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Effects of spinally administered P2X receptor agonists and antagonists on the responses of dorsal horn neurones recorded in normal, carrageenan-inflamed and neuropathic rats

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1 The function and role of P2X receptors in the spinal transmission of nociception was investigated using the selective P2X receptor agonists, α,β-methylene ATP (α,β-me ATP) and β,γ-methylene-L-ATP (β,γ-me-L-ATP) and the P2X receptor antagonists pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonate (PPADS) and suramin.

2 Intrathecal administration of 5 and 50 μg of β,γ-me-L-ATP produced a significant facilitation of the C-fibre evoked response and a tendency towards increased excitability of the post-discharge, but not Aβ-fibre evoked response of dorsal horn neurones recorded in normal animals. Administration of similar doses of α,β-me ATP did not produce an overall change in the response of the neuronal population.

3 Peripheral administration of 20 μg of these agonists into the paw of the rat evoked firing in the dorsal horn neurones.

4 Intrathecal administration of the antagonists, suramin (50 and 500 μg) and PPADS (5, 50 and 500 μg), to normal animals and to animals with a model of neuropathy induced by spinal nerve ligation did not alter the evoked neuronal responses. In contrast, intrathecal administration of 500 μg of suramin to animals 3 h after the induction of carrageenan inflammation produced a significant inhibition of the C-fibre evoked response of the neurones. Similar inhibitions were also seen following high doses of intrathecal PPADS, although this did not reach significance.

5 These results suggest that spinal P2X receptors may play a role in the modulation of spinal nociceptive transmission following the development of inflammation, but that these receptors play at most a minor role in spinal nociceptive processing in normal and neuropathic animals.
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Keywords: P2X; spinal cord; nociception; carrageenan inflammation; neuropathic; suramin; PPADS; α,β-methylene ATP; β,γ-methylene-L-ATP

Abbreviations: α,β-me ATP, α,β-methylene ATP; β,γ-me-L-ATP, β,γ-methylene-L-ATP; DRG, dorsal root ganglia; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonate

Introduction

There has been much interest in the idea that the purine ATP may be involved in spinal nociceptive transmission *via* actions at P2X receptors. P2X receptors, of which seven subunits have been identified ($P2X_{1-7}$), have been shown to be located both in the dorsal horn of the spinal cord and on the DRG/terminals of primary afferents (Collo *et al*., 1996; Surprenant *et al*., 1996; Vulchanova *et al*., 1996; 1997; 1998). One subunit, the $P2X_3$ subunit, is found exclusively in small diameter, capsaicin sensitive sensory ganglia (Chen *et al*., 1995; Lewis *et al*., 1995), making this a possible selective analgesic target.

Studies (predominantly in isolated preparations) have shown that DRG and some spinal dorsal horn neurones respond to ATP (Jahr & Jessell, 1983; Fyffe & Perl, 1984; Salter & Henry, 1985; Bean 1990; Bouvier *et al*., 1991; Li & Perl, 1995; Robertson *et al*., 1996; Bardoni *et al*., 1997) and activation of P2X receptors by ATP has been shown to increase the release of glutamate from primary afferent terminals (Gu & MacDermott, 1997), suggesting that ATP may function as a transmitter or modulator of nociception. Consistent with this, intrathecal administration of the P2X receptor agonist α,β-me ATP produces thermal hyperalgesia (Driessen *et al*., 1994; Tsuda *et al*., 1999), whilst peripheral administration evokes behaviour indicative of pain in animals (Bland-Ward & Humphrey, 1997; Hamilton *et al*., 1999).

The subunit composition of the P2X receptors underlying these effects is not entirely clear. P2X receptors may be either homomeric or heteromeric. Responses of P2X receptors formed from homomeric subunit expression fall into two broad categories. Homomeric expression of $P2X_1$ or $P2X_3$ receptors leads to receptors which desensitize rapidly following application of ATP, and which are sensitive to the agonist α,β-me ATP and the P2X receptor antagonists suramin and PPADS. In contrast, homomeric expression of the other P2X receptors leads to receptors which do not desensitize and which are relatively insensitive to α,β-me ATP, with homomeric $P2X_4$/$P2X_6$ receptors also being insensitive to antagonism by suramin and PPADS (see Ralevic & Burnstock, 1998). However, heteromeric P2X receptors with other characteristics can also be formed (Lewis *et al*., 1995; Lê *et al*., 1998). Indeed, heteromeric P2X receptors comprising $P2X_2$/$P2X_3$ subunits, which form receptors which are non-desensitizing, and sensitive to the agonist α,β-me ATP, have been suggested to have properties which most closely resemble those of native P2X receptors found in adult sensory (nodose) ganglia (Lewis *et al*., 1995). However, other studies suggest that rapidly desensitizing homomeric $P2X_3$ receptors are most likely to be responsible for ATP mediated responses in small diameter nociceptive DRG neurones and their central terminals

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(Robertson *et al*., 1996; Vulchanova *et al*., 1997; Ueno *et al*., 1999). Less is known about the subunit composition of post-synaptic P2X receptors in the dorsal horn of the spinal cord, although all P2X receptors except $P2X_3$ are expressed, with $P2X_2$, $P2X_4$ and $P2X_6$ being most abundant in the dorsal horn (Collo *et al*., 1996; Surprenant *et al*., 1996).

