DEVELOPMENT OF TACTILE ALLODYNIA AND THERMAL HYPERALGESIA BY INTRATHECALLY ADMINISTERED PLATELET-ACTIVATING FACTOR IN MICE

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Abstract

Platelet-activating factor (PAF) is a potent inflammatory lipid mediator in peripheral tissues. However, its role in mediation of nociception in central nervous system is unknown. In the present study, whether PAF plays some role in pain transduction in the spinal cord was studied in mice. Intrathecal injection of PAF induced tactile pain, tactile allodynia at as low as 10 fg ~ 1 pg with a peak response at 100 fg, while lyso-PAF was without effect in the range of doses. Tactile allodynia induced by PAF was blocked by a PAF receptor antagonists, TCV-309, WEB 2086 and BN 50739. The expression of PAF receptor mRNA by RT-PCR was observed in DRG and spinal cord in mice. ATP P2X receptor antagonists, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) and 2',3'-*O*-(2,4,6-trinitrophenyl) adenosine 5-triphosphate (TNP-ATP), NMDA receptor antagonist, MK 801 and nitric oxide synthetase inhibitor, 7-nitroindazole blocked the PAF-induced tactile allodynia. PAF-induced tactile allodynia and thermal hyperalgesia disappeared in neonatally capsaicin-treated adult mice, while tactile allodynia but not thermal hyperalgesia induced by intrathecally injected α,β-methylene ATP, a P2X receptor agonist, was capsaicin-insensitive. The present study demonstrated that PAF is a potent inducer of tactile allodynia and thermal hyperalgesia at the level of the spinal cord. PAF-evoked tactile allodynia is suggested to be mediated by ATP and the following NMDA and NO cascade through capsaicin-sensitive fiber, different from exogenously injected α,β-methylene ATP which is insensitive to capsaicin treatment.

Key words; PAF; tactile allodynia; thermal hyperalgesia; capsaicin; ATP

1. Introduction

Platelet-activating factor (PAF) is an alkyl-phospholipid, described in a variety of immune and inflammatory cells and an important mediator of the inflammatory responses. PAF has also been found in neuronal tissues. PAF is suggested to be implicated in a variety of physiological and pathological states in the central nervous system (CNS) (Ishii and Shimizu, 2000).

Tissue damage or injury of neuron in periphery often cause hyperalgesia and tactile allodynia, the latter is a state in which innocuous tactile stimuli evoke pain. The mechanisms of the production of such pain hypersensitivity is not yet clear, but an increased responsiveness of neurons in the central nervous system is significantly relevant to the development of the pathological state. Prostaglandin is one of the critical mediators for the processing of pain at the spinal cord level and contribute to pain hypersensitivity, in addition to augmenting pain information at peripheral terminals of primary afferent nociceptors (Minami et al., 1999; Uda et al., 1990). Faden and Halt (1992) administered PAF intrathecally to examine its effects in the spinal cord and found that PAF decreased blood flow in the spinal cord, motor function or survival, suggesting the role of PAF in tissue damage after spinal cord injury. PAF antagonist treatment reduces pro-inflammatory cytokine mRNA after spinal cord injury, suggesting that PAF contributes to secondary damage after spinal cord injury

(Hostettler et al., 2002). Actually, PAF levels have been shown to be elevated by 20-fold in spinal cord injured by ischemia and reperfusion (Lindsberg et al., 1990). Although these events suggest PAF as a signaling molecule triggering the inflammatory events in spinal cord, a potential role of PAF in the regulation of pain at spinal cord has not been considered. Recent studies stressed the importance of spinal glial cells for the regulation of pain hypersensitivity and tactile allodynia following peripheral nerve injury (Tsuda et al., 2003; 2004). The number of microglia increased in the dorsal horn on the side of nerve injury model (Aldskogius and Kozlva, 1998). PAF is released from stimulated microglia cells (Jaranowska et al., 1995) and is a potent chemotactic factor of microglia (Aihara et al., 2000). These evidence provide a possibility that PAF may serve as a regulator of pain at spinal cord.

The present study investigated whether PAF plays some role in transducing pain at the spinal cord level by evaluating the development of tactile allodynia and thermal hyperalgesia after intrathecal administration of PAF. Recent studies suggest the central role of ATP/purinoceptors in spinal cord in mediating pain hypersensitivity (Tsuda et al., 1999; 2000; 2003). It has been demonstrated that the NMDA receptor system plays an important role in the development of allodynia by drugs (Minami et al., 1994; 1995). We have recently demonstrated that ATP P2X ionotropic receptors following glutamate/NO systems in spinal cord are involved in the regulation of the development of allodynia. Therefore, the involvement of these systems in the effects of PAF was also studied.

2. Materials and methods

2.1. Animals

Male ICR mice weighing 18-22 g were used for general experiments and female mice at 5-6 weeks of pregnancy were obtained for experiments on neonatal mice. Animals were housed at 22 ± 2 °C and all procedures and handling of the animals were performed according to both the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society and the guidelines of Hiroshima University, Hiroshima, Japan.

2.2 Injections

2.2.1. Intrathecal injection

For intrathecal (i.t.) injection, the head of the mice was placed into plastic cap and the body was held with one hand as described previously (Fukuhara et al., 2000). A 27-gauge needle attached to a Hamilton micro syringe was inserted into the subarachnoid space between the L5 and L6 vertebrate of conscious mice and 5 μl of drug solution was slowly injected as described by Hylden and Wilcox (1980).

