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A-317491, a selective $P2X_3/P2X_{2/3}$ receptor antagonist, reverses inflammatory mechanical hyperalgesia through action at peripheral receptors in rats

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Abstract

The effect of A-317491 (5-({(3-Phenoxybenzyl)[(1*S*)-1,2,3,4-tetrahydro-1-naphthalenyl]amino}carbonyl)-1,2,4-benzenetricarboxylic acid), a recently described selective $P2X_3$ and $P2X_{2/3}$ receptor antagonist, on inflammatory mechanical hyperalgesia was examined. In the rat Freund's complete adjuvant model of inflammatory pain, s.c. administration of A-317491 dose-dependently reversed mechanical hyperalgesia. Maximum percent reversal (72%) was seen 3 h after administration at 10 mg/kg. Substantial plasma concentrations were measured for A-317491 after s.c. dosing 3, 10 and 30 mg/kg. However, the brain-to-plasma concentration ratio, determined 1 h after a 10 mg/kg s.c. dose, indicated limited penetration of A-317491 into the central nervous system. As revealed by neural activity recorded from single C-fiber nociceptive afferent in a Freund's complete adjuvant-inflamed rat skin-nerve preparation, topical application of A-317491 completely blocked afferent activation and mechanical sensitization induced by α,β-methylene ATP, a P2X agonist. These results suggest that A-317491 is a peripherally acting P2X blocker. Its efficacy demonstrates the importance of peripheral $P2X_3/P2X_{2/3}$ receptors in mediating ATP-associated mechanical hyperalgesia following inflammation, confirming previous suggestions of a significant role for $P2X_{2/3}$.

Keywords: P2X receptors; Pain; Hyperalgesia; Inflammation; Peripheral sensitization; Mechanosensitivity

1. Introduction

Extracellular ATP may function as a peripheral pain mediator. For instance, it is known that ATP and its analog α,β-methylene ATP (α,β-meATP) are algesic when administered at high concentrations into the skin of animals and humans (Hamilton et al., 1999; Hamilton and McMahon, 2000; Hilliges et al., 2002; Inoue et al., 2003; Tsuda et al., 2000). A much lower dose is required for such algesic action following inflammation (Hamilton et al., 1999), demonstrating a potentiation of the response under pathological conditions. Electrophysiological studies have shown that ATP and α,β-meATP directly excite a subpopulation of nociceptive sensory neurons (Dowd et al., 1998; Hamilton et al., 2001; Hilliges et al., 2002) and again excitation is potentiated following inflammation (Dowd et al., 1998; Hamilton et al., 2001; Xu and Huang, 2002). P2X receptors belong to a family of nonselective cation channels gated by extracellular ATP. Histological and pharmacological studies have suggested that ATP associated pain and pain modulation are mediated mainly via $P2X_3$ and $P2X_{2/3}$ receptors (Bradbury et al., 1998; Burgard et al., 1999; Cook et al., 1997; Grubb and Evans, 1999; Lewis et al., 1995; Virginio et al., 1998). Additionally, it has been found that reducing $P2X_3$ gene expression using the antisense oligonucleotides or siRNA reduces α,β-meATP-induced nociception (Dorn et al., 2004; Inoue et al., 2003). Furthermore, the electrophysiological and behavioral ATP responses are markedly

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reduced in $P2X_3$ receptor knockout mice (Cockayne et al., 2000; Souslova et al., 2000; Zhong et al., 2001). However, an unexpected and seemingly paradoxical enhancement of inflammation-induced hyperalgesic response has also been reported in $P2X_3$-null mice (Souslova et al., 2000).

Extracellular ATP may be involved particularly in mechanically associated pain under pathological conditions. It is known that ATP can be released from the nerve terminals and/or surrounding cells during noxious mechanical stimulation. As mentioned above, released ATP activates nociceptor terminals via P2X receptors to induce pain. This mechanochemical process may be enhanced under pathological conditions, which could contribute to mechanical hyperalgesia. In favor of this hypothesis, it has been shown in visceral organs like the bladder that ATP is released following stretching and excites afferent nerves (Ferguson et al., 1997). This process is enhanced during inflammation (Sun and Chai, 2004). In the bladder of $P2X_3$ knockout animals, activation of nociceptor terminals by ATP upon mechanical stimulation is substantially impaired, suggesting that this subtype is critical in mediating mechanical activation of afferent nerves (Cockayne et al., 2000; Rong et al., 2002; Vlaskovska et al., 2001). Recently, contribution of extracellular ATP to mechanical hyperalgesia via P2X receptors has been suggested to exist in the skin following inflammation (Dai et al., 2004). By using pERK (phosphorylation of extracellular signal-regulated protein kinase) immunoreactivity as a marker indicating neuronal activation, Dai et al. found that noxious mechanical stimulation activated more sensory neurons in inflamed skin and TNP-ATP (2′ -(or 3′)-*O*-(trinitrophenyl)adenosine 5′ -triphosphate), a nonselective $P2X_3$ and $P2X_{2/3}$ receptor antagonist, significantly blocked the activation and mechanical hyperalgesia.

