British Journal of Pharmacology (2005) 146, 180–188
www.nature.com/bjp

Endogenous opioid mechanisms partially mediate $P2X_3/P2X_{2/3}$-related antinociception in rat models of inflammatory and chemogenic pain but not neuropathic pain

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1 $P2X_3/P2X_{2/3}$ receptors have emerged as important components of nociception. However, there is limited information regarding the neurochemical systems that are affected by antagonism of the $P2X_3/P2X_{2/3}$ receptor and that ultimately contribute to the ensuing antinociception. In order to determine if the endogenous opioid system is involved in this antinociception, naloxone was administered just prior to the injection of a selective $P2X_3/P2X_{2/3}$ receptor antagonist, A-317491, in rat models of neuropathic, chemogenic, and inflammatory pain.

2 Naloxone (1–10 mg kg^{-1}, i.p.), dose-dependently reduced the antinociceptive effects of A-317491 (1–300 μmol kg^{-1}, s.c.) in the CFA model of thermal hyperalgesia and the formalin model of chemogenic pain (2nd phase), but not in the L5–L6 spinal nerve ligation model of neuropathic allodynia. In comparison experiments, the same doses of naloxone blocked or attenuated the actions of morphine (2 or 8 mg kg^{-1}, s.c.) in each of these behavioral models.

3 Injection of a peripheral opioid antagonist, naloxone methiodide (10 mg kg^{-1}, i.p.), did not affect A-317491-induced antinociception in the CFA and formalin assays, suggesting that the opioid component of this antinociception occurred within the CNS. Furthermore, this utilization of the central opioid system could be initiated by antagonism of spinal $P2X_3/P2X_{2/3}$ receptors since the antinociceptive actions of intrathecally delivered A-317491 (30 nmol) in the formalin model were reduced by both intrathecally (10–50 nmol) and systemically (10 mg kg^{-1}, i.p.) administered naloxone.

4 This utilization of the opioid system was not specific to A-317491 since suramin-, a nonselective P2X receptor antagonist, induced antinociception was also attenuated by naloxone.

5 In *in vitro* studies, A-317491 (3–100 μM) did not produce any agonist response at δ opioid receptors expressed in NG108-15 cells. A-317491 had been previously shown to be inactive at the κ and μ opioid receptors. Furthermore, naloxone, at concentrations up to 1 mM, did not compete for [^{3}H] A-317491 binding in 1321N1 cells expressing human $P2X_3$ receptors.

6 Taken together, these results indicate that antagonism of spinal $P2X_3/P2X_{2/3}$ receptors results in an indirect activation of the opioid system to alleviate inflammatory hyperalgesia and chemogenic nociception.

British Journal of Pharmacology (2005) **146,** 180–188. doi:10.1038/sj.bjp.0706346; published online 25 July 2005

Keywords: $P2X_3$; ATP; neuropathic; allodynia; hyperalgesia; inflammation; formalin; opioid

Abbreviations: ATP, adenosine 5′-triphosphate; CFA, complete Freund's adjuvant

Introduction

Adenosine 5′-triphosphate (ATP) is recognized as an important neurotransmitter in nociceptive transmission (Burnstock, 1996; Burnstock & Wood, 1996; Cook & McCleskey, 2002). ATP is a nonselective agonist for each of the seven ionotropic P2X purinoceptor subtypes currently identified (Ralevic & Burnstock, 1998; Bianchi *et al*., 1999; Jacobson *et al*., 2002). Additionally, several receptor subtypes in the metabotropic P2Y receptor family also respond to ATP (Abbracchio *et al*., 2003). While more than one of these P2 receptor subtypes are likely involved in the transmission or modulation of nociceptive signals (Collo *et al*., 1996; Vulchanova *et al*., 1996; 1997; Cockayne *et al*., 2000; Souslova *et al*., 2000; Okada *et al*., 2002; Yoshida *et al*., 2002; Tsuda *et al*., 2003; Wismer *et al*., 2003), the evidence supporting a role for $P2X_3/P2X_{2/3}$ receptors in nociception is perhaps the most compelling (North, 2004).

$P2X_3/P2X_{2/3}$ receptors are highly expressed in small diameter dorsal root ganglion neurons that relay nociceptive signals from the periphery to the spinal cord (Chen *et al*., 1995). Following an injury to the sciatic nerve, neuronal expression of the $P2X_3$ receptor is elevated in both the dorsal root ganglion and the ipsilateral spinal cord (Novakovic *et al*., 1999). Behaviorally, $P2X_3$ (−/−) gene-ablated mice show a significant attenuation in spontaneous pain behaviors following administration of ATP or formalin (Cockayne *et al*., 2000;

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Souslova *et al*., 2000), and animals treated with $P2X_3$ antisense oligonucleotides exhibit reduced thermal hyperalgesia and mechanical allodynia (Barclay *et al*., 2002; Honore *et al*., 2002). Furthermore, it has been recently reported that systemic and site-specific administration of A-317491, the first non-nucleotide antagonist that has high affinity and selectivity for blocking $P2X_3$ homomeric and $P2X_{2/3}$ heteromeric channels, is antinociceptive in rat models of chronic inflammatory and neuropathic pain (Jarvis *et al*., 2002; McGaraughty *et al*., 2003; Wu *et al*., 2004).

Antinociception induced by administration of a $P2X_3/P2X_{2/3}$ receptor antagonist likely does not utilize a single pathway to affect the various forms of pathological nociception. Indeed, it has been demonstrated that A-317491 acts through a different combination of sites to reduce either neuropathic or inflammatory pain (McGaraughty *et al*., 2003). Furthermore, it has been suggested that ATP-induced mechanical allodynia is mediated by $P2X_{2/3}$ receptors on capsaicin-insensitive neurons with slow-desensitizing ATP currents while thermal and nocifensive behaviors are mediated by $P2X_3$ receptors on capsaicin-sensitive neurons with fast-desensitizing ATP responses (Tsuda *et al*., 2000). The utilization of different pathways to affect the various forms of pathological pain likely results in pathway-specific interactions with endogenous neuropharmacological systems. While it is known that both peripheral and spinal application of ATP can trigger the release of glutamate into the spinal dorsal horn (Gu & MacDermott, 1997; Tsuda *et al*., 1999; Wismer *et al*., 2003), there is very limited information regarding the neurochemical systems that are affected by antagonism of the $P2X_3/P2X_{2/3}$ receptor and that ultimately contribute to the ensuing antinociception. Endogenous systems other than glutamate, such as substance P, nitric oxide, GABA, and opioids may also be involved in this $P2X_3/P2X_{2/3}$-related antinociception (Bland-Ward & Humphrey, 1997; Fukuhara *et al*., 2000; Hugel & Schlichter, 2000; Ueda *et al*., 2000; Nakatsuka *et al*., 2001). Thus, even though a clear role for $P2X_3/P2X_{2/3}$ receptors has been demonstrated in many models of pathological nociception, the downstream mechanisms that are recruited following administration of a selective $P2X_3/P2X_{2/3}$ antagonist are not well understood.

In an effort to further delineate the endogenous antinociceptive mechanisms that are activated following an antagonistic block of $P2X_3/P2X_{2/3}$ receptors, the opioid antagonist naloxone was administered prior to A-317491 in animal models of neuropathic, chemogenic, and inflammatory pain. Although A-317491 does not have any significant direct activity at the various opioid receptors (Jarvis *et al*., 2002), it has been demonstrated that the antinociceptive actions of some nonopioid analgesics can be at least partially mediated by an indirect utilization of the endogenous opioid system (Gouarderes *et al*., 1996; Schreiber *et al*., 2000; Tejwani & Rattan, 2000; Vanegas & Tortorici, 2002).