2.2.2. Neonatal capsaicin treatment

Neonatal ICR mice were injected subcutaneously with 50 mg/kg capsaicin or vehicle 2 and 5 days after birth, then 5- to 6- week-old mice were used for the present experiments. To verify desensitization after capsaicin treatment, chemo sensitivity was determined using the wiping test with 33 μM of capsaicin, 1 % ethanol containing saline (Jancsó et al., 1977) and only mice with a lack of chemosensitivity to capsaicin were used for experiments.

2.3. General testing procedures

2.3.1. Touch-evoked tactile allodynia

After the i.t. injection of the drug solution or vehicle, each mouse was placed in an individual plastic cage. Tactile allodynia was assessed every 5 min by lightly stroking the flank of each mouse with a paintbrush. Tactile allodynia response was ranked as described by Minami et al. (1995): 0, no response; 1, mild squeaking with attempts to move away from the stroking probe; 2, vigorous squeaking, biting the stroking probe and strong efforts to escape from the stroking probe. Values were represented by the average of total score evaluated at each time point (possible maximum score at each time point: 2/mouse) during the time course study. In other studies, allodynia was assessed every 5 min over a 60 min period (12 trials) and the values were represented by the average of cumulative score (possible maximum score: 2/mouse at each time

point x 12 trials = 24/mouse). In some experiments, tactile allodynia was evaluated by measuring the paw withdrawal threshold in response to probing with a series of calibrated fine filaments. The animals were placed in a plastic cage. After allowing the animals to adapt to the environment for several min, numbered monofilaments (von Frey filaments; Stoelting, Wood Dale, IL, USA) were applied perpendicularly to the dorsum surface of the hind paws. The smallest filament that caused the animal to flinch or move the paw from the stimulus three times out of three trials was determined to be the mechanical threshold. The filament number was converted to buckling force (gm).

2.3.2. Thermal hyperalgesia

Warm-water tail-flick test and hot-plate test were carried out to assess the effects of agents on the thermal nociceptive threshold. For the warm-water tail-flick test, the tails of mice were immersed in warm water, 48 °C. The latency of tail flick was recorded. For the hot-plate test, mice were placed on a 52 ± 0.1 °C hot plate. The response latency to either a hind-paw lick or jump was recorded. In the absence of responses, animals were removed from the warm water or hot plate after 15 sec and 30 sec, respectively to avoid tissue injury, and 15 sec and 30 sec latencies were assigned as the respective responses.

2.4. PCR analysis of PAF receptor

To analyze expression of PAF receptor, total RNA and then cDNA were prepared

from mouse DRG neurons and dorsal horn using TRIzol RNA extraction and RT-PCR kits.

The tissues were quickly lysed by the addition of TRIzol® reagent (Life Technologies, Rockville, MD). Total RNA was isolated according to the manufacturer's instructions, with the addition of DNase I treatment (RNase-free DNase I; Roche Molecular Chemicals Indianapolis, IN) to eliminate residual genomic DNA. Oligo(dT)-primed first-strand cDNA synthesis was performed with 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Boehringer Mannheim) using 10 μg of total RNA as a template in a total volume of 20 μl. Reaction conditions were as described by the manufacturer. For negative controls without reverse transcription, total RNA was diluted to give the same total RNA concentration as in the cDNA solutions. These control reactions using total RNA as a template were performed to exclude genomic DNA contamination. The reaction solution (20 μl) contained 0.5 μM primer (each for reverse and forward primers), 2 mM $MgCl_2$, 0.2 mM dNTPs (each) and 0.5 units of Taq polymerase (Ampli Taq Gold, PerkinElmer, Roche Molecular System, Branchburry, NJ) in 1 x PCR buffer II supplied by the manufacturer. As a template, 2 μl of total RNA solution or 2 μl of cDNA solution was used. The primers used to amplify mouse PAF receptor were forward, 5'-cctagtgcccaataaggatggct-3'; reverse, 5'-taggagtctggttggctggc-3' (size 530 bp). The cycling conditions were: 95 °C for 10 min, followed by 35 cycles, each consisting of denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec, and extension at 72 °C for 2 min, and a final extension at 72 °C for 10

min. PCR products were separated by migration on 1.6 % (W/V) agarose gels and visualized by staining with ethidium bromide. Products were excised and purified using QIAQuick™ gel extraction kit (Qiagen), then subjected to restriction digest analysis and automated sequencing. PCR products corresponding to the published mouse PAF receptor cDNA sequence were obtained.

2.5. Experimental procedures

The first experiment established whether PAF evokes tactile allodynia and thermal hyperalgesia and whether the responses are mediated by PAF receptors in the spinal cord. On the test day, a group of mice received a 5 μl intrathecal injection of either TCV-309, WEB 2086, BN 50739, PAF receptor antagonists, or vehicle for drugs. Tactile allodynia and thermal behavioral response threshold were assessed before and after the i.t. injection. Animals then received 5 μl of various concentrations of PAF or lyso-PAF, an inactive precursor of PAF. In some experiments, TCV-309 and PAF were simultaneously administered by i.t. injection of a mixed solution of TCV-309 and PAF. In an additional experiment, dorsal root ganglion and spinal cord were isolated from non-treated mice and mRNA was prepared for PCR reaction to identify the PAF receptor mRNA.

