the nociceptive response that lasted about 5 min. The duration of the biting and licking time caused by 0.0625% formalin was significantly inhibited by intrathecal pretreatment with PPADS ($F_{2,26}=16.117$: $P<0.01$; 2.5 and 5 μg mouse^{-1}: $P<0.01$) (Figure 2). The second phase of the nociceptive response of the formalin test has been reported to depend on the signal produced by the first phase of the formalin test. Thus, we evaluated separately the effect of PPADS on the two phases of formalin-induced nociception and found that an intrathecal administration of PPADS (5 μg mouse^{-1}) 5 min after the formalin injection reduced the second phase of the formalin-induced biting and licking response ($F_{1,85}=15.752$, $P<0.01$) (Figure 3a). The total response time of the second phase was also significantly blocked by treatment with PPADS (5 μg mouse^{-1}: $P<0.01$) (Figure 3b), indicating that the inhibitory effect of PPADS on the second phase is not related to that on the first phase of the formalin test. In addition, this treatment did not affect the hindpaw inflammation caused by formalin (Table 1). As shown in Figure 4, intrathecal administration of the selective $P2X_1$, $P2X_3$ and/or $P2X_{2+3}$ receptor antagonist TNP-ATP (1.0 μg mouse^{-1}) 10 min prior to formalin injection significantly suppressed the response time of formalin-induced biting and licking behaviour in the first phase ($P<0.01$). The degree of inhibition of first phase by TNP-ATP was similar to that by PPADS. However, TNP-ATP (1.0 μg mouse^{-1}) did not suppress the second phase of the formalin-induced nociception. This result is confirmed by further experiment that an intrathecal administration of TNP-ATP (1.0 μg mouse^{-1}) 5 min after the formalin injection did not modify the second phase of the formalin-induced biting and licking response (Figure 5), indicating that intrathecal TNP-ATP inhibits only the first phase of the formalin test. The formalin-induced hindpaw inflammation was not affected by pre- and post-treatment with TNP-ATP (Table 1). As shown in Figure 6, the

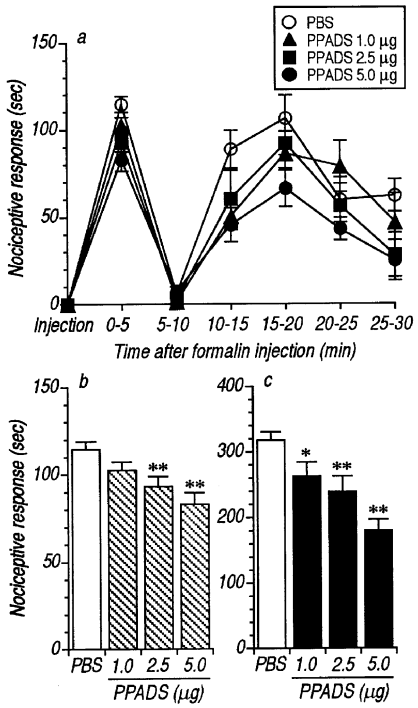

Figure 1 Effect of the intrathecal pretreatment with PPADS on the 2% formalin-induced nociceptive behaviour in mice. (a) Time-course of the formalin-induced biphasic nociceptive response. Each point represents the duration of biting and licking response for 5 min. (b and c) Total duration time of the formalin-induced nociceptive response during each phase. First phase (b) and second phase (c) represent the sum of biting and licking response during 0–5 min and 10–30 min after formalin injection, respectively. Ordinate: time of biting and licking behaviours to formalin-injected hindpaw (s). Mice were injected with PPADS (1.0–5.0 μg mouse^{-1}, i.t.) 10 min before the injection of 2% formalin into left hindpaw. Each point and column represent the mean±s.e.mean of ten mice. *$P<0.05$, ** $P<0.01$ vs PBS-pretreated group.