Methods

Animal preparation

All animal handling and experimental protocols were approved by Abbott's Institutional Animal Care and Use Committee (IACUC). Male Sprague–Dawley rats (260–350 g; Charles River, MA, U.S.A.) were used for all experiments and were housed in a temperature-controlled room with a 12/12-h day/night cycle. Food and water were available *ad libitum*.

Implantation of intrathecal catheters In some cases, animals were implanted with chronic indwelling catheters to permit the direct delivery of study compounds onto the spinal cord. Under halothane inhalation anesthesia, PE-5 catheters (external PE-10, Marsil Enterprises, CA, U.S.A.) were inserted through the cisternal membrane at the base of the skull down to the lumbar enlargement (8.5 cm). Rats were not tested for at least 7 days after surgery. Animals demonstrating motor dysfunction or dehydration at any point following surgery were immediately euthanized.

Behavioral models of pathological nociception

Complete Freund's adjuvant-induced chronic thermal hyperalgesia Chronic inflammatory hyperalgesia was induced by the injection of complete Freund's adjuvant (CFA, 50%, 150 μl) into the plantar surface of the rat's right hindpaw 48 h prior to testing. On the day of testing, animals were acclimatized for 30 min to Plexiglas holding chambers ($18 \times 29 \times 12.5\,cm^3$) that rested on a temperature-regulated (30°C) glass surface. Thermal nociceptive thresholds were determined according to the method described by Hargreaves *et al*. (1988). Briefly, a radiant heat source (8 V, 50 W projector bulb) was focused through the glass surface onto the plantar surface of the hindpaw. Upon paw withdrawal, the heat stimulus was automatically deactivated and the rat's latency to withdraw was recorded to the nearest 0.1 s. Each animal's latency score was an average of two trials, which were separated by at least 5 min. Both the injured and uninjured hindpaws were similarly tested. Withdrawal latencies after injection of vehicle into a hindpaw did not differ from latencies observed in uninjected animals (unpublished observations).

Mean withdrawal latencies were compared within groups (inflamed vs noninflamed paws) and between drug- and vehicle-injected groups. 'Reversal in hyperalgesia' scores for each animal were calculated by the following formula:

$$\frac{(\text{Latency inflamed paw}_{\text{drug}} - \text{Mean latency inflamed paw}_{\text{vehicle}})}{(\text{Mean latency non-inflamed paw}_{\text{vehicle}} - \text{Mean latency inflamed paw}_{\text{vehicle}})} \times 100$$

In cases of negative values, the scores were designated as 0 (no reversal in hyperalgesia).

L5–L6 ligation model of neuropathic allodynia Under halothane inhalation anesthesia, rats received a unilateral tight ligation of the L5 and L6 spinal nerves (Kim & Chung, 1992). Experiments were conducted 1–3 weeks after surgery. Tactile allodynia was determined by measuring paw withdrawal to a series of graded von Frey hair (Stoelting, Wood Dale, IL, U.S.A.) stimulations using the up–down method described by Dixon (1980). Briefly, rats were placed on an elevated mesh bottom floor with a $1.27 \times 1.27\,cm^2$ grid to provide access to the hindpaw plantar surface. An inverted, clear plastic cage ($29 \times 18 \times 12\,cm^3$) was placed over each rat. Each von Frey filament was presented perpendicularly to the hindpaw ipsilateral to the injury, and held in this position for approximately 8 s with enough force to cause a slight buckle

in the filament. Positive responses included sharp withdrawal or flinching behavior during stimulation. Each rat was tested in three sequential trials. Only those rats with a mean baseline threshold score of less than $4.5 \times g$ on the ipsilateral hindpaw were used in this study. Allodynic responses were not observed in any rat following stimulation of the hindpaw contralateral to the injury (maximum force tested was $15 \times g$).

Formalin model of chemogenic nociception Experimentally naïve animals were placed in individual mirrored (45°) Plexiglas cages ($26 \times 22 \times 16\,cm^3$) and allowed to acclimate to the testing environment for 15 min. Formalin (5% in 50 μl) was then injected into the dorsal surface of the right hind paw using a 29.5 gauge needle. The number of nocifensive events (paw flinching, licking, guarding) for each animal was recorded during 1-min periods with each period being separated by 5 min. Flinching behaviors were recorded only during the second phase of the formalin assay.

Drug administration procedures

Naloxone antagonism Three separate experiments were conducted to investigate the actions of naloxone to attenuate $P2X_3/P2X_{2/3}$ antagonist produced antinociception, and whether or not this effect was centrally or peripherally mediated. In one experiment, animals were pretreated with naloxone (1–10 mg kg^{-1}, i.p.) or vehicle 5 min prior to the systemic injection of A-317491 (10–300 μmol kg^{-1}, s.c.) for each of the three behavioral assays. For comparison, the same doses of naloxone were used to antagonize the antinociceptive effects of systemically administered morphine (2–8 mg kg^{-1}, s.c.) in the CFA, neuropathic, and formalin assays. Testing was started 30 min after administration of A-317941 or morphine. A similar administration paradigm was used to measure the effects of naloxone (10 mg kg^{-1}, i.p.) on the nonselective P2X antagonist suramin (40 mg kg^{-1}, s.c.) in the CFA model of thermal hyperalgesia. In another experiment, naloxone methiodide (10 mg kg^{-1}, i.p.) or vehicle was administered 5 min ahead of A-317491 (100 μmol kg^{-1}, s.c.) in the CFA and formalin assays. Finally, naloxone was injected intrathecally (10–50 nmol, 5 min pretreatment), or systemically (10 mg kg^{-1}, i.p., 30 min pretreatment) prior to the intrathecal injection of A-317491 (30 nmol) in the formalin assay. The volume of each intrathecal injection was 10 μl followed by a 10 μl sterile water flush. Testing commenced 5 min after the intrathecal injection of A-317491.

Preparation of compounds For systemic injections, A-317491 (synthesized at Abbott Laboratories, IL, U.S.A.), morphine, naloxone, and naloxone methiodide (all from Sigma-Aldrich, MO, U.S.A.) were dissolved in saline prior to administration. A solution of dimethylsulfoxide (10%), 2-hydroxypropyl-β-cyclodextrin (34%), and saline (at physiological pH) was used as the vehicle for the intrathecal delivery of A-317491, naloxone, and morphine. Suramin (Sigma-Aldrich) was dissolved in water for systemic administration.

Statistics Statistical significance on group means was measured by an ANOVA followed by a Fisher's PLSD *post hoc* analysis ($P<0.05$). Data are presented as mean $\pm$ s.e.m.

In vitro *assays*

The pharmacological selectivity of A-317491 (10 μM) had been previously evaluated by use of standardized assay protocols (Cerep, Celle l'Evescault, France) against each opioid receptor subtype and several other cell-surface receptors, ion channels, transport sites, and enzymes (Jarvis *et al*., 2002). Although A-317491 was inactive at the κ and μ receptors, 10 μM did produce a weak effect at the δ receptor (Jarvis *et al*., 2002). Therefore, a more detailed analysis of A-317491 was conducted to examine its actions at the δ opioid receptor. To this end, cytosolic Ca^{2+} concentrations were measured as described previously (Jarvis *et al*., 2002), to determine the effects of A-317491 on 1 μM [D-Pen(2), D-Pen(5)]-enkephalin (DPDPE)-induced activity in endogenously expressed δ receptors found on NG108-15 cells.

In order to determine if naloxone could prevent A-317491 binding to $P2X_3$ receptors, a binding assay was performed according to Jarvis *et al*. (2004) with 1321N1 cells expressing human $P2X_3$ receptors. Briefly, 3 nM of the [^{3}H] A-317491 radioligand and 20 μg membrane protein were added in each assay tube. A 10 mM stock of naloxone was prepared in dH_2O, and further diluted in dH_2O to obtain ($10\times$) concentrations. A total of 25 ml of the ($10\times$) solutions was added per assay tube. Final assay volume was 250 ml. Incubation time was 30 min at 4°C (on ice). Assay was terminated by rapid filtration over a Whatman GF/B glass fiber filter mat. For comparison, the effects of ATP on [^{3}H] A-317491 binding were also examined. A 10 mM stock solution of ATP was prepared in dH_2O, and diluted with additional dH_2O to obtain ($10\times$) solutions.