Whilst it is clear that both the message and protein for P2X receptor subunits are found within the dorsal horn of the spinal cord (Collo *et al*., 1996; Surprenant *et al*., 1996; Vulchanova *et al*., 1997), and that a subpopulation of dorsal horn neurones respond to exogenously applied ATP (Jahr & Jessell, 1983; Fyffe & Perl, 1984; Salter & Henry, 1985; Li & Perl, 1995; Bardoni *et al*., 1997), studies in a number of isolated systems have failed to show ATP-mediated synaptic transmission in the dorsal horn (Li & Perl, 1995; Li *et al*., 1998). Where evidence of ATP-P2X receptor-mediated synaptic transmission has been seen in the dorsal horn following suppression of other transmitter systems with a cocktail of antagonists, it has been confined to a very small proportion ($<5\%$) of neurones (Bardoni *et al*., 1997). These studies leave the status of ATP as a transmitter of nociceptive information unclear, although ATP has also been proposed to act as a neuromodulator to enhance synaptic transmission in the dorsal horn of the spinal cord at pre- and/or post-synaptic sites (Li & Perl, 1995; Gu & MacDermott, 1997; Li *et al*., 1998). Furthermore, recent studies have suggested that whilst acutely dissociated DRG neurones show robust P2X-purinoceptor mediated responses, only a fraction (6%) of these neurones show responses to ATP when isolated together with the sciatic nerve and dorsal roots (Stebbing *et al*., 1998). These data, together with conflicting data obtained in behavioural studies using intrathecal injection of P2X receptor antagonists (Driessen *et al*., 1994; Li *et al*., 1998), means that the function significance of spinal P2X receptors *in vivo* is unclear.

The present study uses *in vivo* electrophysiological techniques together with spinally administered P2X receptor agonists and antagonists to investigate the functional significance of P2X receptors in spinal nociceptive transmission in normal animals. To elucidate whether the role of endogenous ATP in spinal nociceptive processing alters following nerve injury or the development of peripheral inflammation, P2X receptor antagonists were administered in animals with a tight ligation of the L5–6 spinal nerves or inflammation induced by the injection of carrageenan into the hind paw. Finally, to compare the effects of spinal administration of P2X receptor agonists to the well described peripheral nociceptive actions of ATP, the ability of P2X agonists to evoke firing in dorsal horn neurones following intradermal administration was studied.

Methods

Electrophysiological studies

The experiments were performed in intact halothane anaesthetized rats in accordance with the U.K. Animals (Scientific Procedures) Act, 1986. Male Sprague-Dawley rats (200–250 g) were anaesthetized with halothane (3%) in 33% O_2/66% N_2O and maintained on 2% halothane for the subsequent surgery. Following insertion of a tracheal cannula, the rat was placed in a stereotaxic frame and the vertebral column held in metal clamps. A laminectomy was performed (vertebrae L1–L3) to expose the spinal cord. Before commencement of the electrophysiological recording, the level of halothane was lowered to $\sim$1.6% which was sufficient to maintain a state of complete areflexia. The core temperature of the animal was maintained at 37°C by means of a thermostatically controlled heating blanket.

Single unit extracellular recordings were made with a parylene-coated tungsten electrode from convergent neurones located in the deep dorsal horn (500–1000 μm). These neurones responded to innocuous stimuli such as touch, and to noxious stimuli such as pinch. Electrical stimulation, applied *via* two needles inserted into the peripheral receptive field of the neurone located on the toes of the ipsilateral hind paw, was used as the test stimulus for the experiment. Trials of 16 stimuli (0.5 Hz, 2 ms pulse) were given at three times the C-fibre threshold of each particular neurone. Following a trial of 16 stimuli, the responses of the dorsal horn neurone evoked polysynaptically by C-fibres were separated from A-fibre evoked responses on the basis of latency and quantified. In addition to the C-fibre evoked response occurring in the latency band attributed to afferent C-fibre mediated transmission calculated on the basis of the conduction velocity of C-fibres (90–300 ms post-stimulus), termed 'the C-fibre response', the C-fibre evoked phenomenon of wind-up resulted in the occurrence of neuronal firing beyond the afferent C-fibre latency band, which was termed the post-discharge (300–800 ms). The initial response of the neurone (I), the C-fibre response of the neurone evoked by the first stimulus in the train, was taken as a measure of the non-potentiated response of the neurone prior to augmentation by wind-up.

Control trials were carried out at 10 min intervals and drugs were applied directly to the spinal cord (akin to an intrathecal injection) in a volume of 50 μl following three stable control trials ($<10\%$ variation in the C-fibre evoked response). Following drug administration, the evoked response of the neurone was followed for 60 min. Drugs were given cumulatively at 60 min intervals and the doses shown in the text and in the figures refer to the doses added. Each animal received only one drug and only one neurone was studied per animal.

Induction of carrageenan inflammation

In the carrageenan experiments, inflammation was induced in the anaesthetized animals by the injection of 100 μl of 2% λ-carrageenan (Sigma) into the plantar surface of the ipsilateral hind paw, and the electrically-evoked response of the neurone followed at 10 min intervals for a period of 3 h. Drug administration commenced 3 h after the injection of carrageenan into the paw. In these carrageenan experiments, the three controls immediately prior to administration of the first dose of the drug to the spinal cord were used as controls for the subsequent drug effect.

Preparation of spinal nerve ligated animals

The model of neuropathic pain introduced by Kim & Chung (1992), the spinal nerve ligation model, was used. Briefly, the rats were anaesthetized with halothane in a mixture of O_2 and N_2O (50 : 50). The left L_5 and L_6 spinal nerves were exposed by removing a small piece of the paravertebral muscle and a part of the left spinous process of the L_5 lumbar vertebra. The L_5 and L_6 spinal nerves were then carefully isolated and tightly ligated with 6-0 silk. After checking haemostasis, the muscle, the adjacent fascia and the skin were closed with sutures. Seven to fourteen days after the surgery, the behavioural signs of mechanical allodynia were measured with a series of von Frey hairs as

described previously (Kontinen *et al*., 1998). Briefly, the rats were placed on a metal mesh and von Frey hairs of varying stiffness (bending force 0.2–15 g) were applied to the plantar surface of the paw. Each hair was applied five times at 5 s intervals until the force that induced paw withdrawal in more than half of the stimuli was found. Electrophysiological studies were performed 14–18 days post-surgery.