A-317491 (5-({(3-Phenoxybenzyl)[(1*S*)-1,2,3,4-tetrahydro-1-naphthalenyl]amino}-carbonyl)-1,2,4-benzenetricarboxylic acid) is a recently described pharmacological tool for selective block of $P2X_3$/$P2X_{2/3}$ receptors (Jarvis et al., 2002). A-317491 is selective over other P2 receptors as well as other ion channels and receptors (Jarvis et al., 2002). A-317491 is effective in reducing pain associated behavior in several animal models of inflammatory and neuropathic pain when administered systemically (Jarvis et al., 2002). However, its effect on inflammatory mechanical hyperalgesia has not been previously reported. Here, we have examined this effect using the rat Freund's complete adjuvant model of inflammatory pain in order to further explore the role of $P2X_3$/$P2X_{2/3}$ receptors in mechanically associated pain. In addition, with limited pharmacokinetic data available in the literature for A-317491 we sought to further examine its pharmacokinetic profile including the blood–brain barrier permeability in the present study. Excitation of nociceptor terminals by ATP and its analog has been well characterized previously using a rat skin-nerve preparation under normal and inflammatory conditions (Hamilton et al., 2001). It is found that the excitation is enhanced following inflammation (Hamilton et al., 2001). However, to date, no data have been reported on modulation of the mechanical sensitivity of nociceptor terminals by α,β-meATP in this preparation. Thus, we have studied modulation of nociceptive afferents by α,β-meATP and the effect of A-317491 in this preparation for a better understanding of the underlying neural mechanisms.

2. Materials and methods

2.1. Animals

Male adult Sprague–Dawley rats (Taconic Farms, Germantown, NY) were used. Animals were housed in groups of three in plastic cages with soft bedding under a reversed light/dark cycle of 12:12 h. The Purdue Institutional Animal Care and Use Committee approved all animal procedures according to the guidelines of the Office of Laboratory Animal Welfare.

2.2. Drugs

A-317491 was synthesized at Purdue Pharma (6 Cedar Brook Drive, Cranbury, NJ). For behavioral and pharmacokinetic studies, A-317491 was freshly prepared with 0.01M PBS (phosphate buffer solution, Sigma) unless stated otherwise. For the electrophysiological studies, A-317491 was dissolved in DMSO (dimethyl sulfoxide) as a stock solution (100 mM), and α,β-meATP (Sigma) was dissolved in carbogen (95% O_2, 5% CO_2)-saturated synthetic interstitial fluid (SIF) as a stock solution (40 mM). Aliquots of the stock solutions were stored at −20 °C. On the day of use, they were thawed and kept at 4 °C. Working solutions were made freshly by diluting in carbogen-saturated SIF. Celecoxib (Toronto Research Chemicals, Toronto, Canada) was dissolved in distilled water.

2.3. Freund's complete adjuvant model of inflammatory mechanical hyperalgesia

The Freund's complete adjuvant model of inflammation was used. Under anesthesia with isofluorane (2% in O_2), the rats received an intraplantar injection of 50% Freund's complete adjuvant (50 μl, diluted in saline) to the left hind paw.

Hind paw withdrawal thresholds to a noxious mechanical stimulus were determined using an analygesymeter (model 7200; Ugo Basile, Varese, Italy). Cut-off was set at 250 g and the endpoint was taken as complete paw withdrawal. Paw withdrawal threshold was determined once for each rat at each time point. Baseline paw withdrawal threshold was determined. Twenty-four hours following Freund's complete adjuvant injection, pre-drug paw withdrawal threshold was measured and the rats received a single s.c. dose of A-317491, vehicle (PBS), or a single PO dose of 30 mg/kg

celecoxib (positive control) (n=10–30/group). Paw withdrawal threshold was again determined 1, 3, 5 and 24 h post drug administration. Percent reversal of hyperalgesia for each rat was calculated according to the following equation;

$$\%\,reversal = \frac{\text{post dose threshold} - \text{pre dose threshold}}{\text{base line threshold} - \text{pre dose threshold}}.$$

2.4. Pharmacokinetic studies

Rats pre-implanted with a jugular cannula were fasted overnight and dosed with A-317491 at 3, 10 and 30 mg/kg s.c. (n=3). An additional group of rats (n=3) dosed i.v. at 3 mg/kg using 25% cyclodextran as vehicle allowed the determination of plasma half-life, clearance and volume of distribution for A-317491. Blood was collected pre-dose and 1, 3 and 5 h post-dose. One milliliter of blood was drawn at each time point and replaced with 1 ml of donor blood to prevent any physiological effects associated with volume depletion. The collection tubes contained EDTA as an anticoagulant. Plasma was separated by centrifugation and stored at −20 °C prior to analysis. A-317491 was extracted from the plasma samples via acetonitrile precipitation and centrifugation at 4700×g for 30 min at 4 °C. The supernatant was analyzed on a MicroMass Quattro II triple quadrupole mass spectrometer (Manchester, UK). To study the CNS (central nervous system) permeability, one group of rats (n=3) were administered a single 10 mg/kg s.c. dose of A-317491. Blood and brains were taken 1 h after drug administration and analyzed by separate bioanalytical methods. Brains were diluted ten-fold with water and homogenized on ice for 2 min. The brain homogenate was extracted with 2 ml ethyl acetate followed by centrifugation at 4700×g for 30 min. The supernatant was evaporated under a stream of nitrogen and reconstituted with 100 ml acetonitrile. The plasma and brain samples were analyzed by high performance liquid chromatography on a Waters Alliance 2795 (Milford, MA), coupled with positive electrospray tandem mass spectrometry (MicroMass Quattro II) using a fast liquid chromatography gradient on a Phenomenex 30 mm Synergi Polar RP column (Torrance, CA). Multiple Reaction Monitoring mode was used to achieve selectivity and sensitivity, with limits of quantitation of 5 ng/ml and 50 ng/g for plasma and brain, respectively. A-317491 standard curves were fit to a 1/X weighted quadratic regression and showed linearity. The mean ratio of drug concentration in brain (ng/g of tissue) to plasma (ng/ml) was then calculated. All pharmacokinetic parameters were calculated using the NCA algorithm of Pharsight's WinNonlin Professional v. 3.0 software (Mountain View, CA).