Results

Naloxone antagonism of A-317491-induced antinociception

In CFA animals, significant hyperalgesia ($P<0.01$) was observed on the inflamed but not the noninflamed hindpaw. Withdrawal latencies to thermal stimulation in vehicle-treated rats in the A-317491 experiments were 4.75 ± 0.15 s (inflamed paw) and 9.97 ± 0.28 s (noninflamed paw), and in the morphine experiments the latencies were 3.43 ± 0.36 s (inflamed paw) and 11.14 ± 0.45 s (noninflamed paw). Administration of 30 μmol kg^{-1} (s.c.) of A-317491 was antihyperalgesic (Figure 1a), increasing ($P<0.01$) withdrawal latencies in the inflamed hindpaw. Pretreatment with 10 mg kg^{-1} of naloxone significantly ($P<0.01$) attenuated the A-317491-induced antinociception (Figure 1a). Naloxone (5–10 mg kg^{-1}) also attenuated ($P<0.01$) the antihyperalgesic effects of morphine (2 mg kg^{-1}, s.c., Figure 1b). Withdrawal latencies were unaffected by the injection of naloxone alone (10 mg kg^{-1}, i.p., Figure 1a and b). Pretreatment with the high dose of naloxone (10 mg kg^{-1}, i.p.), also reduced the dose-related efficacy of A-317491 (10–300 μmol kg^{-1}, s.c.) in the CFA assay (Figure 2).

In the second phase of the formalin assay, 70.05 ± 4.06 (A-317491 experiment) and 68.0 ± 3.87 (morphine experiment) instances of nocifensive behaviors were observed from vehicle-treated animals during the recording period. These events were not significantly affected by the administration of naloxone

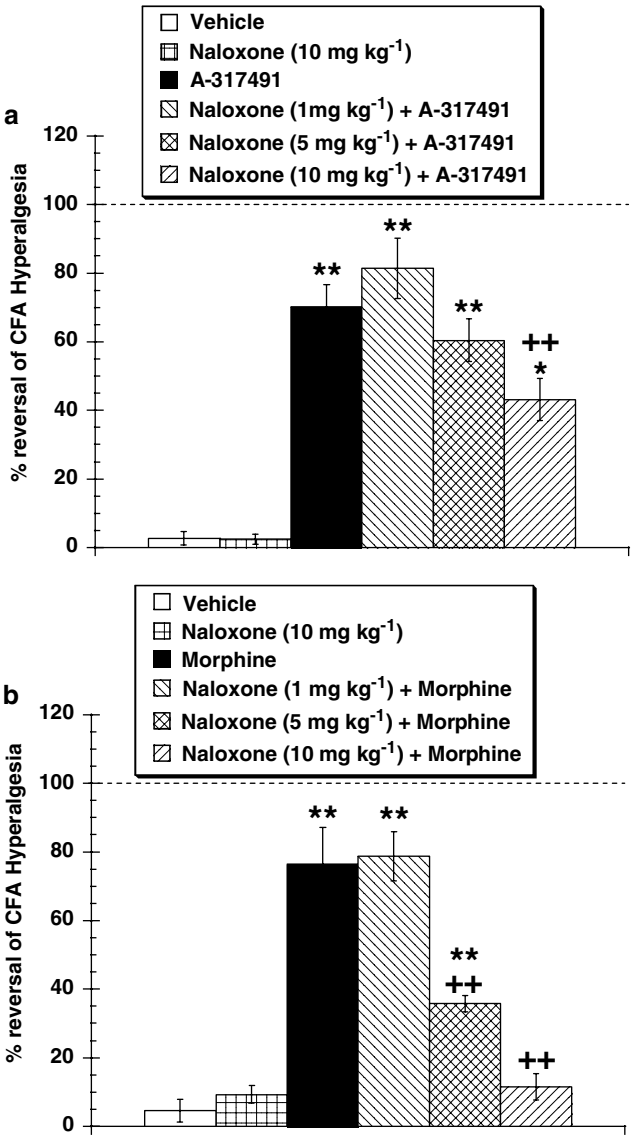

Figure 1 Naloxone (1–10 mg kg^{-1}, i.p.) attenuated, in a dose-related manner, the antihyperalgesic actions of (a) A-317491 (30 μmol kg^{-1}, s.c.) and (b) morphine (2 mg kg^{-1}, i.v.) in the CFA model of thermal hyperalgesia. Withdrawal latencies of the noninflamed hindpaws were unaffected by administration of morphine (11.04 $\pm$ 0.78 s) or A-317491 (10.21 $\pm$ 0.3 s) compared to the vehicle-treated groups (11.14 $\pm$ 0.45 and 9.97 $\pm$ 0.28 s, respectively); $n = 6$–7 per group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle group; $^{++}P < 0.01$ vs A-317491 or morphine group, respectively.

(10 mg kg^{-1}, i.p.), but were reduced ($P < 0.01$) by the injection of A-317491 (100 μmol kg^{-1}, s.c.) or morphine (2 mg kg^{-1}, s.c., Figure 3a and b). The antinociceptive actions of A-317491 and morphine were significantly attenuated by administration of 5 mg kg^{-1} ($P < 0.05$) and 10 mg kg^{-1} ($P < 0.01$) of naloxone.

In vehicle-treated neuropathic rats, the threshold for withdrawal from von Frey hair stimulation to the ipsilateral hindpaw was $2.46 \pm 0.2 \times g$ (A-317491 experiment) and $2.43 \pm 0.47 \times g$ (morphine experiment). Mechanical sensitivity was not affected by the injection of 10 mg kg^{-1}of naloxone (Figure 4a). Administration of A-317491 (100 μmol kg^{-1}, s.c.) raised the withdrawal threshold ($P < 0.05$) to $5.94 \pm 0.66 \times g$,

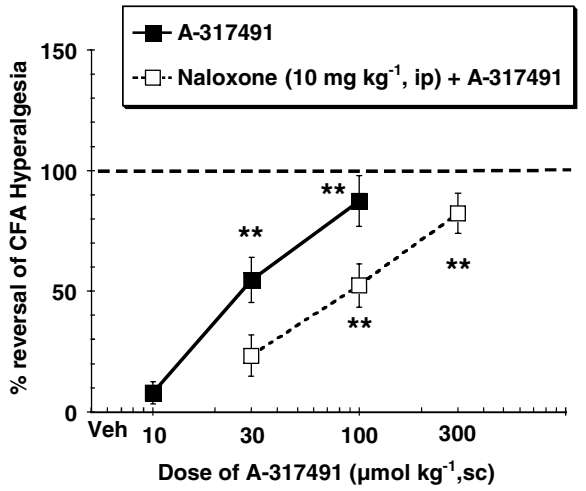

Figure 2 At 10 mg kg^{-1}, i.p. naloxone shifted the A-317491 (10–300 μmol kg^{-1}, s.c.) dose–response curve to the right, thereby decreasing the potency of A-317491 to reduce CFA-induced thermal hyperalgesia on the inflamed hindpaw. Compared to the vehicle control group (10.69 $\pm$ 0.31 s), the noninflamed hindpaw was unaffected by administration of any dose of A-317491 (range of 10.75 $\pm$ 0.6 to 10.76 $\pm$ 0.8 s) or by naloxone-A-317491 (range of 10.3 $\pm$ 0.14 to 11.21 $\pm$ 0.72 s); $n = 6$ per group, $^{**}P < 0.01$ vs vehicle group.

but naloxone pretreatment (10 mg kg^{-1}, i.p.) did not attenuate the antiallodynic action of A-317491 in the L5–L6 nerve ligation model of neuropathic pain (Figure 4a). In contrast, the potent action of morphine (8 mg kg^{-1}, s.c.) was completely blocked by preadministration of 10 mg kg^{-1}of naloxone (Figure 4b).