Peripheral administration of P2X receptor agonists

The ability of the P2X receptor agonists α,β-me ATP and β,γ-me-L-ATP to evoke firing in the dorsal horn neurones when administered into the peripheral receptive field of the neurone was also studied. In these studies, the agonists were administered intradermally in a volume of 20 μl using a 29-gauge needle into the centre of the peripheral receptive field and the firing evoked in the dorsal horn neurone recorded. Saline controls, consisting of the injection of 20 μl of physiological saline into the receptive field of the neurone, were performed prior to the injection of each agonist.

Drugs

The P2X receptor agonists α,β-methylene adenosine 5′-triphosphate dilithium (α,β-me ATP) and β,γ-methylene-L-adenosine 5′triphosphate tetrasodium (β,γ-me-L-ATP) and the P2X receptor antagonists pyridoxal-phosphate-6-azophenyl-2′,5′-disulphonic acid tetrasodium (PPADS) and suramin hexasodium (all Research Biochemicals International) were dissolved in saline.

Statistical analysis

Data are presented as mean percentage of control response ± s.e.mean. Statistical analysis of the data was performed using the paired Student's *t*-test on the raw spike count data to test for significance of drug effects from control and the Mann-Whitney test on normalized data to analyse drug effects between the groups of neurones. A *P* value less than 0.05 was regarded as significant.

Results

Effects of intrathecally administered P2X receptor agonists in normal animals

The effects of four doses (0.1, 1, 5 and 50 μg) of the P2X receptor agonist β,γ-me-L-ATP, administered intrathecally, were tested on the electrically-evoked responses of seven dorsal horn neurones recorded in normal animals. No drug evoked neuronal activity was seen following administration of β,γ-me-L-ATP. However, the noxious evoked responses of six of these neurones were facilitated by β,γ-me-L-ATP whereas the response of one neurone, which did not obviously differ from the others in terms of location or response, was inhibited by all doses of β,γ-me-L-ATP. β,γ-me-L-ATP produced modest facilitations of the C-fibre evoked response of the dorsal horn neurones (Figure 1), with the highest doses (5 and 50 μg) of β,γ-me-L-ATP tested producing a significant facilitation of this response to 129.5 ± 8.7 and 125.5 ± 6.2% of control respectively ($n=6$,

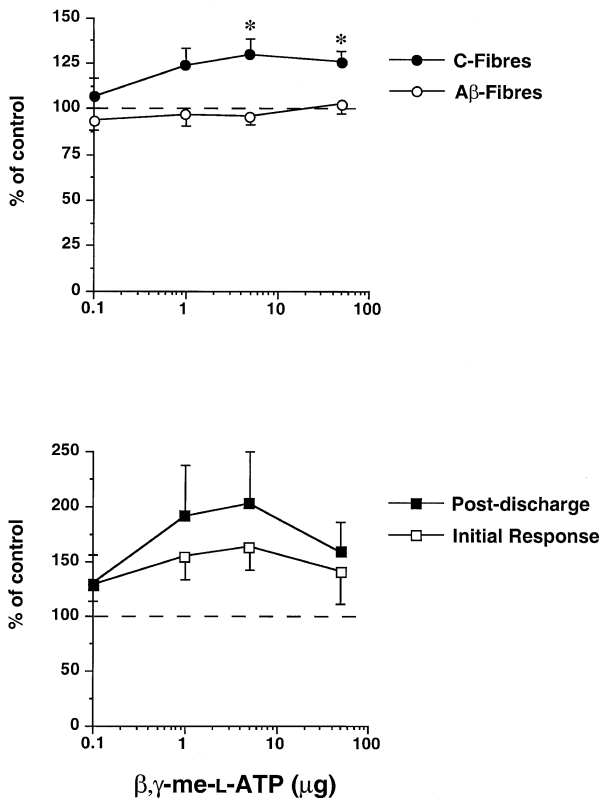

Figure 1 Effects of intrathecal β,γ-me-L-ATP (0.1–50 μg) on the components of the electrically-evoked response of dorsal horn neurones recorded in normal animals. $n=6$ neurones per dose, $^*P<0.05$ compared with control.

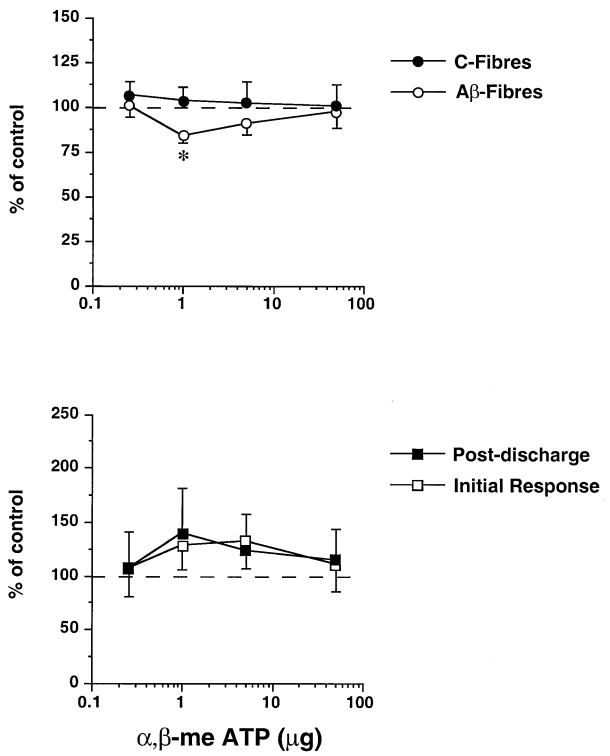

Figure 2 Effects of intrathecal α,β-me ATP (0.25–50 μg) on the components of the electrically-evoked response of dorsal horn neurones recorded in normal animals. All doses were tested on eight neurones, of which five neurones were consistently facilitated, and three inhibited, by all doses α,β-me ATP. $^*P<0.05$ compared with control.