2.5. Electrophysiological studies

Electrophysiological experiments were performed in rats 18 h following Freund's complete adjuvant injection. The time point chosen here was based on a previous report (Brederson et al., 2002) indicating that at this time the skin undergoes inflammation but its physical properties remain suitable for the experimental procedures described below.

For the electrophysiological experiments, a rat hairy skin-saphenous nerve preparation was used as previously described (Kress and Guenther, 1999; Reeh, 1986; Valenzano et al., 2003) with some modifications. Briefly, the rat was sacrificed by exposure to CO_2, and its left hindlimb was removed with a length of the saphenous nerve attached. The entire skin of the paw and lower leg was isolated in ice-cold, carbogen-saturated SIF. SIF consisted of (in mM) 107.7 NaCl, 3.5 KCl, 0.7 $MgSO_4$, 26.2 $NaHCO_3$, 1.7 NaH_2PO_4, 9.6 sodium gluconate, 5.6 glucose, 7.6 sucrose, and 1.5 $CaCl_2$. The skin-nerve preparation was then placed in an organ bath and superfused (15 ml/min) with the carbogen-saturated SIF at 32±0.5 °C.

Neural activity was recorded from functionally identified single C fibers by using teased-fiber recording techniques as previously described (Kress and Guenther, 1999; Reeh, 1986; Valenzano et al., 2003). The activity was differentially amplified (Neurolog, Digitimer) and digitized for real-time sorting of action potentials by template matching (DAPSYS, Johns Hopkins University). Fiber classification was made according to criteria reported previously (Kress and Guenther, 1999; Reeh, 1986; Valenzano et al., 2003). Fibers with a conduction velocity of <1.4 m/s were classified as C fibers. Fibers responsive to 4.56 bar were classified as mechanically sensitive fibers. Fibers were classified as heat sensitive if they responded to a standard radiant heat ramp to 44 °C; otherwise they were classified as heat insensitive. Fibers conducting in the C-fiber range and responsive to both mechanical and heat stimulations were classified as mechano-heat-sensitive C fibers.

Responses of single afferent fibers to standard mechanical pressure were studied using a weight probe (Brederson et al., 2002). The probe consisted of a flat stainless steel tip of 2 mm in diameter; the tip was placed, perpendicularly, onto the skin within the receptive field of the fiber(s) under study so that it presses the skin with weight; 53.4 g (1.7 bar) or 103.4 g (3.2 bar). The probe was manipulated by a micropositioner for up or down excursions at a fixed speed. Each stimulation lasted for 5 s. For studying mechanical sensitization of a particular afferent fiber, a fixed weight was used and the same spot of the receptive field was stimulated every 5 min.

For drug application, a stainless steel ring was placed onto the skin to isolate the receptive field from surrounding bath perfusion. SIF inside the ring was removed 30 s before drug application. During the application, the drug solution inside the ring was continuously bubbled with carbogen to maintain a constant oxygen level and produce turbulence to the solution (Valenzano et al., 2003). A 2-min washout interval followed each drug application. To avoid tachyphylaxis observed with α,β-meATP in pilot tests, no more than one application of this agonist was given to the same fiber in the study.

Responses of single afferent fibers to α,β-meATP or mechanical pressure were quantified by total numbers of action potentials during stimulation. For fibers with spontaneous firing before stimulation, the base level of spontaneous activity was subtracted from activity recorded during the stimulation period to count a response. Fibers generating ≥3 action potentials during α,β-meATP application were counted as responders. For mechanical pressure, the response was considered to be enhanced by α,β-meATP if its magnitude was greater than the mean+2 S.D. of three to four pre-drug responses. Responses were normalized to the size of the first response for each fiber to minimize the inter-fiber variations.

2.6. Statistical analysis

Paw withdrawal threshold data were analyzed using a one-way analysis of variance (ANOVA) followed by Fisher's PLSD for planned comparisons. The neural activity was analyzed using Student's *t*-tests or Mann–Whitney tests as appropriate. All values are given as means±S.E.M. Statistical significance was determined at $P<0.05$.