Contributions from central or peripheral opioids to A-317491-induced antinociception Systemic administration of naloxone methiodide (10 mg kg^{-1}, i.p.), which does not readily cross the blood–brain barrier, did not affect the antinociceptive actions of A-317491 (100 μmol kg^{-1}, s.c.) in either the CFA or formalin assays (Figure 5a). Doses equal to or lower than 10 mg kg^{-1} of naloxone methiodide have been shown to attenuate peripheral-opioid antinociception (Reichert *et al*., 2001; Furst *et al*., 2005). However, intrathecal (30 and 50 nmol, $P < 0.05$ and 0.01, respectively) and systemic (10 mg kg^{-1}, i.p., $P < 0.01$) injections of naloxone reduced the antinociceptive effects of intrathecally delivered A-317491 (30 nmol) on formalin-induced nocifensive behaviors (Figure 5b and c).

Naloxone antagonism of suramin-induced antinociception

In the CFA assay, the nonselective P2X antagonist, suramin (40 mg kg^{-1}, s.c.) significantly raised the withdrawal latencies of the injured paw from 5.28 $\pm$ 0.31 s (vehicle-treated rats) to 8.05 $\pm$ 0.63 s (Figure 6). Pretreatment with 10 mg kg^{-1} (i.p.) of naloxone significantly ($P < 0.01$) attenuated the antihyperalgesic action of suramin.

In vitro *assays*

A-317491 (10 μM) was previously demonstrated to be a highly selective compound with no significant activity at the μ and κ

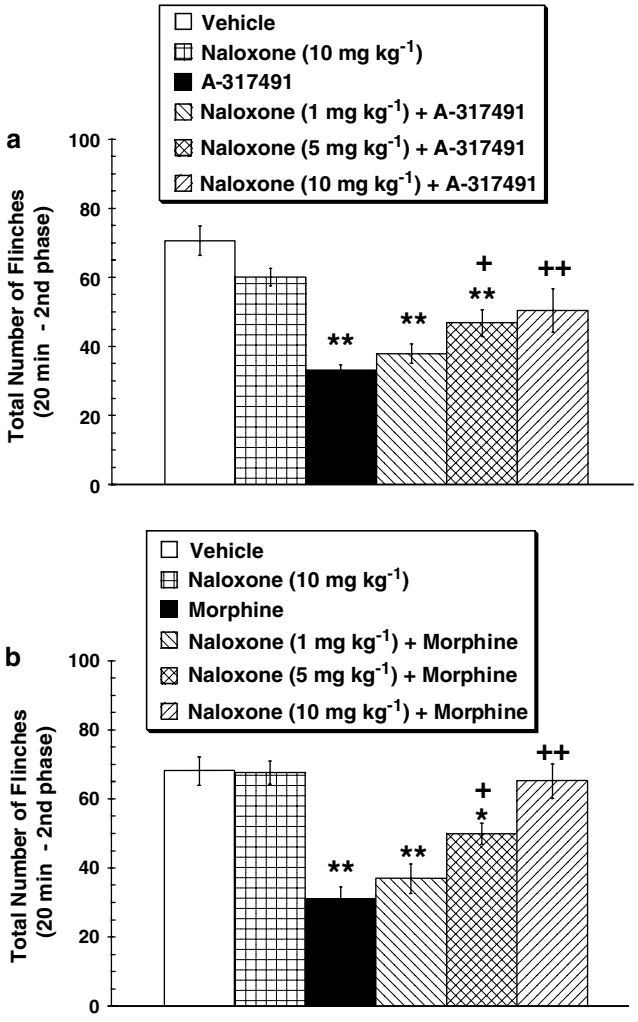

Figure 3 Naloxone (1–10 mg kg^{-1}, i.p.) reduced the antinociceptive actions of (a) A-317491 (100 μmol kg^{-1}, s.c.) and (b) morphine (2 mg kg^{-1}, s.c.) in the formalin model of chemogenic nociception; $n=4$–8 per group, $^{*}P<0.05$, $^{**}P<0.01$ vs vehicle group; $^{+}P<0.05$, $^{++}P<0.01$ vs A-317491 or morphine group, respectively.

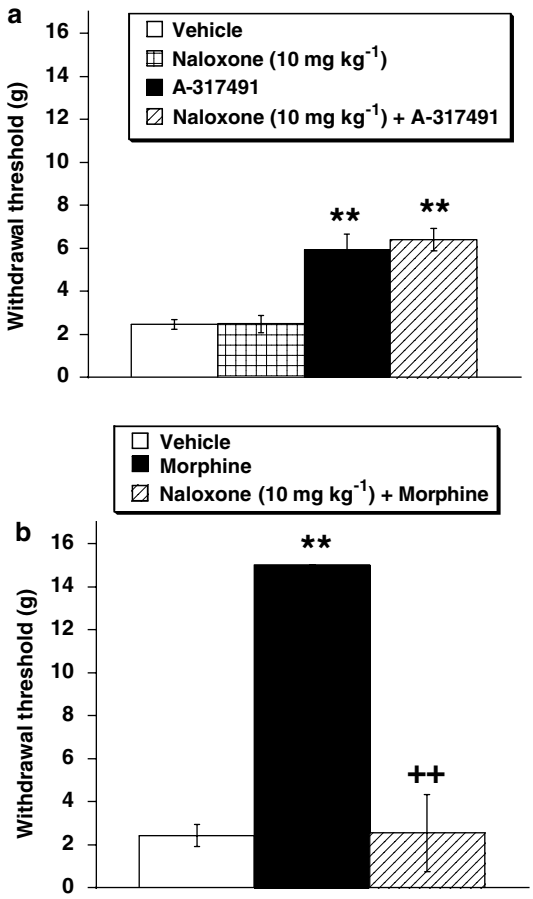

Figure 4 In (a), naloxone (10 mg kg^{-1}, i.p.) did not alter the antiallodynic action of A-317491 (100 μmol kg^{-1}, s.c.) in the L5–L6 ligation model of neuropathic pain. However, in (b) the same dose of naloxone (10 mg kg^{-1}, i.p.) blocked the action of 8 mg kg^{-1} (s.c.) of morphine. Mean withdrawal threshold of the contralateral 'uninjured' hindpaw was 13.76±0.34 g prior to administration of drugs. $n=4$–6 per group, $^{**}P<0.01$ vs vehicle group; $^{++}P<0.01$ vs morphine group.

opioid receptors, but weak activity was observed at the δ receptor (Jarvis *et al*., 2002). Subsequently, by measuring antagonism of DPDPE-evoked (1 μM) cytosolic Ca^{2+} concentrations in NG108-15 cells, the IC_{50} of A-317491 (3–100 μM) was determined to be 20 μM at the δ receptor (Figure 7b). Application of A-317491 at the doses tested did not evoke any agonist activity at the δ receptor (Figure 7a). Additionally, it was determined that naloxone (up to 1 mM) did not compete for specific binding with A-317491 (3 nM) to $P2X_3$ receptors (Figure 8). For comparison, ATP inhibited A-317491 binding with an IC_{50} of 22.7 nM (Figure 8).