Table 1 Effects on the intrathecal treatment with PPADS, TNP-ATP and α,βmeATP on the formalin-induced hindpaw inflammation in mice

Intrathecal treatment	*Thickness of formalin-injected hindpaw* (mm)
PBS	3.6±0.07
PPADS 1 μg mouse^{-1}	3.7±0.10
2.5 μg mouse^{-1}	3.6±0.05
5 μg mouse $^{-1}$	3.6±0.04
PBS	3.8±0.07
PPADS 5 μg mouse^{-1} (Post-treatment)	3.8±0.09
PBS	3.8±0.08
TNP-ATP 1 μg mouse^{-1}	3.8±0.07
PBS	
TNP ATP 1 μg mouse^{-1} (Post-treatment)	3.6±0.05 3.7±0.09
PBS	3.8±0.10
α,βmeATP 5 μg mouse^{-1}	3.8±0.05

Mice were injected intrathecally with PPADS, TNP-ATP and α,βmeATP 10, 10 and 15 min, respectively, prior to the injection of 2% formalin into left hindpaw. In group of Post-treatment with PPADS and TNP-ATP, mice were injected with PPADS and TNP-ATP 5 min after the injection of 2% formalin. Each value represents the mean± s.e.mean of 7–11 mice.

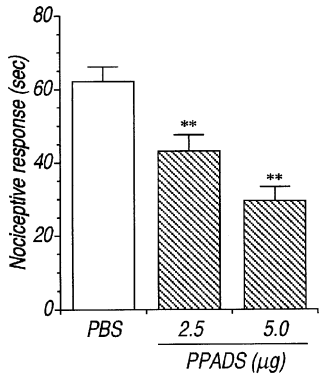

Figure 2 Effect of the intrathecal pretreatment with PPADS on the 0.0625% formalin-induced nociceptive behaviour in mice. Ordinate: total time of biting and licking behaviours to formalin-injected hindpaw during 0–5 min (s). Mice were injected with PPADS (5.0 μg mouse^{-1}, i.t.) 10 min before the injection of 0.0625% formalin into left hindpaw. Each column represents the mean$\pm$s.e.mean of 9–10 mice. $^{**}P<0.01$ vs PBS-pretreated group.

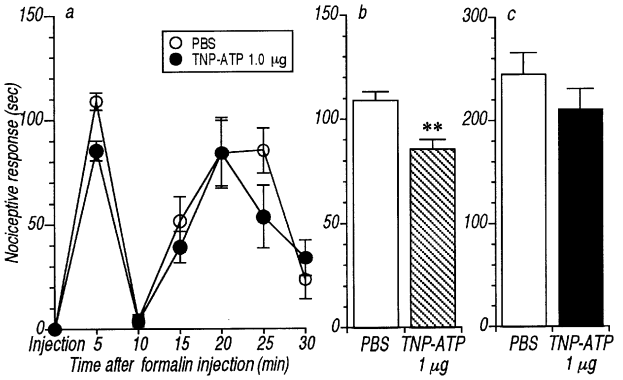

Figure 4 Effect of the intrathecal pretreatment with TNP-ATP on the 2% formalin-induced nociceptive behaviour and inflammation in mice. Explanations of panels in (a), (b) and (c) is similar to the description in Figure 1. Mice were injected with TNP-ATP (1.0 μg mouse^{-1}, i.t.) 10 min before the injection of 2% formalin into left hindpaw. Each point and column represent the mean$\pm$s.e.mean of 7–11 mice. $^{**}P<0.01$ vs PBS-pretreated group.

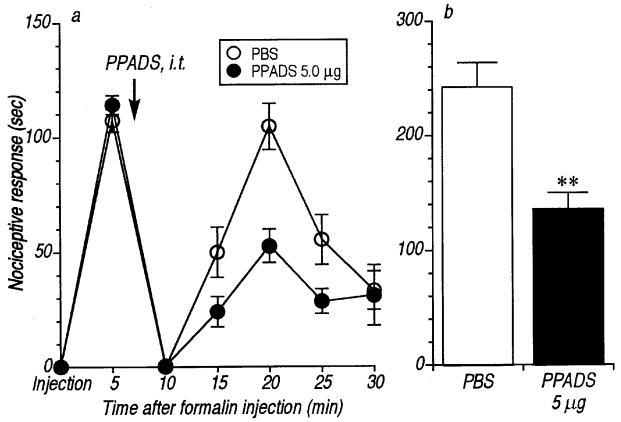

Figure 3 Effect of the intrathecal treatment with PPADS during the first and second phases on the second phase of the 2% formalin-induced nociceptive behaviour in mice. (a) Time-course of formalin-induced biphasic nociceptive response. Each point represents the duration of biting and licking responses for 5 min. (b) Total duration time of formalin-induced nociceptive responses during the second phase (10–30 min). Mice were injected with PPADS (5.0 μg mouse^{-1}, i.t.) 5 min after the injection of 2% formalin into left hindpaw. Each point and column represent the mean$\pm$s.e.mean of 9–10 mice. $^{**}P<0.01$ vs PBS-treated group.