$P<0.05$). The post-discharge and initial response of the neurones was also facilitated (Figure 1), in some cases to a great extent, by β,γ-me-L-ATP, although the magnitude of these facilitations were variable and did not reach significance. In contrast, the Aβ-fibre evoked response of the neurones was not affected by any of the doses of β,γ-me-L-ATP tested (Aβ-fibre evoked response = 95.3 ± 4.1% of control following 5 μg of β,γ-me-L-ATP, $n=6$).

The effects of four doses (0.25, 1, 5 and 50 μg) of the selective P2X receptor agonist α,β-me ATP, administered intrathecally, were tested on the evoked responses of eight dorsal horn neurones recorded in normal animals. α,β-me ATP did not produce consistent effects on the noxious-evoked neuronal responses. The noxious-evoked responses of five of the neurones were facilitated by all doses of α,β-me ATP, whereas the responses of the remaining three neurones were inhibited by α,β-me ATP. With the C-fibre evoked response, this mix of facilitations and inhibitions led to no overall change in the C-fibre evoked response of the neuronal population, but produced a tendency towards a facilitation of the initial response and post-discharge of the neuronal population (Figure 2). Despite the varying effects seen following α,β-me ATP, no obvious differences in the location or neuronal characteristics of neurones facilitated or inhibited by α,β-me ATP were noted. The Aβ-fibre evoked response was not affected by 0.25, 5 or 50 μg of α,β-me ATP (Figure 2), although 1 μg of α,β-me ATP produced a significant inhibition of this response to 83.9 ± 3.9% of control ($P=0.014$).

Responses of dorsal horn neurones evoked by peripherally administered P2X receptor agonists in normal animals

The peripheral administration of 20 μg of α,β-me ATP (39 nmol, $n=6$) or β,γ-me-L-ATP (34 nmol, $n=5$) into the receptive field of the neurone resulted in firing of the dorsal horn neurones which was significantly greater ($P<0.05$) than that produced by the injection of the same volume of saline (Figure 3). The mean duration of neuronal firing evoked by 20 μg of α,β-me ATP and β,γ-me-L-ATP was 163 ± 12 s (1979 ± 514 action potentials) and 126 ± 27 s (1451 ± 374 action potentials) respectively.

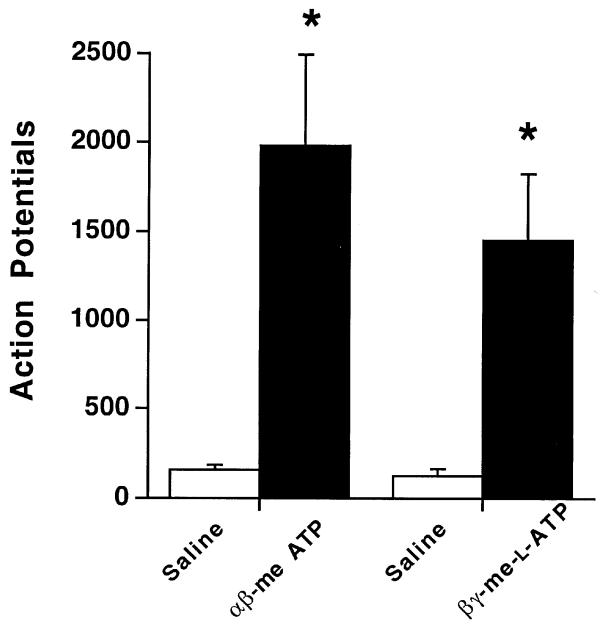

Figure 3 Response evoked in dorsal horn neurones following the intradermal injection of saline or 20 μg of α,β-me ATP ($n=6$) or β,γ-me-L-ATP ($n=5$) into the peripheral receptive field of the neurone. *$P<0.05$ compared with saline.

Effects of intrathecally administered P2X receptor antagonists suramin and PPADS on the evoked neuronal responses recorded in normal animals

To determine whether endogenous ATP acting at P2X receptors plays a role in spinal nociceptive processing, the effects of the intrathecal administration of 50 and 500 μg of the P2X receptor antagonist suramin were tested on the electrically-evoked responses of seven neurones recorded in normal animals. Neither dose of suramin had any overall effect on any of the neuronal measures recorded in normal animals (Figure 4).

The effects of the intrathecal administration of 5, 50 and 500 μg of the P2X receptor antagonist PPADS were tested on the evoked neuronal responses of six neurones recorded in normal animals. As seen with suramin, none of the doses of PPADS produced any overall change in the evoked responses of the neuronal population studied (Figure 5).

Effects of intrathecally administered P2X receptor antagonists suramin and PPADS on the evoked neuronal responses recorded in animals with carrageenan inflammation

To investigate whether the role of ATP acting at P2X receptors is enhanced following the development of peripheral inflammation, the effects of 50 and 500 μg of suramin, administered intrathecally, were tested on the electrically-evoked responses of eight dorsal horn neurones recorded in animals 3 h after the injection of carrageenan into the ipsilateral hind paw. The C-fibre evoked response and post-discharge of the neurones recorded in the carrageenan animals did not differ from those recorded in normal animals (e.g. C-fibre evoked response = 394 ± 49 action potentials in normal animals, and 427 ± 45 action potentials in the carrageenan animals). In these carrageenan animals 500 μg, but not 50 μg, of suramin tended to produce an inhibition of the noxious evoked response of the neurones. Thus, the total C-fibre evoked response (to all 16 stimuli) and particularly the C-fibre response to the first stimulus in the train, the initial response, were inhibited to 84.6 ± 5.8% ($n=8$, $P=0.046$) and 60.6 ± 3.8% ($n=8$, $P<0.0001$) of control respectively (Figure 4). Neither the post-discharge of the neurones, which is generated as a result of wind-up in the dorsal horn neurones, nor the innocuous Aβ-fibre evoked response of the neurones were inhibited by 500 μg of suramin in the carrageenan animals.