Discussion

$P2X_3/P2X_{2/3}$ receptors have emerged as key players in ATP-induced nociception. These receptors are expressed in relevant sensory afferents (Chen *et al*., 1995) and are upregulated following a neuropathic or inflammatory injury (Novakovic *et al*., 1999; Xu & Huang, 2002). Furthermore, gene-ablation or treatment with $P2X_3$ antisense oligonucleotides results in antinociception (Cockayne *et al*., 2000; Souslova *et al*., 2000; Barclay *et al*., 2002; Honore *et al*., 2002). Administration of A-317491, the first potent and selective $P2X_3/P2X_{2/3}$ receptor antagonist, has revealed clear roles for both spinal and peripheral $P2X_3/P2X_{2/3}$ receptors in alleviating pathological nociception in a broad spectrum of animal pain models (Jarvis *et al*., 2002; McGaraughty *et al*., 2003). The present study demonstrates that the endogenous opioid system plays an important role in some forms of antinociception produced by injection of a $P2X_3/P2X_{2/3}$ receptor antagonist. Administration of naloxone, an opioid antagonist, attenuated A-317491-induced antinociception in the CFA and formalin models of pathological nociception. Thus, injection of a potent and selective $P2X_3/P2X_{2/3}$ receptor antagonist appears to utilize the endogenous opioid system to reduce both thermal hyperalgesia and chemogenic nociception.

This opioid-related effect of A-317491 was most likely indirect and due to an activation of 'downstream' opioid mechanisms since A-317491 has little to no direct activity at

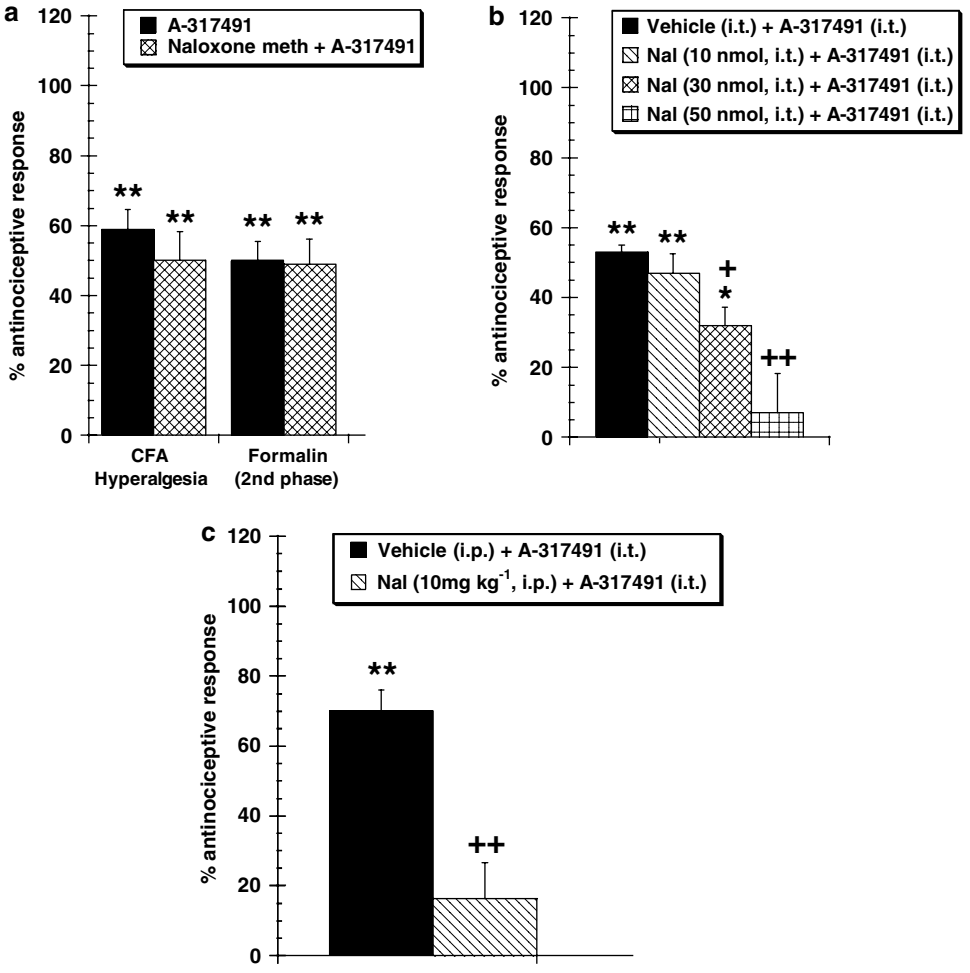

Figure 5 In (a), systemic administration of naloxone methiodide (10 mg kg^{-1}, i.p.), which does not readily cross the blood–brain barrier, did not affect the antinociceptive actions of A-317491 (100 μmol kg^{-1}, s.c.) in the CFA and formalin models, $n = 5$–6 per group. The effects of intrathecal A-317491 (30 nmol) were reduced by either (b) intrathecal (10–50 nmol) or (c) systemic (10 mg kg^{-1}, i.p.) delivery of naloxone in the formalin assay; $^{*}P<0.05$, $^{**}P<0.01$ vs vehicle group; $^{+}P<0.05$, $^{++}P<0.01$ vs respective A-317491 group.

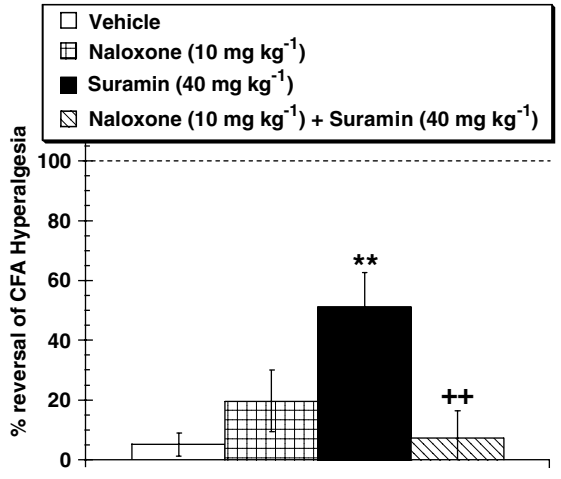

Figure 6 Naloxone (10 mg kg^{-1}, i.p.) reduced the antinociceptive activity of suramin (40 mg kg^{-1}, s.c.), a nonselective P2X receptor antagonist, in the CFA model of thermal hyperalgesia. Withdrawal latencies of the noninflamed hindpaws were unaffected by administration of suramin (10.2 $\pm$ 0.42 s) compared to the vehicle-treated group (10.74 $\pm$ 0.43 s); $n = 6$ per group, $^{**}P<0.01$ vs vehicle group; $^{++}P<0.01$ vs suramin group.

opioid receptors. It has been previously shown that A-317491 is inactive at μ and κ opioid receptors (Jarvis *et al*., 2002). In the current study, at concentrations up to 100 μM, A-317491 did not produce an agonist response at the δ opioid receptor. Although A-317491 was found to have weak antagonist ($IC_{50} = 20\,\mu$M) activity at the δ opioid receptor, it is unlikely that free plasma concentrations of A-317491 ever reached sufficient levels to affect the δ receptor. Systemic bioavailability of A-317491 is approximately 80%, but the compound is highly (>99%) protein bound (Jarvis *et al*., 2002). Thus, the estimated free plasma concentration following systemic injection of 100 μmol kg^{-1} of A-317491 is approximately 300 nM, which is approximately 10-fold less than that required to block δ receptors *in vitro*. Furthermore, the antinociceptive effect of suramin, a structurally dissimilar nonselective P2X receptor antagonist, was also reduced by naloxone.