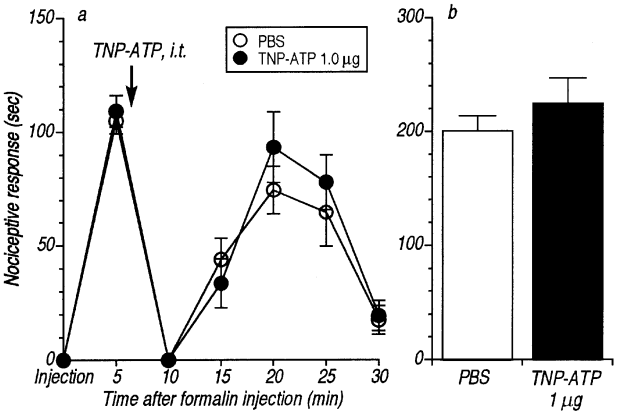

Figure 5 Effect of the intrathecal treatment with TNP-ATP during the first and second phases on the second phase of the 2% formalin-induced nociceptive behaviour in mice. (a) Time-course of formalin-induced biphasic nociceptive response. Each point represents the duration of biting and licking responses for 5 min. (b) Total duration time of formalin-induced nociceptive responses during the second phase (10–30 min). Mice were injected with TNP-ATP (1.0 μg mouse^{-1}, i.t.) 5 min after the injection of 2% formalin into left hindpaw. Each point and column represent the mean$\pm$s.e.mean of 8–9 mice.

first phase, but not the second phase of the formalin-induced nociceptive response was significantly reduced by pretreatment with α,βmeATP (5.0 μg mouse^{-1}) 15 min before the formalin injection ($P<0.05$). We recently demonstrated that this schedule of intrathecal pretreatment with α,βmeATP (5.0 μg mouse^{-1}) caused the desensitization of α,βmeATP-sensitive P2X receptors (perhaps $P2X_3$ receptors subtype) in mouse spinal cord *in vivo* (Tsuda *et al*., 1999). The formalin-induced hindpaw inflammation was not affected (Table 1).

Effects on the capsaicin-induced nociceptive behaviour in mice

Mice receiving an injection of capsaicin (1.6 μg paw^{-1}) exhibited licking and biting of the injected hindpaw, and this nociceptive behaviour lasted for 5 min which is similar to previous results described by Sakurada *et al*. (1992). Pretreatment with intrathecal PPADS (5 μg mouse^{-1}) significantly inhibited the capsaicin-induced nociceptive response ($P<0.01$) (Table 2). Very similar inhibition of capsaicin-induced nociception was obtained when mice were intrathecally pretreated with TNP-ATP at a dose of 1.0 μg mouse^{-1} ($P<0.01$). The degree of maximum inhibition of capsaicin-induced nociceptive response by each antagonist was approximately 50%. Furthermore, the capsaicin-induced nociceptive response was also significantly reduced in mice that had been injected intrathecally with α,βmeATP (5.0 μg mouse^{-1}) 15 min prior to capsaicin injection ($P<0.01$).

Effects on the rotarod performance test in mice

The motor coordination evaluating rotarod performance test was not changed by intrathecal pretreatment with PPADS (5 μg mouse^{-1}) or TNP-ATP (1.0 μg mouse^{-1}). Time on rotarod (s)/60 s; saline: 56.3$\pm$2.5 s ($n=7$), PPADS: 58.4$\pm$1.6 s ($n=7$), TNP-ATP: 60.0$\pm$0 s ($n=6$).

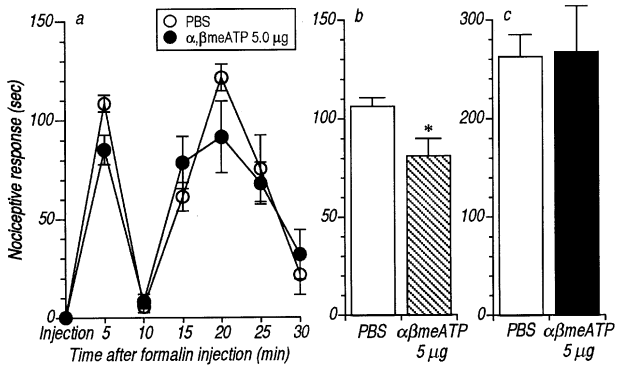

Figure 6 Effect of the desensitization of P2X receptors sensitive to α,βmeATP on the 2% formalin-induced nociceptive behaviour in mice. Explanations of panels in (a), (b) and (c) is similar to the description in Figure 1. Mice were injected with α,βmeATP (5.0 μg mouse^{-1}, i.t.) 15 min before the injection of 2% formalin into left hindpaw. Each point and column represent the mean±s.e.mean of 7–8 mice. *$P<0.05$ vs PBS-pretreated group.