The effects of 5, 50 and 500 μg of PPADS were also tested on the evoked responses of dorsal horn neurones recorded in animals 3 h after the injection of carrageenan into the ipsilateral hind paw ($n=7$). The evoked neuronal responses recorded in the carrageenan animals were not significantly influenced by any of the doses of PPADS (Figure 5). However, the highest doses of PPADS tested tended to produce an inhibition of the initial response of the neurones, with 500 μg of PPADS reducing this response to 63.6 ± 19.4% of control.

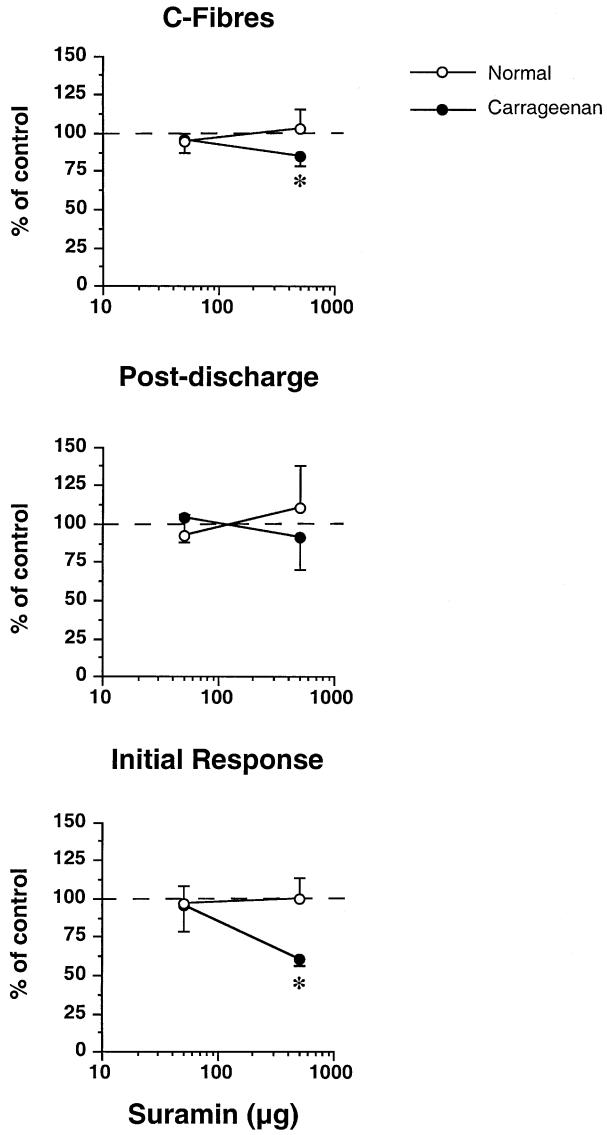

Figure 4 Effects of intrathecal suramin (50 and 500 μg) on the electrically-evoked response of dorsal horn neurones recorded in normal animals, and in animals 3 h after the injection of carrageenan into the hind-paw. $n=7-8$ neurones per dose, $^*P<0.05$ compared with control.

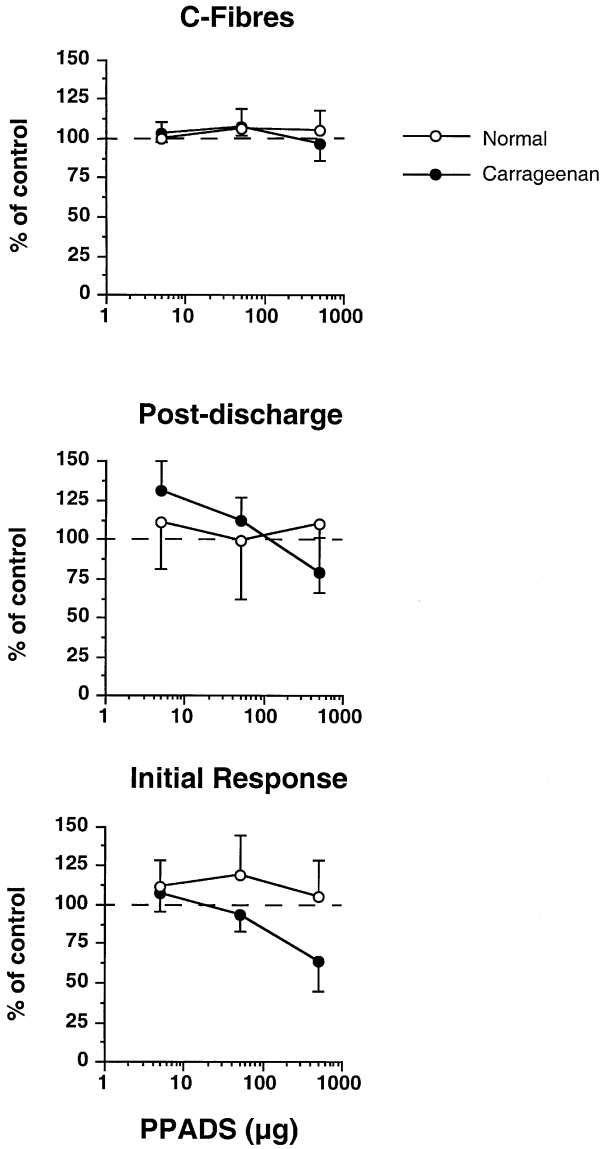

Figure 5 Effects of intrathecal PPADS (5–500 μg) on the evoked responses of dorsal horn neurones recorded in normal animals, and in animals 3 h after the injection of carrageenan into the hind-paw. $n=6-7$ neurones per dose.