The indirect utilization of the endogenous opioid system to attenuate nociception has been demonstrated following administration of several pharmacological agents including nonsteroidal anti-inflammatory drugs (NSAIDS), antidepressants, α_2-adrenoreceptor agonists, and neuropeptide FF agonists (Gouarderes *et al*., 1996; Schreiber *et al*., 2000; Tejwani & Rattan, 2000; Vanegas & Tortorici, 2002). Thus,

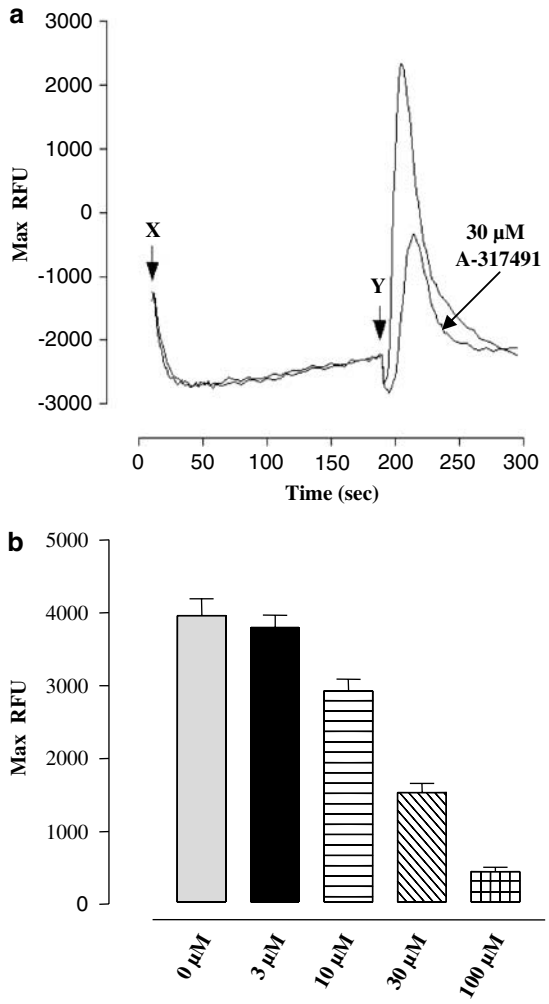

Figure 7 (a) Representative changes in intracellular calcium concentrations in NG108-15 cells by A-317491 and DPDPE. Following pipetting equilibration, application of 30 μM A-317491 (X) alone did not produce a significant increase in intracellular calcium concentrations. Similar application of 1 μM DPDPE (Y), in the absence of A-317491, resulted in a rapid increase in intracellular calcium concentrations (large peak). Application of 30 μM A-317491 significantly attenuated the amplitude of the DPDPE-evoked calcium response. (b) A-317491 produced a concentration-dependent block of 1 μM DPDPE-evoked increase in intracellular calcium concentrations ($IC_{50} = 20\,\mu$M).

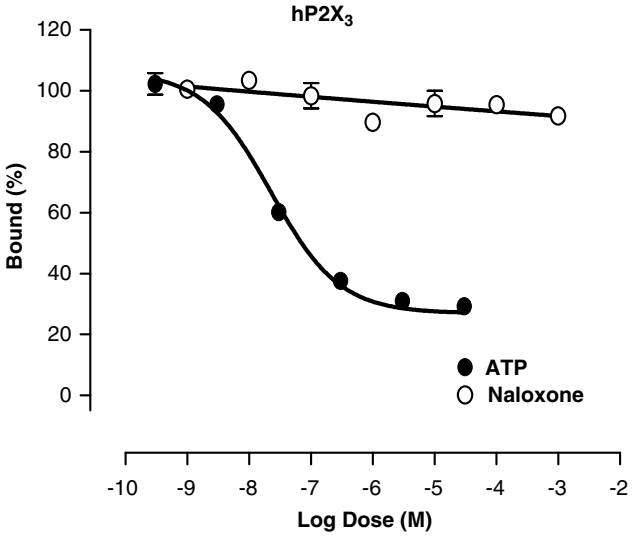

Figure 8 Representative concentration–effect curves for the ability of ATP and naloxone to compete for 3 nM [^{3}H] A-317491 binding to 1321N1 cells expressing human $P2X_3$ receptors. Naloxone, at concentrations up to 1 mM, did not compete for [^{3}H] A-317491 binding. In contrast, ATP inhibited 3 nM [^{3}H] A-317491 binding in a concentration-dependent fashion with an IC_{50} of 22.7 nM.

pharmacologically diverse compounds can indirectly tap into the advantageous actions of endogenous opioids. Along with the pharmacological diversity, there is likely a large diversity in mechanisms and regions that are used to trigger the opioid activity. Although the current experiments do not explain how antagonism of the $P2X_3/P2X_{2/3}$ receptors indirectly initiates opioid activity to decrease nociception, the mechanism may include enhancing the release of opioids from primary afferents or second-order spinal neurons (Zadina *et al*., 1999; Przewlocki & Przewlocka, 2001), or limiting the effects of antiopioid transmitters like CCK (Wiesenfeld-Hallin *et al*., 2002). Nevertheless, whatever mechanism triggered the activation of the opioid system, it did not result in tolerance to A-317491, since the antihyperalgesic effectiveness of A-317491 does not diminish with repeated dosing (Jarvis *et al*., 2002).

Inflammatory hyperalgesia can be reduced by endogenous opioids acting at both central and peripheral sites (Przewlocki & Przewlocka, 2001). However, the contributions from endogenous opioids following antagonism of $P2X_3/P2X_{2/3}$ receptors appear to be both initiated and mediated by central sites. Administration of 10 mg kg^{-1} of naloxone methiodide, which acts as a peripheral opioid antagonist when given systemically, did not attenuate the antinociceptive actions of systemic A-317491 in either the CFA or formalin models of pathological nociception suggesting that the opioid contribution to the antinociception was mediated by central mechanisms. Doses lower than or equal to 10 mg kg^{-1} of naloxone methiodide have been demonstrated to attenuate peripherally mediated opioid antinociception (Reichert *et al*., 2001; Furst *et al*., 2005). The opioid utilization could also be triggered by activity at central $P2X_3/P2X_{2/3}$ receptors, since the antinociceptive actions of intrathecally delivered A-317491 were attenuated by systemic administration of naloxone. A central interaction between these mechanisms was confirmed by the effectiveness of intrathecally administered naloxone to reverse the actions of intrathecal A-317941 in the formalin assay.

The most likely site of A-317491 action to induce the opioid effects is the superficial laminae of the spinal dorsal horn (Cook *et al*., 1997; Vulchanova *et al*., 1997). However, a recent paper has suggested that when administered systemically, A-317491 is a peripherally acting $P2X_3/P2X_{2/3}$ antagonist (Wu *et al*., 2004). The authors base this assertion upon a low plasma-to-brain ratio (0.00825) for A-317491 following subcutaneous injections. Despite this very low ratio, the authors also measured 242.9 ± 3.2 ng g^{-1} tissue of A-317491 (430 nM) in the brain following a 10 mg kg^{-1} dosing (about 16 μmol kg^{-1}). Considering that the K_i value for A-317491 at the rat $P2X_3$ receptor is 22 ± 8 nM (Jarvis *et al*., 2002), the levels of A-317491 entering the central nervous system following systemic administration, particularly at doses higher than 16 μmol kg^{-1},

should be sufficient to produce spinal-mediated antinociception and trigger opioid utilization.

In contrast to the antihyperalgesic effects in the CFA and formalin models, the antiallodynic actions of A-317491 in the L5–L6 ligation model of neuropathic pain were not attenuated by naloxone. This suggests that antagonism of $P2X_3/P2X_{2/3}$ receptors can reduce at least one form of pathological nociception without triggering endogenous opioid mechanisms. Interestingly, a similar profile exists for the indirect interaction between neuropeptide FF and endogenous opioids (Altier *et al*., 2000). Naloxone is able to block the antinociceptive effects of neuropeptide FF in models of inflammation but not neuropathy (Altier *et al*., 2000). This lack of opioid utilization by neuropeptide FF and A-317491 in models of neuropathic pain may be related to the decreased efficacy of spinal opioids following nerve injury (Ossipov *et al*., 1995), particularly when measuring tactile allodynia (Bian *et al*., 1995; Lee *et al*., 1995). Thus, $P2X_3/P2X_{2/3}$-mediated antinociception is not totally reliant on contributions from endogenous opioids. It is likely that both opioid-independent and opioid-dependent mechanisms were involved in reducing CFA and formalin-related nociception, since naloxone, at least at the doses tested, did not completely block the actions of A-317491.