3. Results

3.1. Behavioral studies

Intraplantar injection of 50 μl Freund's complete adjuvant into the hind paw of rats resulted in the development of mechanical hyperalgesia as indicated by a decreased paw withdrawal threshold to a noxious mechanical stimulus (Fig. 1A). s.c. administration of A-317491 produced a dose-dependent reduction in mechanical hyperalgesia 1 h ($F_{(7,161)}$=11.117, $p<0.0001$), 3 h ($F_{(7,161)}$=13.058, $p<0.0001$) and 5 h ($F_{(7,161)}$=15.593, $p<0.0001$) post-administration (Fig. 1A). At the 1 h time point, statistically significant increases in paw withdrawal threshold were seen at the 3, 10 and 30 mg/kg doses (Fig. 1A). The dose-dependent reversal of hyperalgesia at the 1 h time point estimated an ED_{50} value, defined as the dose that produces 50% of the maximum reversal, of 2.3 mg/kg with a saturation of effect at the 10 mg/kg dose (Fig. 1B). At the 3 and 5 h time points, statistically significant increases in paw withdrawal threshold were also seen at the 3, 10 and 30 mg/kg doses (Fig. 1A). The maximum percent reversal (72.2±9.3%) was achieved 3 h following the 10 mg/kg dose. Oral administration of the cyclo-oxygenase-2 inhibitor, celecoxib (positive control) also resulted in a statistically significant reversal of the hyperalgesia (data not shown).

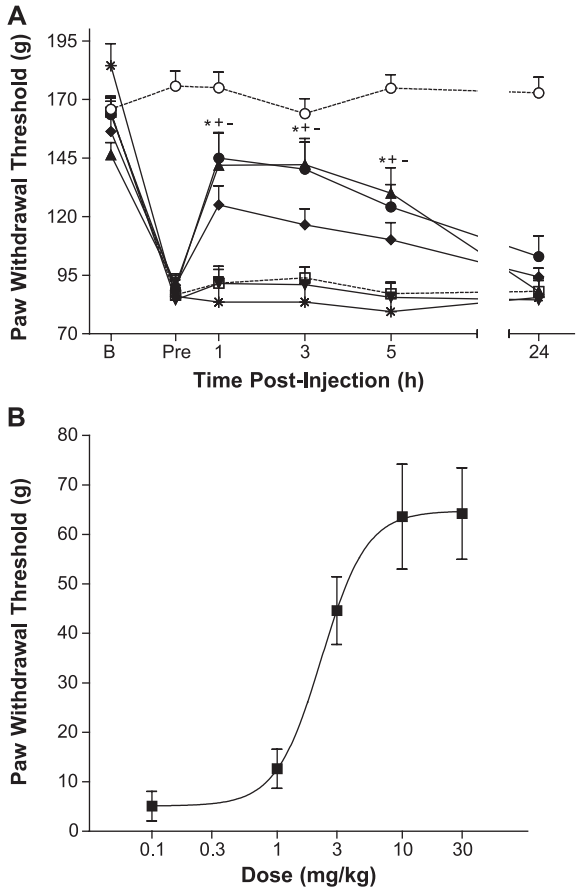

Fig. 1. A-317491 dose-dependently reverses inflammatory mechanical hyperalgesia. Rats received an intraplantar injection of saline (○) or Freund's complete adjuvant (all other groups) into the hind paw, followed by s.c. administration of A-317491 (0.1mg/kg: *; 1mg/kg: ▼; 3 mg/kg: ◆; 10 mg/kg: ▲ or 30 mg/kg: ●) or vehicle (□) 24 h later. Hind paw withdrawal thresholds to a noxious mechanical stimulus were determined using an analygesymeter and are plotted versus time (A). The dose–response curve for reversal of mechanical hyperalgesia by A-317491 is shown at the 1 h time point (B). Cut-off was set at 250 g. * indicates a significant difference of the 30 mg/kg dose group as compared to the vehicle group, + indicates a significant difference of the 10 mg/kg dose, and – indicates a significant difference of the 3 mg/kg dose (Fisher's PLSD post-hoc test; $P<0.05$). B=Baseline measurement, Pre=Predrug measurement. Data shown are the mean±S.E.M. (n=10–30 rats/group).

3.2. Pharmacokinetic studies

In order to examine its pharmacokinetic profile, A-317491 was administered to rats via either the s.c. or i.v. route. As shown in Fig. 2A, high plasma concentrations of A-317491 (>1000 ng/ml) were measured at 1, 3 and 5 h after s.c. administration of 10 and 30 mg/kg and 1 and 3 h after the 3 mg/kg dose. The plasma concentrations were dose-dependent. Penetration into the CNS was determined 1 h after a 10 mg/kg s.c. dose of A-317491. At this time, the plasma concentrations again reached a high level (29446.4±527.2 ng/ml) but the measured brain concentration was considerably lower (242.9±3.2 ng/g tissue, Fig. 2B). The mean brain-to-plasma ratio for A-317491 was calculated at 0.00825, indicating that A-317491 does not significantly penetrate into the CNS. In addition, mean A-317491 plasma concentrations were determined following a single 3 mg/kg i.v. dose. From the measured plasma concentrations the plasma half-life was calculated at 7.38