A separate group of mice was used in the second experiment to determine the influence of P2X receptor antagonists, pyridoxalphosphate-6-azophenyl-2',4'-

disulfonic acid (PPADS) and 2',3'-*O*-(2,4,6-trinitrophenyl) adenosine 5-triphosphate (TNP-ATP), NMDA receptor antagonist, MK 801, and NO synthetase inhibitor, 7-nitroindazole (7-NI) on PAF-induced tactile allodynia. On the test day, animals received spinal injection of 5 μl of either artificial cerebrospinal fluid (ACSF) or 9 nmol of MK 801; or they received intraperitoneal injection of 25 mg/kg of 7-NI. One hundred ng of PAF were then injected intrathecally in a volume of 5 μl. Tactile allodynia response was assessed before and after administration of PAF. A combination of PPADS or TNP-ATP with 100 ng of PAF was administered as a 5 μl mixed solution of the two agents. Tactile allodynia response was assessed before and after the injection of PPADS or TNP-ATP alone and in combination with PAF.

In the third experiment, neonatal mice were treated with capsaicin to evaluate the effect of capsaicin treatment on PAF- or α,β-methylene ATP -induced tactile allodynia and thermal response threshold. Either ACSF, 100 ng of PAF or 1 μg of α,β-methylene ATP was injected intrathecally in adult mice neonatally treated with the mixture of 10 % ethanol and 10 % Tween-80 (vehicle for capsaicin) or capsaicin. Tactile allodynia and thermal behavioral response threshold were assessed 15 min after the injection.

2.6. Drugs

PAF, lyso-PAF, PPADS, α,β-methylene ATP, MK 801, 7-NI, capsaicin were obtained commercially. TCV-309, WEB 2086 and BN 50739 were donated from

Takeda Pharmaceutical Co., Boehringer Ingelheim KG, and Institute Henri Beaufour, respectively. PAF was dissolved in ethanol, the ethanol was removed from an aliquot in a siliconized tube by introducing nitrogen gas into the tube and then dissolved in ACSF. Capsaicin was dissolved in the mixture of 10 % ethanol and 10 % Tween-80 in sterile saline, while 7-NI was dissolved in the mixture of 10 % dimethyl sulfoxide (DMSO) and 30 % propylene glycol in distilled water. BN 50739 dissolved in DMSO and diluted appropriately (final concentration of DMSO was 0.1 %). Other reagents were dissolved in ACSF. ACSF composition (in mM) was NaCl 142, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, NaH_2PO_4 1.25, D-glucose 10, HEPES 10, 0.05% fatty acid-free bovine serum albumin, pH 7.4.

2.7. Statistical analysis

The results are presented as mean ± S.E.M. Thermal hyperalgesia data were analyzed by parametric ANOVA and significance ($p<0.05$) was further examined by Duncan's test. Data for tactile allodynia were analyzed by non-parametric ANOVA and significance ($p<0.05$) was further examined by Fisher's protected least significant difference (Fisher's PLSD) test for multiple-comparison. Student's *t* test was also used. $p<0.05$ was considered significant.

3. Results

3.1. Effect of i.t. PAF on mechanical tactile allodynia and thermal hyperalgesia

The i.t. administration of PAF at 100 fg per mouse induced prominent agitation responses, such as biting, vocalization and escape from the probe, when tactile stimuli with a paintbrush was applied to the flank of each mouse. Strong responses were evoked shortly after the injection when the maximal score was obtained, persisted for 60 min, then gradually recovered over a 10 hr period (Fig. 1A). Tactile allodynia was evaluated by measuring the paw withdrawal threshold in response to probing with a series of calibrated fine filaments (von Frey hairs) (Fig. 1B). The threshold was markedly reduced after the administration of PAF and the time-course was similar to that of agitation responses. The administration of artificial cerebrospinal fluid did not induce any allodynic responses. These results indicate that the intrathecally injected PAF evokes strong pain by innocuous tactile stimuli.

In the warm-water tail-flick test and hot-plate test, non-treated mice and ACF-treated control mice responded with constant latencies. PAF at a dose of 100 fg shortened these response times (response time in non-treated mice, ACSF-treated mice and PAF 100 fg-treated mice were 1.4 ± 0.1, 1.5 ± 0.2 and 0.6 ± 0.1 sec in warm-water tail-flick test, and those are 9.8 ± 0.6, 9.6 ± 0.8 and 4.7 ± 0.4 sec in hot-plate test, respectively. Significantly different from control, $P<0.01$, $n=10$). These findings

demonstrated that mice injected with PAF had developed thermal hyperalgesia.

Intrathecal administration of PAF as low as 10 to 100 fg dose-dependently produced tactile allodynia in mice with peak response at 100 fg to 1 pg. Lyso-PAF was without effect in this range of dosages (Fig. 2A). Mice treated with more than 1 μg of PAF died, although lyso-PAF did not cause death at doses up to 10 μg. Tactile allodynic response induced by 100 fg PAF was antagonized by the simultaneous administration of a PAF receptor antagonist, TCV-309, 15 fmol, and blocked completely by the pretreatment of TCV-309, WEB 2086 and BN 50739 (Fig. 2B). Thermal hyperalgesic response to PAF was also blocked by PAF receptor antagonists (data not shown). Thus, the tactile allodynia and thermal hyperalgesia induced by PAF are mediated by PAF receptor stimulation.