In conclusion, antagonism of $P2X_3/P2X_{2/3}$ receptors results in an indirect utilization of the opioid system to alleviate pathological nociception in animal models of inflammatory hyperalgesia and chemogenic pain, but not in a model of neuropathic allodynia. Moreover, our results suggest that this antinociception is triggered by direct antagonism of spinal $P2X_3/P2X_{2/3}$ receptors and is mediated by the spinal opioid system.

References

ABBRACCHIO, M.P., BOEYNAEMS, J.M., BARNARD, E.A., BOYER, J.L., KENNEDY, C., MIRAS-PORTUGAL, M.T., KING, B.F., GACHET, C., JACOBSON, K.A., WEISMAN, G.A. & BURNSTOCK, G. (2003). Characterization of the UDP-glucose receptor (re-named here the P2Y(14) receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol. Sci.,* **24,** 52–55.

ALTIER, N., DRAY, A., MENARD, D. & HENRY, J.L. (2000). Neuropeptide FF attenuates allodynia in models of chronic inflammation and neuropathy following intrathecal or intracerebroventricular administration. *Eur. J. Pharmacol.,* **407,** 245–255.

BARCLAY, J., PATEL, S., DORN, G., WOTHERSPOON, G., MOFFATT, S., EUNSON, L., ABDEL'AL, S., NATT, F., HALL, J., WINTER, J., BEVAN, S., WISHART, W., FOX, A. & GANJU, P. (2002). Functional downregulation of P2X3 receptor subunit in rat sensory neurons reveals a significant role in chronic neuropathic and inflammatory pain. *J. Neurosci.,* **22,** 8139–8147.

BIAN, D., NICHOLS, M.L., OSSIPOV, M.H., LAI, J. & PORRECA, F. (1995). Characterization of the antiallodynic efficacy of morphine in a model of neuropathic pain in rats. *Neuroreport,* **6,** 1981–1984.

BIANCHI, B.R., LYNCH, K.J., TOUMA, E., NIFORATOS, W., BURGARD, E.C., ALEXANDER, K.M., PARK, H.S., YU, H., METZGER, R., KOWALUK, E., JARVIS, M.F. & VAN BIESEN, T. (1999). Pharmacological characterization of recombinant human and rat P2X receptor subtypes. *Eur. J. Pharmacol.,* **376,** 127–138.

BLAND-WARD, P.A. & HUMPHREY, P.P. (1997). Acute nociception mediated by hindpaw P2X receptor activation in the rat. *Br. J. Pharmacol.,* **122,** 365–371.

BURNSTOCK, G. (1996). A unifying purinergic hypothesis for the initiation of pain. *Lancet,* **347,** 1604–1605.

BURNSTOCK, G. & WOOD, J.N. (1996). Purinergic receptors: their role in nociception and primary afferent neurotransmission. *Curr. Opin. Neurobiol.,* **6,** 526–532.

CHEN, C.C., AKOPIAN, A.N., SIVILOTTI, L., COLQUHOUN, D., BURNSTOCK, G. & WOOD, J.N. (1995). A P2X purinoceptor expressed by a subset of sensory neurons. *Nature,* **377,** 428–431.

COCKAYNE, D.A., HAMILTON, S.G., ZHU, Q.M., DUNN, P.M., ZHONG, Y., NOVAKOVIC, S., MALMBERG, A.B., CAIN, G., BERSON, A., KASSOTAKIS, L., HEDLEY, L., LACHNIT, W.G., BURNSTOCK, G., MCMAHON, S.B. & FORD, A.P. (2000). Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature,* **407,** 1011–1015.

COLLO, G., NORTH, R.A., KAWASHIMA, E., MERLO-PICH, E., NEIDHART, S., SURPRENANT, A. & BUELL, G. (1996). Cloning of P2X5 and P2X6 receptors and the distribution and properties of an extended family of ATP-gated ion channels. *J. Neurosci.,* **16,** 2495–2507.

COOK, S.P. & MCCLESKEY, E.W. (2002). Cell damage excites nociceptors through release of cytosolic ATP. *Pain,* **95,** 41–47.

COOK, S.P., VULCHANOVA, L., HARGREAVES, K.M., ELDE, R. & MCCLESKEY, E.W. (1997). Distinct ATP receptors on pain-sensing and stretch-sensing neurons. *Nature,* **387,** 505–508.

DIXON, W.J. (1980). Efficient analysis of experimental observations. *Annu. Rev. Pharmacol. Toxicol.,* **20,** 441–462.

FUKUHARA, N., IMAI, Y., SAKAKIBARA, A., MORITA, K., KITAYAMA, S., TANNE, K. & DOHI, T. (2000). Regulation of the development of allodynia by intrathecally administered P2 purinoceptor agonists and antagonists in mice. *Neurosci. Lett.,* **292,** 25–28.

FURST, S., RIBA, P., FRIEDMANN, T., TIMAR, J., AL-KHRASANI, M., OBARA, I., MAKUCH, W., SPETEA, M., SCHUTZ, J., PRZEWLOCKI, R., PRZEWLOCKA, B. & SCHMIDHAMMER, H. (2005). Peripheral *versus* central antinociceptive actions of 6-amino acid-substituted derivatives of 14-*O*-methyloxymorphone in acute and inflammatory pain in the rat. *J. Pharmacol. Exp. Ther.,* **312,** 609–618.

GOUARDERES, C., JHAMANDAS, K., SUTAK, M. & ZAJAC, J.M. (1996). Role of opioid receptors in the spinal antinociceptive effects of neuropeptide FF analogues. *Br. J. Pharmacol.,* **117,** 493–501.

GU, J.G. & MACDERMOTT, A.B. (1997). Activation of ATP P2X receptors elicits glutamate release from sensory neuron synapses. *Nature,* **389,** 749–753.

HARGREAVES, K., DUBNER, R., BROWN, F., FLORES, C. & JORIS, J. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain,* **32,** 77–88.

HONORE, P., KAGE, K., MIKUSA, J., WATT, A.T., JOHNSTON, J.F., WYATT, J.R., FALTYNEK, C.R., JARVIS, M.F. & LYNCH, K. (2002). Analgesic profile of intrathecal $P2X_3$ antisense oligonucleotide treatment in chronic inflammatory and neuropathic pain states in rats. *Pain,* **99,** 11–19.

HUGEL, S. & SCHLICHTER, R. (2000). Presynaptic P2X receptors facilitate inhibitory GABAergic transmission between cultured rat spinal cord dorsal horn neurons. *J. Neurosci.,* **20,** 2121–2130.

JACOBSON, K.A., JARVIS, M.F. & WILLIAMS, M. (2002). Perspective: purine and pyrimidine (P2) receptors as drug targets. *J. Med. Chem.,* **45,** 4057–4093.

JARVIS, M.F., BIANCHI, B., UCHIC, J.T., CARTMELL, J., LEE, C.H., WILLIAMS, M. & FALTYNEK, C. (2004). [^{3}H]A-317491, a novel high-affinity non-nucleotide antagonist that specifically labels human P2X2/3 and P2X3 receptors. *J. Pharmacol. Exp. Ther.,* **310,** 407–416.

