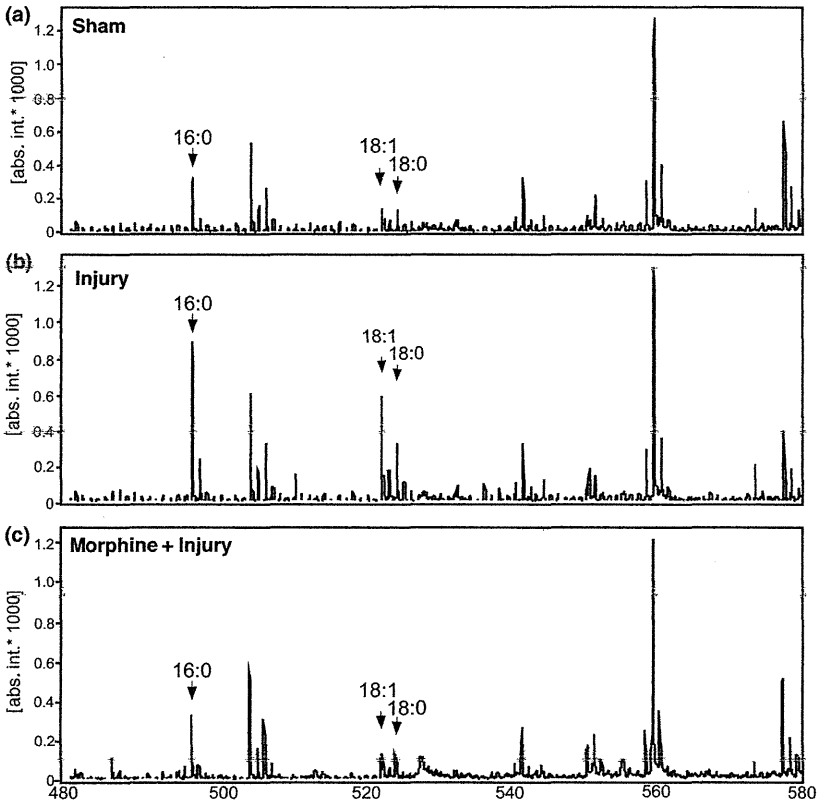

Fig. 6 Pre-emptive inhibition of injury-induced LPC production by morphine. The representative chart of mass spectra of spinal cord preparations treated with sham-operation (a), SCNI (b) and morphine administration (3 nmol, i.c.v.) 30 min prior to SCNI (c) are shown.

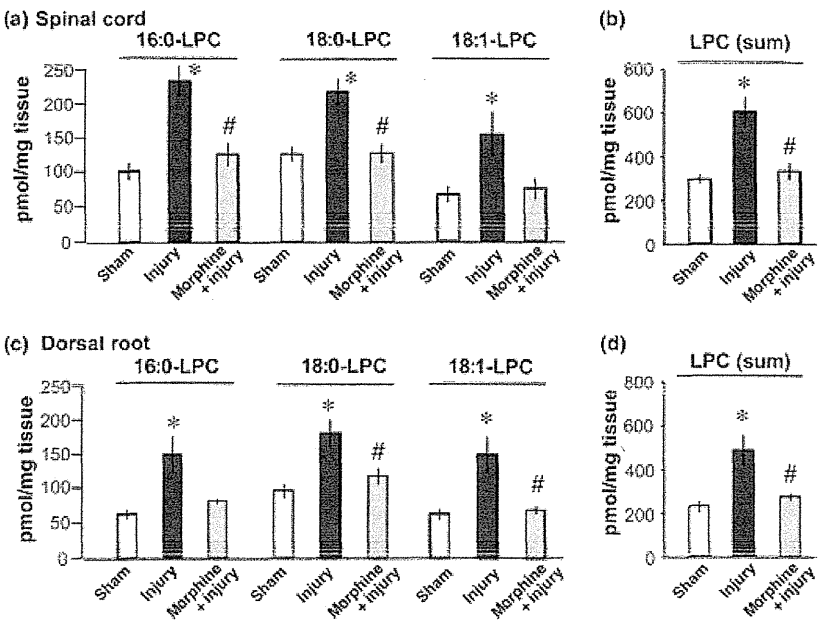

Fig. 7 Quantitative analysis of injury-induced LPC production and its inhibition by morphine pre-treatment. The tissue content of LPC species in spinal cord (a, b) or dorsal root preparations (c, d) treated with injury with or without morphine pre-treatment are quantified. Data are expressed as the mean ± SEM from experiments using 3–6 mice. $^{*}p < 0.05$, versus sham-operation preparations. $^{\#}p < 0.05$, versus injury-operation preparations.