3.2. Expression of PAF receptor

To evaluate the expression of PAF receptor mRNA in DRG and spinal cord, RT-PCR was performed using specific primers for mouse PAF receptor designed using the known sequence of PAF receptor. RT-PCR demonstrated specific amplification of 530 bases expected size of PAR-receptor transcript in DRG and spinal cord to a lesser extent (Fig. 3). As a positive control, the lung where the density of the PAF receptor expression is known to be very high showed high amplification. There was no amplification in spleen where PAF receptor is known to be unexpressed (data not

shown). There was no amplification without RT reaction in each tissue. The similar results were obtained from 3 independent total RNA derived from each tissue.

3.3. Effects of blockers for P2X receptor, NMDA receptor and NOS

ATP P2X receptors, NMDA receptors and NO have been suggested to be involved in the development of tactile allodynia. To test whether PAF-induced tactile allodynia is mediated through these signaling cascatde, the effects of various inhibitors of this cascade on PAF-induced tactile allodynia were evaluated. PPADS, an antagonist of P2XR subtypes $P2X_{1,2,3,5,7}R$ reduced by about 70 % of PAF-induced tactile allodynia at 0.3 nmol and showed no further reduction even at a higher dose of 5 nmol, while TNP-ATP, an antagosint of P2XR subtypes $P2X_{1\text{-}4}R$ completely inhibited the tactile allodynia (Fig.4). NMDA receptor antagonist, MK 801 and an NO synthetase inhibitor, 7-NI, all inhibited the production of tactile allodynia induced by PAF. Therefore, PAF-induced tactile allodynia may be mediated by purine and NMDA receptor, and NO systems.

3.4. Effects of capsaicin treatment on PAF-induced thermal hyperalgesia and tactile allodynia

Whether the intrathecal injection of PAF produces thermal hyperalgesia was

examined in warm-water tail-flick test (Fig. 5A) and hot-plate test in normal mice and capsaicin-treated mice. Non-treated mice and ACSF-treated control mice responded to the thermal stimuli with constant latencies of 1.4 ± 0.1 and 1.5 ± 0.2 sec in warm-water test. PAF shortened tail-flick response time (0.6 ± 0.1 sec) at a dose of 100 fg (Fig. 5A). The i.t. administration of 1 μg of α,β-methylene ATP, tested as a reference compound, reduced the latency period of tail-flick response time (1.1 ± 0.2 sec).

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is a neurotoxin that causes the destruction of mainly unmyelinated, small-diameter fibers when administered to neonatal animals. In mice that had been neonatally treated with capsaicin, the response times to thermal stimuli in ACSF-injected control mice (1.5 ± 0.2 sec) were prolonged to 2.7 ± 0.3 sec on tail-flick test (Fig. 5A). Neither PAF nor α,β-methylene ATP affected the response time on tail-flick test in capsaicin-treated mice (Fig. 5A). There were no effects of PAF and α,β-methylene ATP on hot-plate test either in capsaicin-treated mice (data not shown). These results demonstrated that mice had developed thermal hyperalgesia mediated by capsaicin-sensitive fiber. To test whether PAF-induced tactile allodynia is mediated by capsaicin-sensitive fiber, mice intrathecally injected with PAF were challenged with innocuous stimuli. In normal mice intrathecally injected with PAF 100 fg, an innocuous tactile stimuli applied to the flank of the mouse with a paint-brush evoked an allodynic response. In capsaicin-treated mice, PAF failed to evoke tactile allodynia, while α,β-methylene ATP still evoked tactile allodynia (Fig. 5B). In the paw withdrawal response to probing with a series of

von Frey hairs, the threshold was markedly reduced in PAF-treated mice in comparison to that in ACSF-treated control mice. PAF failed to reduce the threshold in capsaicin-treated mice (Fig. 5C).

These results show that PAF-induced tactile allodynia was capsaicin-sensitive as well as PAF-induced thermal hyperalgesia, while α,β-methylene ATP-induced tactile allodynia is capsaicin-insensitive.

4. Discussion

Although PAF injected into the rat hindpaw causes thermal hyperalgesia, there is dissociation between the induction of edema and that of hyperalgesia with regard to the doses of PAF required (PAF induced edema from 5 ng, and hyperalgesia from 1.25 μg), time course of the phenomena (hyperalgesia is a delayed response than edema) and in the drugs able to antagonize their development (Bonnet et al., 1981; Dallob et al., 1987; Vargaftig and Ferreira, 1981). In healthy volunteers, PAF injected intradermally induced weheal and flare responses and subsequently erythema and cellular infiltration. The effects of PAF were much potent than those of PGE_2, but there was no hyperalgesia demonstrated by PAF, although intradermal PGE_2 induced hyperalgesia (Sciberras et al., 1987). Thus, the role of PAF in pain production does not seem striking compared to its pro-inflammatory action in the periphery. The present study demonstrated that intrathecal injection of PAF evoked potent and long-lasting tactile pain and thermal hyperalgesia in mice. The effects of PAF seem to be mediated by PAF receptor, because

TCV-309, a selective PAF receptor antagonist (Terashita et al., 1992) as well as WEB 2086 and BN 50739 blocked the behavioral response to PAF. Lyso-PAF, an inactive intermediate for PAF receptor, was without effect at this range of dosage. The distribution of PAF receptor has been demonstrated in a variety of cells and tissues including the CNS (Ishii and Shimizu, 2000). Actually RT-PCR demonstrated the specific amplification in mouse DRG neuron and spinal cord in the present study.