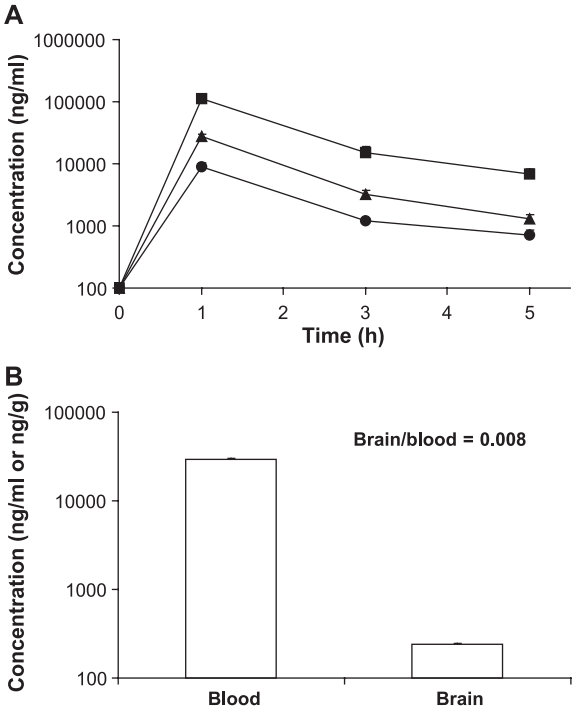

Fig. 2. Pharmacokinetic profile of A-317491. In (A), A-317491 was dosed to rats subcutaneously (3 mg/kg: ●; 10 mg/kg: ▲; or 30 mg/kg: ■) and plasma samples were drawn at the time points indicated. In (B), plasma and brain samples were drawn 1 h after administration of A-317491 (10 mg/kg, s.c.). Samples were processed as described in Methods. Data shown are mean±S.E.M.; error bars represent S.E.M. (n=3 rats/data point).

h using a non-compartmental model. The clearance rate was 1.83 l/h/kg, and the volume of distribution was 0.17 l/kg.

3.3. Electrophysiological studies

All the recordings were made in the rat skin inflamed with Freund's complete adjuvant. Fifty-seven single mechanically sensitive C-fiber afferents were identified. Their conduction velocities ranged from 0.39 to 1.00 m/s (0.59±0.01, n=57). A large proportion of the C afferents (34/57 fibers) had spontaneous activity.

At 0.5 mM, α,β-meATP activated the majority of mechano-heat-sensitive C fibers (75%, 9/12 fibers). An example of the activation is shown in Fig. 3A. Such activation was concentration-dependently inhibited by A-317491. Fibers treated with 1 mM A-317491 had no activation during application of 0.5 mM α,β-meATP as shown in a representative record (Fig. 3B) and by the data pooled from all fibers tested (Fig. 3C). Overall, the response was 0.3±0.31 action potentials/5 min (n=8) in the presence of 1 mM A-317491 vs. 13.2±6.09 action potentials/5min (n=12) in control (P=0.01; Mann–Whitney test). A concentration–effect curve for the inhibition of α,β-meATP-induced activation by A-317491 is shown in Fig. 3D. It indicated a trend that less fibers were activated by α,β-meATP when treated with higher concentrations of A-317491. No statistically significant difference was observed