Effects of the P2X receptor antagonists suramin and PPADS on the evoked responses of dorsal horn neurones recorded in animals following ligation of the L5–6 spinal nerves

To investigate whether ATP acting at P2X receptors might have an altered role in neuropathic pain states, the effects of 50 and 500 μg of suramin (eight rats) or 5, 50 and 500 μg of PPADS (seven rats), administered intrathecally, were tested on the evoked responses of dorsal horn neurones recorded in rats 14–18 days after a tight ligation of their L5–6 spinal nerves. Behavioural testing of the animals prior to the electrophysiological recordings demonstrated that all animals included in this study displayed signs of mechanical allodynia as has been reported previously (Kontinen *et al*., 1998). Thus, 14 days after surgery the von Frey hair force that induced paw withdrawal in the ipsilateral paw was 5.5 ± 1.9 g (median, median absolute deviation, $n=15$), whereas applying the strongest hair used (15.1 g) did not cause paw withdrawal in the contralateral paw. In the electrophysiological studies in these animals, electrical stimulation of the peripheral receptive field of the neurones evoked responses which were similar in magnitude to those evoked in normal animals i.e. average C-fibre evoked response $=394\pm49$ and 350 ± 42 action potentials and post-discharge $=294\pm73$ and 202 ± 49 action potentials in normal and neuropathic animals respectively.

None of the doses of suramin (Figure 6) or PPADS (Figure 7) tested in these spinal nerve ligated animals produced a significant overall change in the evoked responses of the population of neurones tested, although the noxious evoked responses of some individual neurones were sensitive to the effects of the drugs. Thus, the C-fibre evoked response, initial response and post-discharge of two of the seven neurones tested with PPADS and one of the eight neurones tested with suramin were inhibited in a dose-related manner, with the highest dose of each drug reducing these responses to approximately 50% of control. These neurones did not obviously differ from the other neurones,

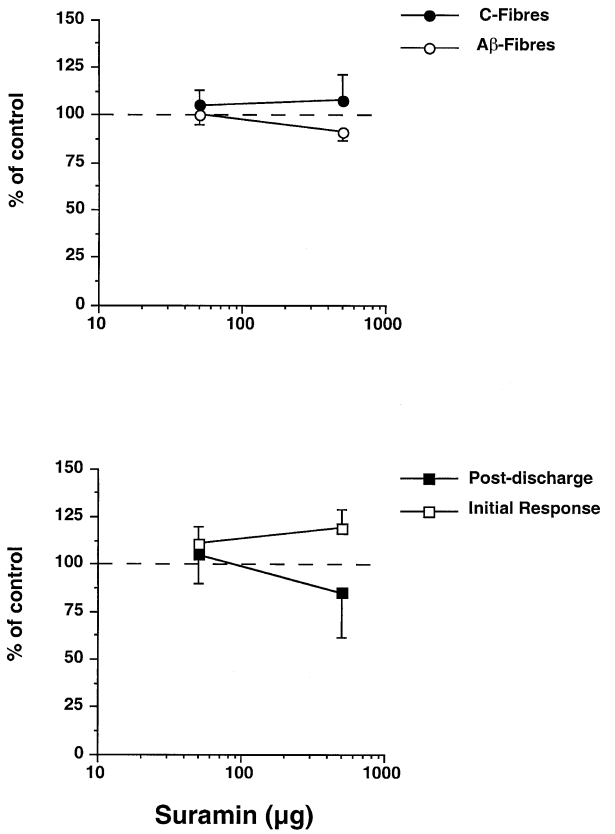

Figure 6 Effects of intrathecal suramin (50–500 μg) on the electrically-evoked response of dorsal horn neurones recorded in animals 14–18 days after ligation of their L5–6 spinal nerves. $n=8$ neurones per dose.

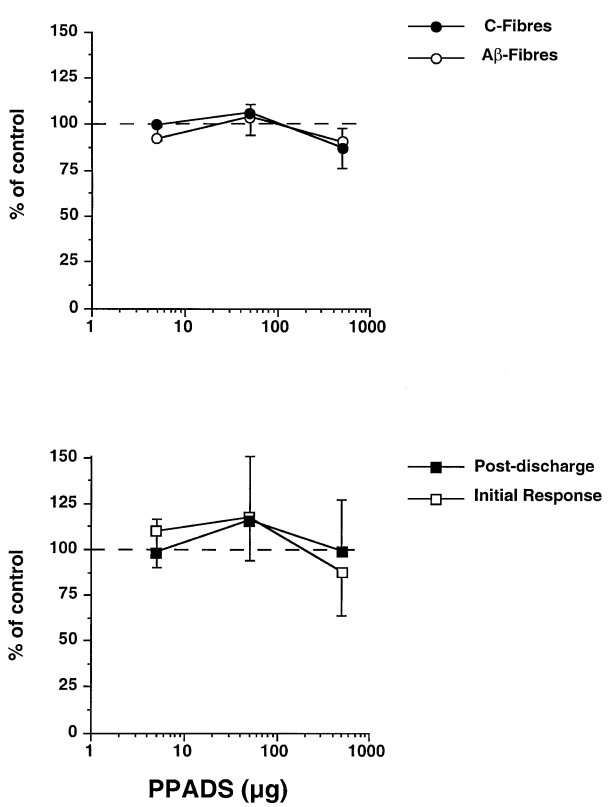

Figure 7 Effects of intrathecal PPADS (5–500 μg) on the electrically-evoked response of dorsal horn neurones recorded in animals 14–18 days after ligation of their L5–6 spinal nerves. $n=7$ neurones per dose.

which were unaffected by the drugs, in terms of their location or response, nor did the animals from which these neurones were recorded differ in terms of their behaviour post-operatively. The Aβ-fibre evoked responses of these three neurones were not similarly inhibited by the antagonists.