PAF is suggested to serve as a transmitter in the central nervous system based on the formation of PAF upon stimulation of nervous tissue, the presence of PAF receptor and the ability of PAF to activate the functions of neuronal cells (Bito et al., 1992; Catalan et al., 1992; Honda et al., 1991; Sogos et al., 1990). We have previously observed that PAF by itself did not evoke any secretory response in adrenal chromaffin cell where the expression of PAF receptor was demonstrated, but markedly potentiated ACh-induced catecholamine release (Morita et al., 1995), suggesting the modulating role of PAF on secretory response. As PAF receptor mRNA is detected in DRG, if PAF receptor expressed in nerve terminal of primary afferent neuron in spinal cord, the stimulation of the PAF receptors could contribute to facilitate the release of neurotransmitters for pain evoked by peripheral nociceptive stimulation as discussed below.

ATP has been proposed as a transmitter candidate for primary afferent neurons and may be involved in spinal nociceptive transmission (Driessen et al., 1994; Li et al., 1998) as well as in peripheral nerve ending (for review, see Burnstock and Wood, 1996;

Ralevic and Burnstock, 1998). Recent study demonstrated that tactile allodynia could be induced by plantar or intrathecal application of α,β-methylene ATP, an agonist of P2X receptor subtypes $P2X_{1,3}R$ and PPADS, an antagonist of P2XR subtypes $P2X_{1,2,3,5,7}R$ blocked tactile allodynia induced by α,β-methylene ATP (Fukuhara et al., 2000; Tsuda et al., 2000), suggesting that $P2X_{1,3}$ receptors in the spinal cord is involved in the development of tactile allodynia. PPADS, reduced but did not block completely PAF-induced tactile allodynia even at high dosage, while TNP-ATP, an antagosint of P2XR subtypes $P2X_{1-4}R$ completely inhibited the tactile allodynia. Thus, it may be suggested that PAF-induced tactile allodynia is mediated by stimulation of several P2X receptor subtypes such as $P2X_{1,3,4}$ receptors in the spinal cord. It has been reported that ATP is co-released with other neurotransmitter such as GABA in the spinal cords (Jo and Schlichter, 1999). Whether PAF stimulates ATP release remains to be elucidated.

Although the mechanisms for drug-induced tactile allodynia remain unclear, activation of the glutamate/NO system is thought to play an important role in drug-induced tactile allodynia. This idea is based on the following evidence; 1) drugs-induced tactile allodynia is blocked by NMDA receptor antagonists (Fukuhara et al., 2000; Ishikawa et al., 2000; Minami et al., 1994; 1995; 2001; Onaka et al., 1996; Turnbach and Randich, 2002), NO synthetase inhibitor (Fukuhara et al., 2000; Minami et al., 1994; 1995) or eliminated in mice genetically disrupted with GluR subtype (Minami et al., 2001), 2) intrathecal administration of glutamate and NO donors induced tactile allodynia in conscious mice (Minami et al., 1994), 3) PGE_2 stimulated

glutamate release (Malmberg et al., 1995; Nishihara et al., 1995) and NO release (Sakai et al., 1998) in the spinal cord. PAF-induced tactile allodynia was also inhibited by the intrathecal injection of MK 801, a noncompetitive antagonist of NMDA receptor, and of 7-NI, an inhibitor of NO synthetase. Consistent with these previous findings, the present results suggested that the activation of spinal PAF receptors elicits tactile allodynia via the pathway of P2X receptors, NMDA receptors and NO.

Systemic administration of capsaicin to neonatal mice is known to selectively destroy capsaicin-sensitive neurons (Jancsó et al., 1977). Tsuda et al. (2000) reported that thermal hyperalgesia induced by α,β-methylene ATP injection into the rat hindpaw were lost in neonatally capsaicin-treated adult rats, while mechanical tactile allodynia remained, suggesting that the mechanical tactile allodynia is signaled by distinct primary afferent neurons from that for thermal hyperalgesia. We have also observed that thermal hyperalgesia induced by intrathecally administered α,β-methylene ATP was sensitive to capsaicin-treatment while tactile allodynia was insensitive. However, tactile allodynia as well as thermal hyperalgesia induced by intrathecal PAF were disappeared in capsaicin-treated mice, nevertheless PAF-induced tactile allodynia is suggested to be mediated by spinal ATP/P2X receptor activation. One exponent for the difference between PAF- and α,β-methylene ATP-induced tactile allodynia in capsaicin sensitivity could be possible as follow. Several ATP receptor subtypes were expressed both presynaptically in the terminals of primary afferent fibers where the activation of presynaptic P2X receptors elicits glutamate release (Gu and MacDermott, 1997) and

postsynaptically in dorsal horn (Brake et al., 1994; Chen et al., 1995; Cook et al., 1997). Therefore, exogenously administered α,β-methylene ATP into the spinal cord can act on both pre-synaptic P2X receptors located in either capsaicin-sensitive or insensitive neurons and post-synaptic P2X receptors. This may explain the insensitivity of α,β-methylene ATP-induced tactile allodynia to capsaicin. The present evidence that PAF-induced tactile allodynia was lost in capsaicin-treated mice would suggest that ATP is selectively released by PAF from capsaicin-sensitive neurons or endogenously derived ATP by PAF acts on capsaicin-sensitive neurons. The evidence that neonatal capsaicin treatment eliminated NMDA-induced tactile allodynia (Minami et al., 2001) further support NMDA-mediation of PAF-induced allodynia via capsaicin-sensitive neuron.