Table 2 Effects of the intrathecal pretreatment with PPADS, TNP-ATP and αβmeATP on the capsaicin-induced nociceptive response in mice

Pretreatment (intrathecal)	*Nociceptive response* (s)
PBS	71.3±5.7
PPADS 5 μg mouse $^{-1}$	37.9±2.5**
PBS	79.2±7.5
TNP-ATP 1 μg mouse $^{-1}$	36.8±3.6**
PBS	73.7±6.2
α,βmeATP 5 μg mouse^{-1}	42.9±5.4**

Mice were injected intrathecally with PPADS, TNP-ATP and α,βmeATP 10, 10 and 15 min, respectively, 10 min prior to the injection of capsaicin (1.6 μg paw^{-1}) into the left hindpaw. Each value represents the mean±s.e.mean of 8–10 mice. **$P<0.01$ vs PBS-injected group.

Discussion

The present study shows the first experimental evidence that the blockade of spinal P2X receptors produced a pronounced antinociceptive effect in two models of chemical-induced nociception. This strongly supports the proposal that ATP may be spinal endogenous substrates inducing the nociceptive response *in vivo*. We found that the first phase of the formalin-induced nociceptive response, which has been considered to be neurogenic pain related to direct activation of primary afferent fibres (Puig & Sorkin, 1996; McCall *et al*., 1996), was reduced by intrathecal pretreatment with PPADS in a dose-dependent fashion. Although the degree of inhibition by PPADS in the first phase is weak, ability to suppress the first phase by PPADS is confirmed by the results that intrathecal pretreatment with PPADS showed a clear inhibition of the nociceptive response caused by the injection into the hindpaw of a low concentration of formalin. Low concentrations of formalin have been reported to produce only the first phase of the nociceptive response and to be useful in evaluating the first phase of the formalin test (Rosland *et al*., 1990; Sakurada *et al*., 1993). Furthermore, the selective antagonist for $P2X_1$, $P2X_3$ and/or $P2X_{2+3}$ receptors TNP-ATP (Virginio *et al*., 1998) also produced an inhibitory effect on formalin-induced nociceptive response in the first phase. The inhibitory effects of PPADS and TNP-ATP are not related to non-specific central depression from the observation that intrathecal injection of neither PPADS (5 μg mouse^{-1}) nor TNP-ATP (1 μg mouse^{-1}) produced sedation and motor incoordination as evaluated by the rotarod performance test. These findings suggest the involvement of endogenous ATP and the $P2X_1$, $P2X_3$ and/or $P2X_{2+3}$ receptor subtypes in formalin-induced neurogenic pain. This is substantiated by further experiments using the capsaicin test (Sakurada *et al*., 1992). The capsaicin test has been proposed as a model of neurogenic pain because capsaicin excites small-diameter primary afferent fibres *via* activation of the vanilloid receptor, VR1 (Caterina *et al*., 1997), thereby causing intense pain (Simone *et al*., 1989). In this model of neurogenic pain using capsaicin, intrathecal pretreatment with PPADS and TNP-ATP showed clear inhibitory effects on capsaicin-induced nociceptive behaviour similar to the results in the formalin test. Therefore, endogenous ATP and the $P2X_1$, $P2X_3$ and/or $P2X_{2+3}$ receptor subtypes in the spinal cord may be generally involved in neurogenic pain caused by excess activation of primary afferent fibres.