JARVIS, M.F., BURGARD, E.C., MCGARAUGHTY, S., HONORE, P., LYNCH, K., BRENNAN, T.J., SUBIETA, A., VAN BIESEN, T., CARTMELL, J., BIANCHI, B., NIFORATOS, W., KAGE, K., YU, H., MIKUSA, J., WISMER, C.T., ZHU, C.Z., CHU, K., LEE, C.H., STEWART, A.O., POLAKOWSKI, J., COX, B.F., KOWALUK, E., WILLIAMS, M., SULLIVAN, J. & FALTYNEK, C. (2002). A-317491, a novel potent and selective non-nucleotide antagonist of P2X3 and P2X2/3 receptors, reduces chronic inflammatory and neuropathic pain in the rat. *Proc. Natl. Acad. Sci. U.S.A.,* **99,** 17179–17184.

KIM, S.H. & CHUNG, J.M. (1992). An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain,* **50,** 355–363.

LEE, Y.W., CHAPLAN, S.R. & YAKSH, T.L. (1995). Systemic and supraspinal, but not spinal, opiates suppress allodynia in a rat neuropathic pain model. *Neurosci. Lett.,* **199,** 111–114.

MCGARAUGHTY, S., WISMER, C.T., ZHU, C.Z., MIKUSA, J., HONORE, P., CHU, K.L., LEE, C.H., FALTYNEK, C.R. & JARVIS, M.F. (2003). Effects of A-317491, a novel and selective P2X3/P2X2/3 receptor antagonist, on neuropathic, inflammatory and chemogenic nociception following intrathecal and intraplantar administration. *Br. J. Pharmacol.,* **140,** 1381–1388.

NAKATSUKA, T., MENA, N., LING, J. & GU, J.G. (2001). Depletion of substance P from rat primary sensory neurons by ATP, an implication of P2X receptor-mediated release of substance P. *Neuroscience,* **107,** 293–300.

NORTH, R.A. (2004). P2X3 receptors and peripheral pain mechanisms. *J. Physiol.,* **554,** 301–308.

NOVAKOVIC, S.D., KASSOTAKIS, L.C., OGLESBY, I.B., SMITH, J.A., EGLEN, R.M., FORD, A.P. & HUNTER, J.C. (1999). Immunocytochemical localization of P2X3 purinoceptors in sensory neurons in naive rats and following neuropathic injury. *Pain,* **80,** 273–282.

OKADA, M., NAKAGA, W.A.T., MINAMI, M. & SATOH, M. (2002). Analgesic effects of intrathecal administration of P2Y nucleotide receptor agonists UTP and UDP in normal and neuropathic pain model rats. *J. Pharmacol. Exp. Ther.,* **303,** 66–73.

OSSIPOV, M.H., LOPEZ, Y., NICHOLS, M.L., BIAN, D. & PORRECA, F. (1995). The loss of antinociceptive efficacy of spinal morphine in rats with nerve ligation injury is prevented by reducing spinal afferent drive. *Neurosci. Lett.,* **199,** 87–90.

PRZEWLOCKI, R. & PRZEWLOCKA, B. (2001). Opioids in chronic pain. *Eur. J. Pharmacol.,* **429,** 79–91.

RALEVIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.,* **50,** 413–492.

REICHERT, J.A., DAUGHTERS, R.S., RIVARD, R. & SIMONE, D.A. (2001). Peripheral and preemptive opioid antinociception in a mouse visceral pain model. *Pain,* **89,** 221–227.

SCHREIBER, S., BACKER, M.M., HERMAN, I., SHAMIR, D., BONIEL, T. & PICK, C.G. (2000). The antinociceptive effect of trazodone in mice is mediated through both mu-opioid and serotonergic mechanisms. *Behav. Brain Res.,* **114,** 51–56.

SOUSLOVA, V., CESARE, P., DING, Y., AKOPIAN, A.N., STANFA, L., SUZUKI, R., CARPENTER, K., DICKENSON, A.H., BOYCE, S., HILL, R., NEBENUIS-OOSTHUIZEN, D., SMITH, A.J., KIDD, E.J. & WOOD, J.N. (2000). Warm-coding deficits and aberrant inflammatory pain in mice lacking P2X3 receptors. *Nature,* **407,** 1015–1017.

TEJWANI, G.A. & RATTAN, A.K. (2000). Antagonism of antinociception produced by intrathecal clonidine by ketorolac in the rat: the role of the opioid system. *Anesth. Analg.,* **90,** 1152–1156.

TSUDA, M., KOIZUMI, S., KITA, A., SHIGEMOTO, Y., UENO, S. & INOUE, K. (2000). Mechanical allodynia caused by intraplantar injection of P2X receptor agonist in rats: involvement of heteromeric P2X2/3 receptor signaling in capsaicin-insensitive primary afferent neurons. *J. Neurosci.,* **20,** RC90.

TSUDA, M., SHIGEMOTO-MOGAMI, Y., KOIZUMI, S., MIZOKOSHI, A., KOHSAKA, S., SALTER, M.W. & INOUE, K. (2003). P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature,* **424,** 778–783.

TSUDA, M., UENO, S. & INOUE, K. (1999). *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. *Br. J. Pharmacol.,* **127,** 449–456.

UEDA, H., MATSUNAGA, S., INOUE, M., YAMAMOTO, Y. & HAZATO, T. (2000). Complete inhibition of purinoceptor agonist-induced nociception by spinorphin, but not by morphine. *Peptides,* **21,** 1215–1221.

VANEGAS, H. & TORTORICI, V. (2002). Opioidergic effects of nonopioid analgesics on the central nervous system. *Cell Mol. Neurobiol.,* **22,** 655–661.

VULCHANOVA, L., ARVIDSSON, U., RIEDL, M., WANG, J., BUELL, G., SURPRENANT, A., NORTH, R.A. & ELDE, R. (1996). Differential distribution of two ATP-gated channels (P2X receptors) determined by immunocytochemistry. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 8063–8067.

VULCHANOVA, L., RIED, M.S., SHUSTER, S.J., BUELL, G., SURPRENANT, A., NORTH, R.A. & ELDE, R. (1997). Immunohistochemical study of the P2X2 and P2X3 receptor subunits in rat and monkey sensory neurons and their central terminals. *Neuropharmacology,* **36,** 1229–1242.

WIESENFELD-HALLIN, Z., XU, X.J. & HOKFELT, T. (2002). The role of spinal cholecystokinin in chronic pain states. *Pharmacol. Toxicol.,* **91,** 398–403.

WISMER, C.T., FALTYNEK, C.R., JARVIS, M.F. & MCGARAUGHTY, S. (2003). Distinct neurochemical mechanisms are activated following administration of different P2X receptor agonists into the hindpaw of a rat. *Brain. Res.,* **965,** 187–193.

WU, G., WHITESIDE, G.T., LEE, G., NOLAN, S., NIOSI, M., PEARSON, M.S. & ILYIN, V.I. (2004). A-317491, a selective P2X(3)/P2X(2/3) receptor antagonist, reverses inflammatory mechanical hyperalgesia through action at peripheral receptors in rats. *Eur. J. Pharmacol.,* **504,** 45–53.

XU, G.Y. & HUANG, L.Y. (2002). Peripheral inflammation sensitizes P2X receptor-mediated responses in rat dorsal root ganglion neurons. *J. Neurosci.,* **22,** 93–102.

YOSHIDA, K., NAKAGAWA, T., KANEKO, S., AKAIKE, A. & SATOH, M. (2002). Adenosine 5′-triphosphate inhibits slow depolarization induced by repetitive dorsal root stimulation *via* P2Y purinoceptors in substantia gelatinosa neurons of the adult rat spinal cord slices with the dorsal root attached. *Neurosci. Lett.,* **320,** 121–124.

ZADINA, J.E., MARTIN-SCHILD, S., GERALL, A.A., KASTIN, A.J., HACKLER, L., GE, L.J. & ZHANG, X. (1999). Endomorphins: novel endogenous mu-opiate receptor agonists in regions of high mu-opiate receptor density. *Ann. NY Acad. Sci.,* **897,** 136–144.

(Received March 26, 2005
Revised May 4, 2005
Accepted June 22, 2005
Published online 25 July 2005)