For the source of PAF in spinal cord, glial cells or immune and inflammation related cells could be responsible in pathological states. PAF levels have been shown to be elevated in spinal cord injured (Lindsberg et al., 1990) and contributes to the robust inflammatory responses in acute phase and spread of secondary injury (Faden and Halt, 1992; Hostettler and Carlson, 2002). The significant relevance of microglia to pain hypersensitivity has been demonstrated; the number of microglia accumulated in the dorsal horn on the side of nerve injury model (Aldskogius and Kozlva, 1998), and an activation of P2X4 receptors in spinal hyperactive microglia mediates tactile allodynia after nerve injury via the activation of p38MAPK signaling pathway (Tsuda et al., 2003; 2004). Stimulated microglia cells produces PAF (Jaranowska et al., 1995). PAF

receptor mRNA and functional expression are the most abundant in microglia (Mori et al., 1996) and PAF functions as a potent chemotactic factor of microglia (Aihara et al., 2000). In addition, PAF receptors function to stimulate the production of NO (Cardile et al., 1996), PGE_2 (Teather and Wurtman, 2003) or NGF (Brodie, 1995) in astrocytes. The expression of mRNA of PAF receptor by RT-PCR in spinal cord in the present study may reflect the gene of glial cells. Thus, there is a possibility that the interaction between glia and neuron mediated by PAF contributes to form network for pain signals for hypersensitivity in pathological states. Taken together, PAF may be produced in a variety of cells in spinal cord under pathological conditions and be a candidate for mediator of noxious stimuli.

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Figure legends

Fig. 1. The time course of tactile allodynia induced by intrathecally injected PAF in mice.

A: Tactile allodynia was assessed by light stroking of the flank of each mouse with a paintbrush. Each point represents the average of score evaluated at each time point. B: Tactile allodynia was assessed by measuring the paw withdrawal threshold in response to probing with von Frey hairs. Control mice were injected with vehicle; artificial cerebrospinal fluid (ACSF). N=12-19 mice per group.

Fig.2. The dose-response relationship of PAF- and lyso-PAF-induced tactile allodynia in mice (A). Tactile allodynia was assessed by light stroking of the flank of each mouse with a paintbrush. Each point represents the average of cumulative score evaluated every 5 min for 60 min. B: Blockade of PAF-induced tactile allodynia by PAF receptor antagonists. Antagonists were injected intrathecally in a solution containing PAF (simultaneously) or 60 min before the injection of PAF. N=9-20 mice per group. **$p<0.01$ compared with the vehicle (i.t.)-injected group (Fisher's PLSD test).

Fig.3. RT-PCR analysis of the expression of mouse PAF receptor mRNA

cDNA fragments of mouse PAF receptor (530 bp) were amplified from DRG (lane 1 and 5), dorsal horn (SP; lane 2 and 6), and lung (lane 3 and 7) cDNA, as described in

Materials and Methods. Lanes 5, 6, and 7; negative controls, in which RT was omitted. Lane 4; positive control, cDNA of mouse PAF receptor.

Fig.4. Effects of P2X receptor antagonists (PPADS and TNP-ATP), NMDA receptor antagonist (MK 801) and NO synthase inhibitor (7-NI) on PAF-induced tactile allodynia in mice. Tactile allodynia was assessed by light stroking of the flank of each mouse with a paintbrush after the intrathecal injection of PAF, 0.1 pg. Each point represents the average of cumulative score evaluated every 5 min for 60 min. Intrathecal injection of PPADS and TNP-ATP were performed simultaneously with PAF. MK 801 was injected intrathecally 60 min before the injection of PAF. 7-nitroindazole (7-NI) was injected intraperitoneally 15 min before the injection of PAF. N=10-20 mice per group. **$p<0.01$ compared with the vehicle-injected group (Fisher's PLSD test).

Fig. 5. Effects of PAF and α,β-methylene ATP on thermal hyperalgesia and tactile allodynia in control and neonatally capsaicin-treated adult mice. A: Thermal hyperalgesia was evaluated by warm-water tail-flick test 15 min after the intrathecal injection of ACSF, PAF and α,β-methylene ATP (α,β-me-ATP). B: Tactile allodynia was assessed by light stroking of the flank of the mice with paintbrush after the intrathecal injection of ACSF, PAF and α,β-me-ATP. Each point represents the average of cumulative score evaluated every 5 min for 60 min. C: Tactile allodynia was assessed by measuring paw withdrawal threshold in response to probing with von Frey

hairs at 15 min after the intrathecal injection of ACSF, PAF and α,β-me-ATP. N=8-30 mice per group. #p<0.05, ##p<0.01 compared with the respective ACSF (i.t.)-injected group (Duncan's test). **p<0.01 compared with the respective ACSF (i.t.)-injected group (Fisher's PLSD test). ††p<0.01 compared with the respective vehicle (s.c.) treated group (Student's *t* test).