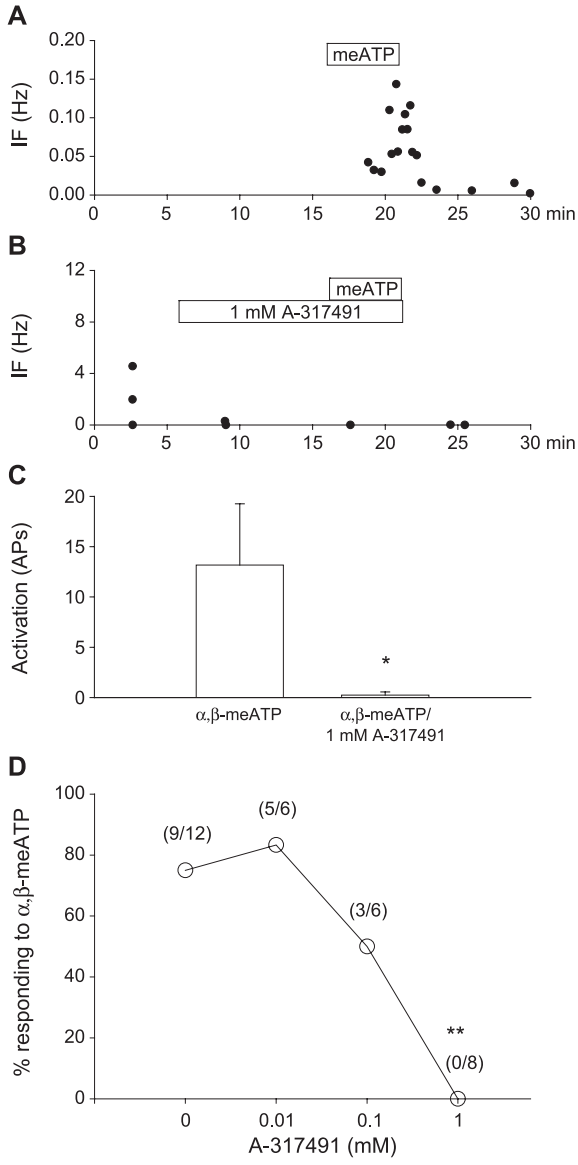

Fig. 3. Blocking effect of A-317491 on responses of mechano-heat-sensitive C fibers to 0.5 mM α,β-meATP in inflamed skin. A rat skin-nerve preparation was used to record single fiber activity 18 h after Freund's complete adjuvant treatment. In (A), a representative response of a single C fiber to α,β-meATP applied to the receptive field for 5 min is illustrated. Its conduction velocity is 0.67 m/s. Each dot represents an action potential plotted as instantaneous frequency (IF). In (B), an example of the blockade of the α,β-meATP response by 1 mM A-317491 in another single fiber with a conduction velocity of 0.68 m/s is shown. Note that this fiber had spontaneous activity. In (C), afferent activation induced by α,β-meATP is compared in all fibers tested between α,β-meATP alone (n=12) and in the presence of 1 mM A-317491 (n=8). Activation is measured as numbers of action potentials during agonist application. The difference is statistically significant between two groups (*P<0.05; Mann–Whitney test). Data shown are mean±S.E.M. In (D), a concentration–effect curve is plotted for the inhibition of α,β-meATP-induced afferent activation by A-317491. Note that the percentage of fibers responsive to the agonist declines with increasing concentrations of the antagonist. Numbers of responsive fibers/total fibers studied are presented in parenthesis. The difference is statistically significant for proportions of responsive fibers between the 1 mM group and control (**P<0.01, Fisher Exact test).

for the 0.01 and 0.1 mM concentrations in either percentage of responders or the magnitude of activation (37±17.84 action potentials/5 min, n=6 and 10±4.89 action potentials/5 min, n=6 for the 0.01 and 0.1 mM concentrations, respectively) compared to control, indicating that at these two lower concentrations A-317491 was less effective in blocking α,β-meATP excitation.

At 0.1 mM, a less proportion of mechano-heat-sensitive C fibers (28.6%, 2/7 fibers) were directly activated by α,β-meATP. However, this concentration of α,β-meATP sensitized a subset of mechanically sensitive C fibers to mechanical pressure of 1.7 or 3.2 bar in inflamed skin. One example is illustrated in Fig. 4. The sensitization was characterized by an increase in the number of action potentials generated in response to the same mechanical pressure during α,β-meATP application. Six out of nine fibers showed such sensitization. The data pooled from all six sensitized fibers are shown in Fig. 5A. Overall, a 1.5-fold increase in the size of the mechanical response was observed as compared to control (P=0.013; paired Student's t-test). This increase appeared to be short lasting. Of the six fibers that were sensitized to mechanical stimulation, two were activated by α,β-meATP and the other four were not, suggesting that mechanical sensitization is not restricted to fibers showing direct activation. Furthermore, α,β-meATP-induced mechanical sensitization was blocked by 0.1 mM A-317491 (Fig. 5B). In a separate group, six mechanically sensitive C-fibers were pretreated with 0.1 mM A-317491. In the presence of this concentration of A-317491, no increase in the mechanical response was seen in any fiber during application of 0.1 mM α,β-meATP. The difference between the proportion of sensitized fibers (0/6 fibers) observed with A-317491 and the proportion of sensitized fibers (6/9 fibers) without the antagonist was significant (P=0.03; Fisher Exact test), suggesting that the sensitization was completely blocked by A-317491. However, A-317491 did not affect basal mechanical responses of studied fibers in inflamed skin (Fig. 5B). In addition, no block of α,β-meATP-induced mechanical sensitization was observed by a lower concentration (0.01 mM) of A-317491. Three additional mechanically sensitive C fibers were examined and two showed sensitization to mechanical stimulation during application of 0.1 mM α,β-meATP in the presence of 0.01 mM A-317491. The magnitude of sensitization of the two fibers (a 1.6- and 1.7-fold increase, respectively) was similar to that observed without A-317491, demonstrating that at this low concentration, A-317491 did not block mechanical sensitization.

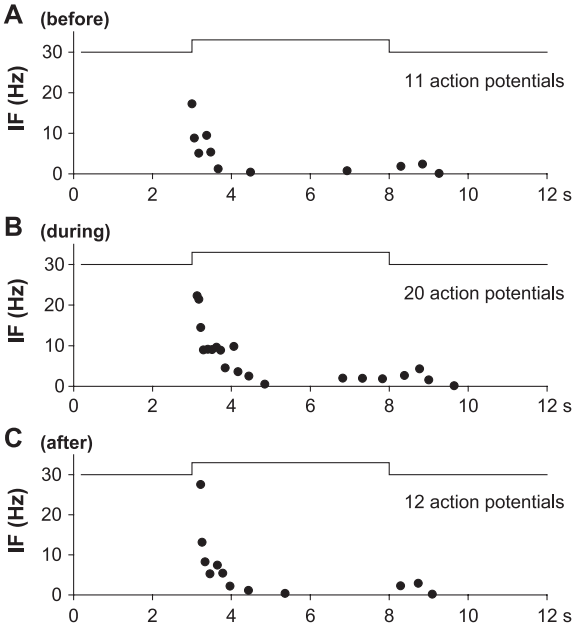

Fig. 4. Example of the responses of a single mechano-heat-sensitive fiber to mechanical pressure in inflamed skin. Three individual responses, before (A), during (B) and after (C) 0.1 mM α,β-meATP, respectively, are shown. In each recording, the fiber fired in response to mechanical pressure applied to the same spot inside its receptive field, with the same force (1.7 bar) and for the same duration (5 s), as indicated by the upper trace. Numbers of action potentials recorded during stimulation are presented. The response was almost doubled during application of α,β-meATP. The response showed accommodation over the time.