Discussion

In recent years, there has been much interest in the possibility that ATP acting at P2X receptors functions as a transmitter/modulator of nociception. In the present *in vivo* study, performed in adult rats, spinal administration of the selective P2X receptor agonist α,β-me ATP failed to produce clear effects on the noxious or innocuous evoked responses of dorsal horn neurones. The agonist, by contrast, evoked a powerful excitatory response in the same population of neurones when administered into the periphery. These results do not support a major role of ATP as a transmitter or modulator of nociception in the dorsal horn of the spinal cord. This may at first seem surprising given that a number of studies have shown that α,β-me ATP causes depolarization of small diameter, capsaicin sensitive DRG neurones (Stebbing *et al*., 1998; Ueno *et al*., 1999), and ATP has been shown to modulate the release of glutamate from primary afferent fibres (Gu & MacDermott, 1997; Li *et al*., 1998), suggesting that P2X receptors, located presynaptically on primary afferent terminals, have the potential to modulate nociceptive transmission. The agonist was applied intrathecally in the present study, so would have access to receptors located on primary afferent terminals in the dorsal horn of the spinal cord, one possible destination for P2X receptors synthesized in DRG. Indeed $P2X_3$ and $P2X_2$ receptor immunoreactivity of primary afferent origin is seen in the superficial laminae of the dorsal horn, consistent with a presynaptic localization of these receptors on primary afferent terminals (Vulchanova *et al*., 1997; 1998). Thus, recording the responses of deep dorsal horn neurones would allow any functional consequences of P2X receptor mediated activity on primary afferent terminals or indeed interneurones and output pathways in the dorsal horn to be observed.

However, to date many of the electrophysiological studies examining P2X receptor-mediated responses in dorsal root ganglion neurones have been performed on isolated neurones, often obtained from immature animals, so the relevance of these results to nociceptive transmission in intact, adult animals is not clear. Furthermore, there is evidence that when recordings are made from intact DRG neurones isolated together with the peripheral nerve, only a small proportion (6%) respond to ATP in contrast to acutely dissociated DRG neurones, where almost all neurones (90%, including 100% of small diameter neurones) respond (Stebbing *et al*., 1998). This observation that dissociation of DRG neurones can lead to an upregulation in P2X receptor-mediated responses in these neurones is supported by the finding that a larger proportion of DRG neurones in primary culture express $P2X_3$ immunoreactivity than is seen in sections of DRG tissue (Novakovic *et al*., 1999).

There are however a number of other reasons why the use of α,β-me ATP in the present study may have failed to point to a role of P2X receptors in the spinal transmission or modulation

of nociception which need to be considered. Firstly, α,β-me ATP, although widely used as a P2X receptor agonist, is known to act predominantly at homomeric $P2X_1/P2X_3$ receptors and heteromeric $P2X_{2/3}$ receptors. It is known that native P2X receptors on primary afferent terminals are most likely to be heteromeric $P2X_{2/3}$ receptors or homomeric $P2X_3$ receptors (Lewis *et al*., 1995; Robertson *et al*., 1996; Ueno *et al*., 1999), which would respond to α,β-me ATP. Indeed, the finding in the present study that peripherally administered α,β-me ATP evoked firing in the dorsal horn neurones suggests that functional P2X receptors responding to α,β-me ATP are present on the peripheral terminals of primary afferent fibres in these animals. However, the post-synaptic P2X receptors expressed in the dorsal horn of the spinal cord are predominantly $P2X_2$, $P2X_4$ and $P2X_6$ receptor subtypes (Collo *et al*., 1996), which when expressed as homomeric receptors are relatively insensitive to α,β-me ATP (see Ralevic & Burnstock, 1998), although $P2X_{2/4}$ heteromeric receptors with moderate sensitivity to α,β-me ATP have recently been reported (Lê *et al*., 1998). Indeed, acutely dissociated dorsal horn neurones have been shown to be insensitive to α,β-me ATP (Bardoni *et al*., 1997), consistent with P2X receptors comprising $P2X_2$, $P2X_4$ or $P2X_6$ subunits. Thus the lack of clear effects of α,β-me ATP in the present study does not discount a role for other subtypes of P2X receptors in spinal nociceptive processing, although less than 5% of acutely dissociated dorsal horn neurones have been reported to be sensitive to ATP itself, which would activate all P2X receptors (Bardoni *et al*., 1997).

Despite the lack of consistent effects with the P2X receptor agonist α,β-me ATP, intrathecal administration of similar doses of the P2X receptor agonist β,γ-me-L-ATP produced facilitations in the noxious evoked responses of the majority of neurones tested. Unlike α,β-me ATP, which activates both $P2X_1$ and $P2X_3$ homomeric receptors, β,γ-me-L-ATP has been reported to be selective for $P2X_1$ receptors (Trezise *et al*., 1995; Rae *et al*., 1998). Whether this difference in the selectivity of the two agonists is responsible for the differences seen is unclear. Dorsal root ganglion neurones taken from neonatal rats have been reported to be almost insensitive to β,γ-me-L-ATP (Rae *et al*., 1998), which could suggest that the effects of this drug in the present study are most likely to represent actions on post-synaptic receptors in the dorsal horn of the spinal cord. However, the firing evoked in dorsal horn neurones by the peripheral administration of β,γ-me-L-ATP in the present study suggests the presence of β,γ-me-L-ATP-responsive P2X receptors on primary afferents.

Further reasons for a possible lack of clear effects of α,β-me ATP in this study include rapid desensitization of the receptors by the agonist, which could lead to α,β-me ATP acting as a functional antagonist of endogenous ATP in some cases (although some of the currents evoked in small DRG neurones by ATP and α,β-me ATP do not desensitize), and a saturation of P2X receptor-mediated responses by endogenous ATP released by the suprathreshold stimulus used in the present studies. Behavioural studies have reported pronociceptive effects of similar doses of α,β-me ATP (0.3–30 μg) in rats and mice following intrathecal administration (Driessen *et al*., 1994; Tsuda *et al*., 1999), which suggests that desensitization of the receptors is not the reason for the lack of effect seen in the present study. The suprathreshold stimuli used in the present study may lead to a saturation of P2X receptor-mediated responses by endogenous ATP which may not occur with the threshold stimuli employed in the behavioural study. If this is the reason for the lack of P2X receptor mediated effects, then this should be revealed by the use of P2X receptor antagonists.