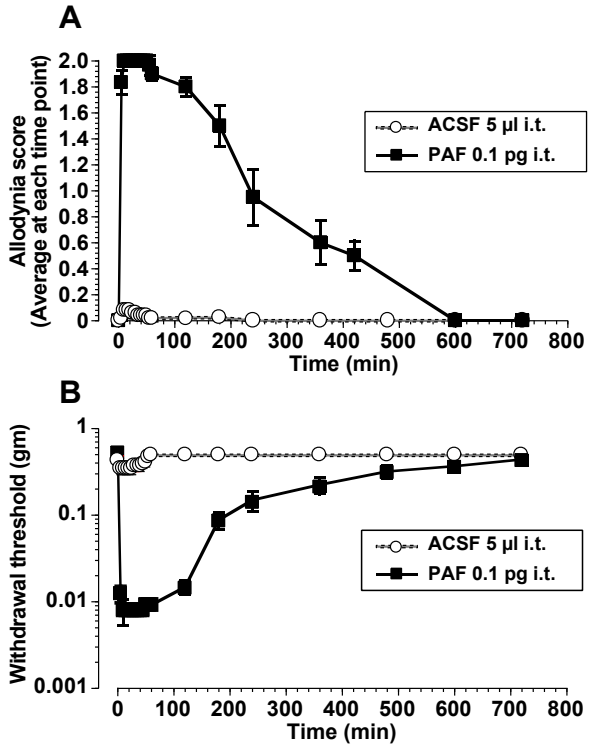
A
Allodynia score
(Average at each time point)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
600
700
800
Time (min)
ACSF 5 µl i.t.
PAF 0.1 pg i.t.
B
Withdrawal threshold (gm)
1
0.1
0.01
0.001
0
100
200
300
400
500
600
700
800
Time (min)
ACSF 5 µl i.t.
PAF 0.1 pg i.t.

Fig. 1.

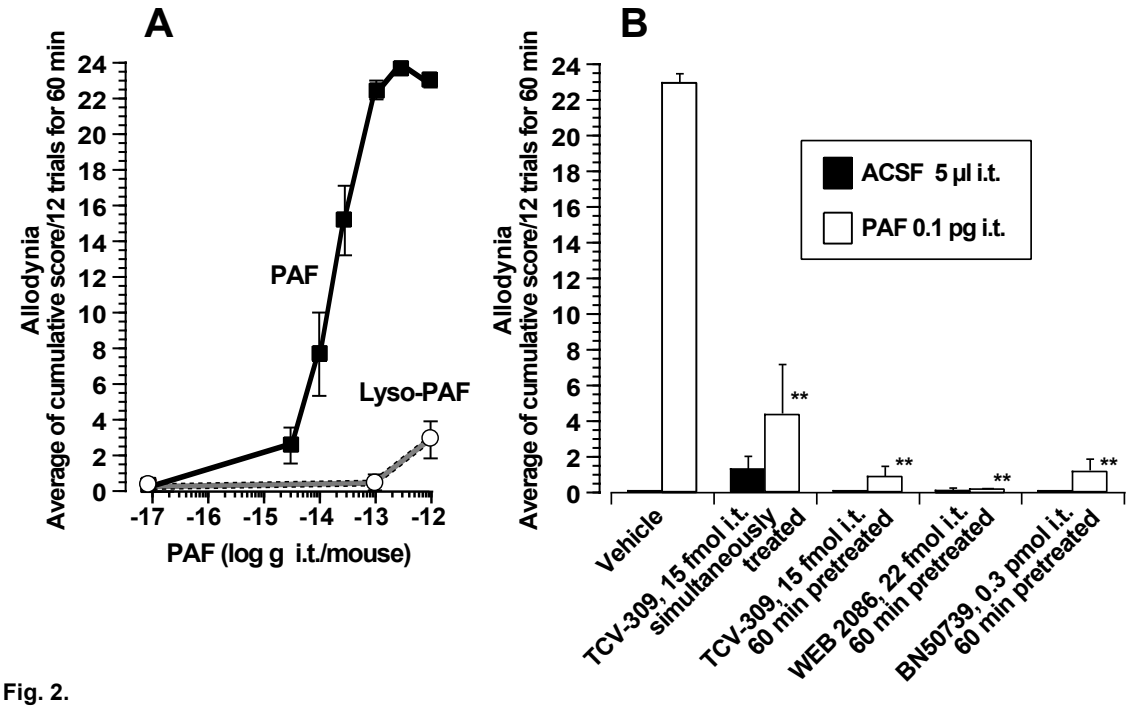
A
Allodynia
Average of cumulative score/12 trials for 60 min
PAF
Lyso-PAF
PAF (log g i.t./mouse)
B
ACSF 5 µl i.t.
PAF 0.1 pg i.t.
Vehicle
TCV-309, 15 fmol i.t. simultaneously treated
TCV-309, 15 fmol i.t. 60 min pretreated
WEB 2086, 22 fmol i.t. 60 min pretreated
BN50739, 0.3 pmol i.t. 60 min pretreated

Fig. 2.