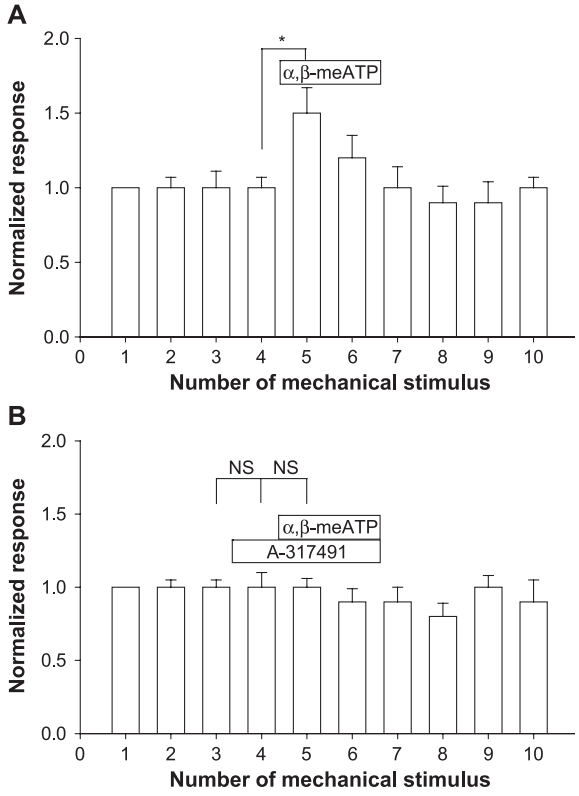

Fig. 5. Blocking effect of A-317491 on α,β-meATP-induced mechanical sensitization in inflamed skin. In (A), a set of mechanically sensitive C-fibers (n=6) showed an increase in their responses to a standard mechanical pressure (1.7 or 3.2 bar for 5 s) during application of 0.1 mM α,β-meATP to the receptive fields. In (B), no such enhancement was observed when 0.1 mM A-317491 was present as tested in a separate group of mechanically sensitive C-fibers (n=6). For each fiber, 10 sequential stimuli were applied with an interstimulus interval of 5 min, and responses were normalized to the first response. (*P<0.05, NS=not significant; paired Student's t-test). Data shown are mean±S.E.M.

4. Discussion

A-317491 is the first non-nucleotide antagonist available for blocking $P2X_3/P2X_{2/3}$ receptors with high potency and selectivity (Jarvis et al., 2002). In the present study, we observed significant reduction of mechanical hyperalgesia by s.c. administration of A-317491 in the Freund's complete adjuvant-induced model of chronic inflammatory pain. This finding supports the conclusion of a recent study using the two P2X receptor antagonists TNP-ATP and IP5I (diinosine pentaphosphate) (Dai et al., 2004). TNP-ATP exhibits selectivity for $P2X_1$, $P2X_3$, and $P2X_{2/3}$ receptors whereas IP5I preferentially blocks $P2X_1$ and $P2X_3$ receptors but not $P2X_{2/3}$ receptors (Dunn et al., 2000). Dai et al. (2004) found that TNP-ATP but not IP5I reverses mechanical hyperalgesia following intraplantar injection of the Freund's complete adjuvant. Their work demonstrates that extracellular ATP is involved in mechanical hyperalgesia during cutaneous inflammation and, when considered in light of selectivity (Dunn et al., 2000), suggests that the effect is mediated mainly by $P2X_{2/3}$ heteromultimers. This is confirmed here using A-317491 as a selective $P2X_3$ and $P2X_{2/3}$ receptor antagonist.

A good bioavailability has been reported for A-317491 when administered systemically (Jarvis et al., 2002). To better understand the systemic efficacy of A-317491, we further examined its pharmacokinetic profile following s.c. administration. In agreement with the previous report (Jarvis et al., 2002), s.c. A-317491 showed substantial and sustained plasma concentrations, which correlates well with the time course of behavioral effects observed here and in previous studies (Jarvis et al., 2002). However, we noticed that very little A-317491 was measured in the brain following s.c. administration. The obtained brain-to-blood concentration ratio indicates that A-317491 poorly penetrates into the CNS. This limited CNS permeability strongly suggests that A-317491 is a peripherally acting blocker, which seems in conflict with some central mechanisms suggested for its antihyperalgesic effects by comparison of intrathecal and intraplantar efficacy using behavioral assessments (McGaraughty et al., 2003). It was reported that both intrathecal and intraplantar administration effectively reversed thermal hyperalgesia following either inflammation or nerve injury in rats. In contrast, nerve injury-associated tactile allodynia was reduced by intrathecal but not intraplantar A-317491 (McGaraughty et al., 2003). While it is clear that an effect of A-317491 occurs following intrathecal administration, based on our observation that A-317491 poorly penetrates into the brain we believe that following s.c. administration, the antihyperalgesic effect is predominantly due to inhibition of receptors located on peripheral neurons. However, several alternatives would also explain our observations. (1) The brain permeability was determined in naïve animals, and it is possible that A-317491 does not penetrate into the brain in normal conditions but gets into the CNS through an impaired blood–brain barrier under pathological circumstances (Huber et al., 2001). To clarify this possibility, pharmacokinetic profiling could be performed under those pathological conditions. (2) The brain permeability was determined 1 h after s.c. administration, which does not exclude the possibility that A-317491 may have reached the CNS before this time point. However, the time course of the pharmacological effects observed here and elsewhere (Jarvis et al., 2002) does not seem consistent with this possibility. (3) We did not measure A-317491 in the spinal cord; it is possible that A-317491 may penetrate into the spinal cord but not the brain. However, we feel this possibility is unlikely based on physiology (i.e. the blood–brain barrier surrounds both brain and spinal cord) and the fact that there are no examples of a compound that exhibits such anatomical selection.