Which P2X ($P2X_1$, $P2X_3$ and $P2X_{2+3}$) receptor subtypes are involved in the inhibitory effects of PPADS and TNP-ATP on neurogenic pain caused by formalin and capsaicin? The functional characteristics of these P2X receptors are clearly different. The ATP-evoked inward current is dramatically decreased by repeated application of ATP in the case of $P2X_1$ or $P2X_3$ receptors, but there is little or no such decrease in the case of $P2X_{2+3}$ receptors (Lewis *et al*., 1995; Ueno *et al*., 1998). Utilizing the differences in these P2X receptors, we have previously demonstrated a reduction in the putative $P2X_3$-mediated thermal hyperalgesia by intrathecal pretreatment with α,βmeATP (5 μg mouse^{-1}) (Tsuda *et al*., 1999). In the present study, a group of mice that had previously received α,βmeATP intrathecally showed reduced formalin (first phase)- and capsaicin-induced nociceptive behaviours. This result is inconsistent with a previous study in which pretreatment with intrathecal α,βmeATP did not affect formalin nociception in rats (Driessen *et al*., 1994). Although we can not clearly explain this discrepancy at present, it is possible that this may be due to differences in the animal species (mouse vs rat), the concentration of formalin (2% vs 5%) and/or the methods of nociceptive response measurement (duration vs score of nociceptive behaviours). The same degree of inhibitory effect following pretreatment with PPADS, TNP-ATP and α,βmeATP on the formalin (first phase)- and capsaicin-induced nociceptive behaviours in the present study indicate a contribution of the $P2X_1$ or $P2X_3$ receptors subtype, which are desensitized by pretreatment with α,βmeATP. However, the ATP- or α,βmeATP-evoked inward current in rat DRG neurons (Ueno *et al*., 1999) and rat dorsal horn neurons in the spinal cord (Li & Perl, 1995; Bardoni *et al*., 1997) are not the same as those in cells transfected with $P2X_1$ receptors (Werner *et al*., 1996; Parker, 1998). Furthermore, we have previously demonstrated that treatment with an intrathecal β,γmethylene-L-ATP, the potent $P2X_1$ receptor agonist, does not cause any nociceptive behaviour and hyperalgesia in mice (Tsuda *et al*., 1999). Therefore, we propose an important role for the $P2X_3$ receptor subtype rather than for the $P2X_1$ receptor.

Li *et al*. (1998) have found in an electrophysiological study using spinal cord slices that PPADS reduces excitatory postsynaptic currents (EPSCs) in the superficial dorsal horn neurons evoked by electrical stimulation of primary afferent fibres, although no residual EPSCs are detected in the presence of glutamate antagonists. Few of the dorsal horn neurons have been reported to respond to α,βmeATP (Bardoni *et al*., 1997).

These authors have proposed a presynaptic neuromodulatory role of endogenous ATP and P2X receptors to enhance synaptic transmission in the spinal cord. The presynaptic excitatory role of P2X receptors has also been demonstrated previously in co-culture of DRG and dorsal horn neurons (Gu & MacDermott, 1997). The presynaptic neuromodulatory role of endogenous ATP is of particular interest in the light of findings in the present study concerning the putative role of the spinal $P2X_3$ receptor subtype in neurogenic pain. This is because (1) $P2X_3$ receptors have been shown to be located in the central presynaptic terminal sites of capsaicin-sensitive primary afferent fibres (Guo *et al*., 1999; Llewellyn-Smith & Burnstock, 1998; Vulchanova *et al*., 1997; 1998); (2) ATP has been shown to be released by application of capsaicin from primary afferent nerve terminals in the spinal cord (White *et al*., 1985; Sweeny *et al*., 1989); and (3) injection of formalin into the hindpaw produces activation of C-fibre during the first phase (Puig & Sorkin, 1996; McCall *et al*., 1996). Therefore, our present behavioural findings suggest that within minutes of the injection of formalin or capsaicin, ATP is released from central termini of capsaicin-sensitive primary afferent fibres and that the released ATP activates presynaptic $P2X_3$ subtype located on its fibre which, in turn, enhances the release of neurotransmitters such as glutamate (Li *et al*., 1998; Malmberg & Yaksh, 1995a; Ueda *et al*., 1993; Tsuda *et al*., 1999). This pathway, through endogenous ATP and $P2X_3$ receptors in the spinal cord, would be necessary for the full expression of neurogenic pain caused by excess activation of primary afferent fibres.