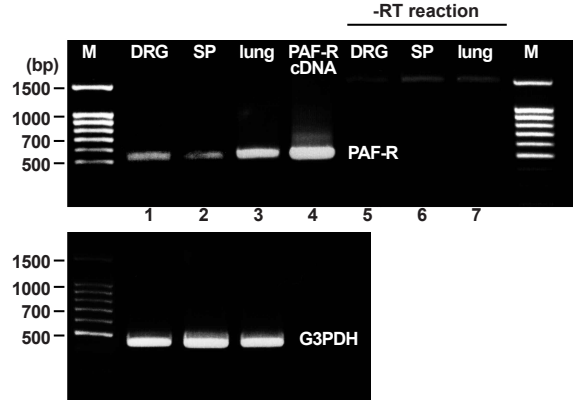
-RT reaction
M
DRG
SP
lung
PAF-R cDNA
DRG
SP
lung
M
(bp)
1500
1000
700
500
PAF-R
1
2
3
4
5
6
7
1500
1000
700
500
G3PDH

Fig. 3.

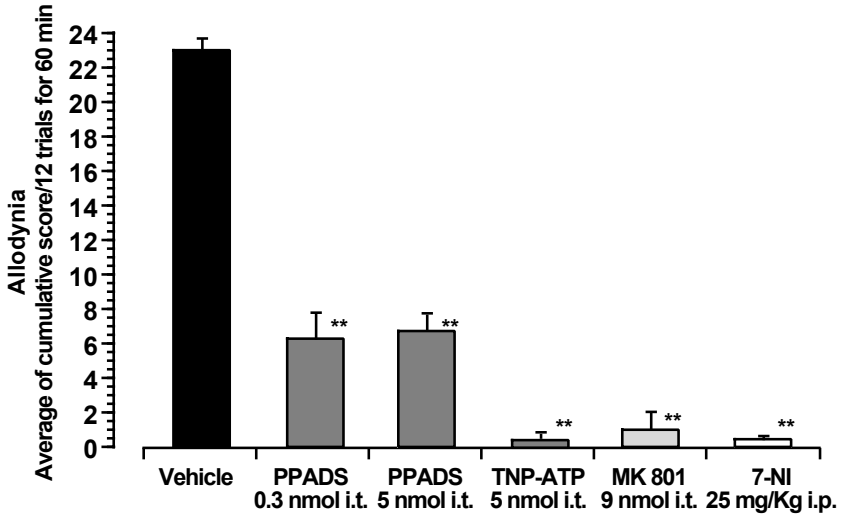
Allodynia
Average of cumulative score/12 trials for 60 min
24
22
20
18
16
14
12
10
8
6
4
2
0
**
**
**
**
**
Vehicle
PPADS
0.3 nmol i.t.
PPADS
5 nmol i.t.
TNP-ATP
5 nmol i.t.
MK 801
9 nmol i.t.
7-NI
25 mg/Kg i.p.

Fig. 4.

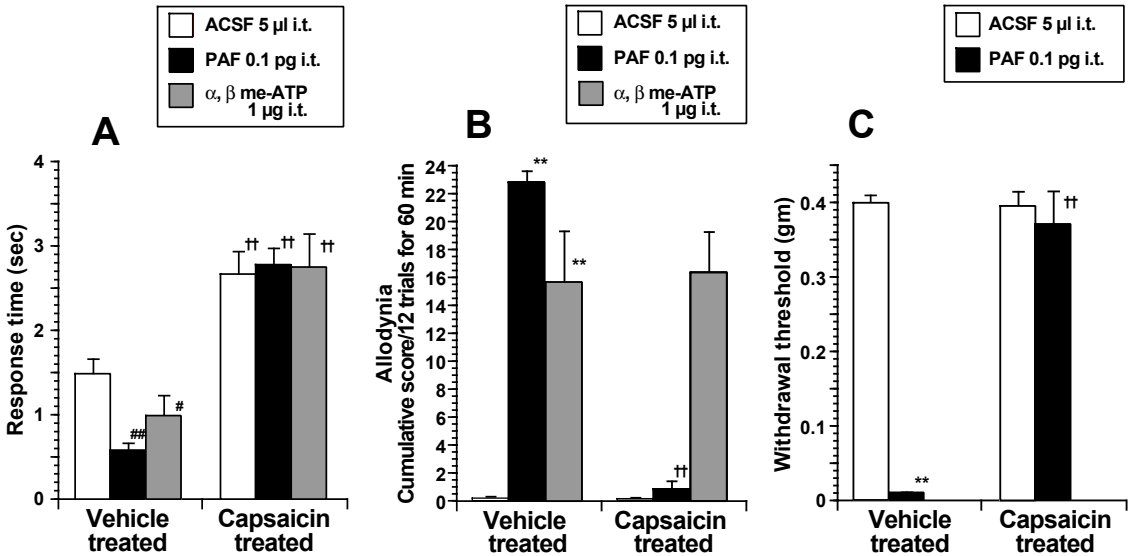
ACSF 5 µl i.t.
PAF 0.1 pg i.t.
α, β me-ATP 1 µg i.t.
A
Response time (sec)
0
1
2
3
4
##
#
††
††
††
Vehicle treated
Capsaicin treated
B
Allodynia Cumulative score/12 trials for 60 min
0
2
4
6
8
10
12
14
16
18
20
22
24
**
**
††
Vehicle treated
Capsaicin treated
C
Withdrawal threshold (gm)
0
0.1
0.2
0.3
0.4
**
††
Vehicle treated
Capsaicin treated

Fig. 5.