The possible role of endogenous ATP in the spinal transmission and/or modulation of nociception was investigated using the P2X receptor antagonists suramin and PPADS. In normal animals, neither antagonist produced a marked effect on the population of neurones. The P2X receptor antagonists suramin and PPADS do not block all P2X receptors, notably $P2X_4$ and $P2X_6$ receptors (Ralevic & Burnstock, 1998), which are thought to make up a large proportion of post-synaptic P2X receptors in the dorsal horn of the spinal cord (Collo *et al*., 1996). However, it has been shown that 85% of all responses evoked by ATP in dorsal horn neurones are sensitive to PPADS/suramin (Bardoni *et al*., 1997). Taken together with the agonist studies, these results suggest that ATP acting at P2X receptors in the spinal cord is unlikely to be playing a major role in spinal nociceptive processing in normal animals. It is possible that the strong glutamatergic drive produced by the suprathreshold stimuli used in the present study may mask a weak purinergic contribution to nociceptive transmission. However, the data obtained in the present study with the P2X receptor antagonists are consistent with behavioural studies of nociceptive thresholds where intrathecally administered PPADS has been reported to be without effect in tests of acute nociception (Driessen *et al*., 1994; Li *et al*., 1998). Suramin has been reported to produce antinociception in some studies (Ho *et al*., 1992; Driessen *et al*., 1994) however, the lack of effect of the more selective agent PPADS makes it unlikely that this effect was mediated solely by a blockade of P2X receptors.

It is well known that a number of spinal transmitter systems undergo plastic changes following the development of a protracted pain state such as those produced by inflammation or peripheral nerve injury, with these changes often related to the nature of the condition (see Dickenson *et al*., 1997). A recent study has shown an upregulation in the expression of $P2X_3$ receptors in the dorsal root ganglia and spinal cord of rats with neuropathic injury induced by chronic constriction of the sciatic nerve (CCI model) (Novakovic *et al*., 1999), suggesting that ATP may play a role in the altered nociceptive processing seen in this condition. However, the lack of overall effect seen in the present study following the intrathecal administration of the P2X receptor antagonists suramin and PPADS in animals with peripheral neuropathy induced by the tight ligation of the L5–6 spinal nerves suggests that ATP acting at P2X receptors sensitive to these antagonists (including $P2X_3$ receptors) does not play an enhanced role in spinal nociceptive processing in neuropathic states, at least at this time point in this model. Indeed, Novakovic *et al*. (1999) found an upregulation in the levels of $P2X_3$ expression in the DRG following nerve injury regardless of whether the rats developed behavioural signs of neuropathy, suggesting the two events are not causally related.

In contrast, high spinal doses of the P2X receptor antagonists PPADS and suramin did produce inhibitions of the noxious evoked responses of dorsal horn neurones recorded in animals 3 h after the induction of carrageenan inflammation. This suggests that the spinal release of ATP may be increased following the development of peripheral inflammation leading to a P2X receptor mediated facilitation of the C-fibre evoked response. Although non-P2X receptor-mediated effects have been suggested to underlie some of the actions of suramin in some other studies (Driessen *et al*., 1994, and see Gu *et al*., 1998), both PPADS and suramin produced the same pattern of effect in the present study in the carrageenan animals, strongly implicating a block of P2X receptors in their actions. The initial response of the neurones

was most sensitive to inhibition by the P2X receptor antagonists, consistent with an action on primary afferent terminals where ATP may act to increase the release of glutamate as demonstrated by Gu & MacDermott (1997). The exact P2X receptor subtype underlying these actions cannot be determined from the present studies. However, suramin/PPADS sensitive $P2X_3$ receptors are known to be associated with the terminals of non-peptide C-fibres (Vulchanova *et al*., 1998), making these receptors a likely candidate.

Intrathecally administered suramin has been reported to produce antinociception in the formalin test, another model of inflammatory nociception, although here non-P2X receptor mediated effects may have contributed to the antinociceptive effects seen (Driessen *et al*., 1994). Another study has also shown an antinociceptive effect of suramin in the formalin test following local administration into both the ipsilateral and contralateral paw, indicating a systemic drug effect, suggesting that an increased central but not peripheral role of ATP contributes to inflammatory nociception in this model (Sawynok & Reid, 1997).

An increased spinal release of ATP following inflammation has yet to be demonstrated, but an altered balance of spinal transmitters could also account for the enhanced role of ATP in spinal nociceptive processing following the development of inflammation. ATP has been shown to be co-released with the inhibitory neurotransmitter GABA in the spinal cord, with the inhibitory actions of GABA predominating under normal conditions (Jo & Schlichter, 1999). It is possible that the balance switches in favour of the excitatory action of ATP following the development of inflammation.

In conclusion, to date there is no compelling *in vivo* evidence for a major role of ATP acting at spinal suramin/PPADS sensitive P2X receptors in the transmission or modulation of nociception under normal conditions or under conditions of peripheral nerve injury. However, as seen in *in vitro* studies, a small proportion of spinal nociceptive neurones are sensitive to these P2X receptor ligands, suggesting ATP may play a minor or specialized role in spinal nociceptive processing. There are enhanced, but relatively modest, actions of ATP acting at suramin/PPADS sensitive P2X receptors in nociceptive processing in the spinal cord following the development of a peripheral inflammatory state.

The relative balance between the peripheral and central roles of a number of receptors synthesized in the dorsal root ganglia of sensory neurones is variable. Opioid receptors have a mainly central function, whereas in the case of receptors for bradykinin and prostanoids, although having spinal effects, their excitatory role appears to be predominantly peripheral (Dray, 1997). What determines this balance remains to be established but the actions of ATP at P2X receptors would appear to fall into the latter category.

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