Another important finding obtained from the present study is that intrathecal pretreatment with PPADS produced a pronounced inhibition of the second phase of formalin-induced nociceptive behaviour. The second phase of formalin-induced nociception has been considered to be dependent on (1) the sensitization of dorsal horn neurons due to the initial barrage of primary afferent input during the first phase and (2) the inflammation-induced hyperactivity of primary afferent neurons input to dorsal horn neurons (Tjølsen *et al*., 1992; Coderre *et al*., 1993). To clarify the former possibility, we investigated whether PPADS produces a clear suppression of nociceptive response in the second phase when PPADS was injected intrathecally after the appearance of the first phase. If an inhibitory effect of PPADS on the second phase was solely dependent on the PPADS-induced inhibition of the first phase, then PPADS would be expected not to inhibit the formalin-induced nociceptive behaviour in the second phase. The results obtained showed that a similar degree of inhibition of formalin nociception in the second phase by PPADS was observed in mice that had been injected intrathecally with PPADS before or after the first phase. This finding indicates that PPADS can suppress the development of the second phase of the formalin-induced nociceptive response without dependence on the inhibitory effect of the first phase. Thus, it appears that the latter possibility is responsible for the PPADS-induced inhibition of the second phase. However, the formalin-induced hindpaw inflammation itself was not attenuated by intrathecal pretreatment with PPADS at any dose. Therefore, it is conceivable that the suppression of the second phase by PPADS may be related to an inhibitory effect on the hyperexcitability of primary afferent neurons by inflammation input to dorsal horn neurons during the second phase. Electrophysiological studies have found an increase in the activities of Aδ- and C-fibres located at the injection and adjacent sites during the second phase of the formalin-induced pain (Puig & Sorkin, 1996; McCall *et al*., 1996). Activity of these fibres (particularly C-fibre) has been considered to play a key role in the generation of the second phase from the demonstration that formalin-induced nociceptive behaviour in the second phase is suppressed in neonatal capsaicin-treated rat in which the capsaicin-sensitive primary afferent nerves have been destroyed (Hua *et al*., 1996; Peterson *et al*., 1997). This evidence leads us to the expectation that TNP-ATP can inhibit the second phase of formalin nociception as well as the first phase. However, such an inhibitory effect of TNP-ATP was not seen. Although the failure to suppress the second phase by TNP-ATP is surprising because of the common contribution of capsaicin-sensitive fibres to the first and second phases of formalin nociception, this may be explained by the hypothesis that the $P2X_3$ receptors have already been desensitized by endogenous ATP-induced excess activation after the first phase. This hypothesis is strongly supported by further demonstration that the effect of TNP-ATP on the first and second phases was similar in mice that had desensitized $P2X_3$ receptors by intrathecal pretreatment with α,βmeATP. Although at present we cannot comment on the involvement of the receptor subtype from the several candidate P2X receptors sensitive to PPADS and insensitive to TNP-ATP in the second phase of formalin pain, this will be clarified by the development of new selective compounds for the P2X receptor subtypes. In addition, we have not excluded the possible involvement of P2Y receptors in the second phase of formalin pain. In fact, PPADS is known to possess an antagonistic activity to P2Y receptors (reviewed in Ralevic & Burnstock, 1998). Several lines of evidence have shown that ATP, 2-methylthio-ATP and uridine triphosphate (UTP) through P2Y receptors evoke an increase in the intracellular level of free Ca^{2+} concentration in astroglial cells from dorsal spinal cord (Salter & Hicks, 1994; Ho *et al*, 1995; Idestrup & Salter, 1998). Chen & Chen (1998) have found that ATP evokes the release of arachidonic acid in cultured astrocytes that may cause the release of eicosanoids such as prostaglandin E_2 (PGE_2). Release of PGE_2 in the spinal cord has been known to be associated with the processing of nociceptive information in the second phase of formalin pain (Malmberg & Yaksh, 1995b).

In conclusion, our present data provide the first evidence that general P2 receptor and selective P2X receptor antagonists produced pronounced antinociceptive activities in formalin and capsaicin models of pain in mice. These behavioural findings suggest that ATP in the spinal cord is endogenous substrates inducing pain resulting from the direct activation of primary afferent fibres by irritants (neurogenic pain) and from hyperactivity of primary afferent fibres by inflammation (inflammatory pain). More importantly, evidence in our study reveals that the PPADS- and TNP-ATP-sensitive P2X receptor subtype (perhaps the $P2X_3$ receptor subtype) which is desensitized by α,βmeATP plays an important role in neurogenic pain and that the PPADS-sensitive and TNP-ATP-insensitive P2X (and/or P2Y) receptors may be involved in the inflammatory pain caused by formalin.

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