The possible peripheral mechanisms for A-317491 were further investigated in our electrophysiological studies using a skin-nerve preparation. We observed that the cutaneous nerve terminals of C-fiber nociceptors were activated by α,β-meATP in Freund's complete adjuvant-inflamed skin. Interestingly, α,β-meATP also sensitized the nerve terminals to mechanical stimulation in inflamed skin. Both α,β-meATP-induced nociceptor activation and mechanical sensitization were completely blocked by A-317491 applied topically to the receptive fields of the nociceptors. These electrophysiological results provide further evidence to suggest that $P2X_3$ and $P2X_{2/3}$ receptors present in the cutaneous terminals of nociceptors are critical in mediating modulation of mechanical sensitivity of nociceptors by extracellular ATP following inflammation. Enhanced excitation of nociceptor terminals by ATP and its analog has been reported previously in carrageenan-inflamed skin using similar preparations (Hamilton et al., 2001). In the present study, we have demonstrated for the first time that C-fiber nociceptors are sensitized to mechanical stimulation by α,β-meATP in inflamed skin. Page et al. (2000) have reported that mucosal afferent fibers are sensitized to mechanical stimulation by ATP during oesophageal inflammation. Thus, it seems that nerve terminals in both somatic and visceral organs are subject to purinergic modulation of their sensitivity to mechanical stimulation following inflammation. Blockade of ATP-induced activity in the peripheral terminals of primary nociceptive afferents has been previously reported using the nonselective P2X receptor antagonist PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium) in vitro (Kress and Guenther, 1999) and in vivo (Dowd et al., 1998). Interestingly, it is found that ATP-induced heat sensitization of mechano-heat-sensitive fibers is also blocked by PPADS (Kress and Guenther, 1999).

Although the reversal of mechanical hyperalgesia by A-317491 observed in our behavioral assessments suggests a role of the endogenous ATP in the maintenance of such hyperalgesia following inflammation, we did not notice any modulation of basal mechanical responses of nociceptive C-afferent fibers by A-317491 in recordings from isolated skin

inflamed with Freund's complete adjuvant. This may relate to different experimental arrangements used here. In the skin-nerve preparation, the skin was continuously superfused with a large volume of the buffer solution. Accordingly, ATP and other substances tonically released from the inflamed tissue would be continuously washed out in this experimental arrangement. This could then explain the lack of A-317491's effect on the basal response in this preparation under an assumption that mechanical sensitization requires continuous exposure of nerve terminals to ATP released tonically from the inflamed tissue. In the behavioral experiments, however, continuous ATP exposure should be well maintained without disturbance described above. In fact, using an experimental arrangement similar to that used in our behavioral studies, Dai et al. (2004) have shown that mechanical sensitization of primary sensory neurons innervating inflamed skin is blocked by PPADS and TNP-ATP, indicating that inflammation-induced mechanical sensitization of sensory neurons is indeed maintained by locally released endogenous ATP.

The mechanisms for sensitization of nociceptors to mechanical stimulation by activation of P2X receptors following inflammation remain unknown. However, it was found that expression of P2X receptors in primary sensory neurons increased following inflammation (Xu and Huang, 2002). In addition, inflammatory mediators can alter the phosphorylation status of the receptors (Paukert et al., 2001). Furthermore, an increased calcium influx of nociceptive neurons has been suggested to be associated with thermal hyperalgesia by ATP via P2X receptors (Kress and Guenther, 1999). Thus, through facilitated P2X receptors, ATP, released upon mechanical stimulation, may induce further calcium influxes into the neuron to potentiate excitability and induce hyperalgesia following inflammation.

In summary, we have demonstrated that systemic administration of A-317491 is effective in reversing mechanical hyperalgesia associated with chronic inflammatory pain. Furthermore, we have shown that systemically administered A-317491 results in substantial and sustained plasma levels with a very low concentration of this compound in the brain. Finally, we have demonstrated that A-317491 blocks α,β-meATP-induced excitation and mechanical sensitization of inflamed cutaneous terminals of nociceptors. Taken together, our results strongly suggest that the systemic efficacy of A-317491 is primarily mediated through action at peripheral receptors and confirms that peripheral $P2X_3$ and largely $P2X_{2/3}$ receptors are critical in modulation of mechanically associated pain following inflammation.

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