Discussion

The present study shows that the successful simultaneous measurement of several species of LPC molecules by use of NALDI-TOF-MS analysis, which has been developed recently as a matrix-free/surface-assisted alternative to matrix-assisted laser desorption/ionization-TOF-MS (Wyatt *et al.* 2010). This new MS analysis enables to minimize the

background signal ion-peaks in the low molecular region observed in the case with matrix-assisted laser desorption/ionization-TOF-MS (Wyatt *et al.* 2010) and seems to have some advantages in terms of simple and rapid procedures, compared to the case with liquid chromatography–MS, which requires the determination of separation condition for each species of LPC molecules (Morishige *et al.* 2010). In terms of resolution and identification of each species of LPC molecules, there was no substantial problem by use of MS–MS analysis of each signal ion-peak and following data base search. Most important issue was the relatively high yield of recovery from the fresh tissues (spinal cord and dorsal root). By simplifying the extraction and purification processes, we succeeded in the establishment of 70–89% recovery. The species of LPC molecules we obtained from these preparations were saturated LPC molecules such as 16:0-LPC, 18:0-LPC and 18:1-LPC, but not highly unsaturated LPC molecules such as 18:2-LPC, 18:3-LPC or 20:4-LPC or LPC molecules with longer chain, which are detected in plasma (Takatera *et al.* 2006). There is a report that saturated and unsaturated fatty acids are mainly located at the position of sn-1 and sn-2, respectively (Yamashita *et al.* 1997). Thus, it is speculated that phospholipase A_2 (PLA_2) plays important roles in the injury-induced generation of LPCs than PLA_1. This speculation is consistent to previously observation that nerve injury causes an activation of PLA_2 in the spinal cord within 60 min (Ma *et al.* 2010b).

A series of our studies have revealed that LPA and LPA_1 receptor signaling play key roles in the initiation of nerve injury-induced neuropathic pain and its underlying mechanisms such as demyelination of dorsal root, up-regulation of $Ca_v\alpha2\delta1$ in DRG and protein kinase Cγ in the dorsal horn (Inoue *et al.* 2004; Ueda 2006, 2008). Most recently, we have reported that LPA production evaluated by biological assay using B103 cells expressing LPA_1 occurs only in the ipsilateral dorsal horn and dorsal root, but not in the spinal and sciatic nerve or in corresponding contralateral regions (Ma *et al.* 2010b). These findings are consistent with the finding that nerve injury-induced LPA_1-mediated demyelination was only observed in the ipsilateral dorsal root, but not spinal nerve or sciatic nerve (Ma *et al.* 2010b; Nagai *et al.* 2010), suggesting that LPA produced in the spinal cord is transported, but does not diffuse to the dorsal root. In addition, we observed that the LPA production was also reproduced in the *in vitro* study using spinal cord slices by the combined application of substance P and NMDA, which are supposed to mimic intense nociceptive transmission (Inoue *et al.* 2008b). As the presence of ATX was essential for LPA production in these studies, the ATX-mediated conversion of LPC appears to be involved in the LPA production, being consistent to the findings that nerve injury-induced demyelination and neuropathic pain were significantly attenuated in $atx^{+/-}$-mice (Inoue *et al.* 2008a; Ma *et al.* 2009b; Nagai *et al.* 2010). A recent report demonstrated that significant amounts of ATX are present in the cerebrospinal fluid, but the levels of LPC and LPA are very low, suggesting that extracellular LPC production would be a rate-limiting step for LPA production (Nakamura *et al.* 2009). In addition, as the measurement of LPA requires negative mode in MS analysis, which is much less sensitive than the case with positive mode used for LPA measurement (Tanaka *et al.* 2004), we were unable to directly measure LPA in the present study.

For these reasons, we attempted to measure the change in LPC levels after the nerve injury. In the time–course study, it was revealed that the levels of 16:0-LPC, 18:0-LPC and 18:1-LPC in the spinal cord and dorsal horn were all significantly increased at 75 min after the nerve injury, but followed by rapid decline. Most interestingly, these levels were further increased at 120 min in $atx^{+/-}$-mice, although they were substantially equivalent to those in WT-mice at 75 min. The fact that ATX-mediated conversion of LPC to LPA (deduced from the detected losses of LPC) is late may be explained by the view that the step of transport of newly generated LPC to ATX in extracellular space or CSF is time-consuming process. But it is unlikely that the activation of ATX is time-consuming, because ATX in CSF from naive mice already possesses activities to convert LPC to LPA (Inoue *et al.* 2008b). This view is consistent to the previous finding that *in vitro* LPA production using spinal cord slices absolutely requires the addition of exogenous ATX (Inoue *et al.* 2008b). The injury-induced increase of LPC in the ipsilateral dorsal root/mouse was approximately 30% of ipsilateral dorsal half of spinal cord. Thus, it is suggested that LPC in its form is transported to and converted to LPA at the dorsal root, taking into account the previous findings that injury-induced LPA production and LPA_1-mediated demyelination were only observed in the dorsal root, but not spinal nerve or sciatic nerve (Ma *et al.* 2010b; Nagai *et al.* 2010). However, the possibility that injury-induced LPC production occurs in the dorsal root as well as spinal cord cannot be excluded. The fact that peak effects of LPC production in $atx^{+/-}$-mice were observed at 120 min after the injury was consistent with the pharmacological study to see the blockade of nerve injury-induced neuropathic pain using Ki16425, a short-lived LPA_1 antagonist (Ma *et al.* 2009a).

The most important finding is that the pre-treatment with morphine abolished the nerve injury-induced elevation of LPC levels. Nerve injury-induced neuropathic pain has a nature to sustain for at least several weeks. It is accepted that this nature is closely related to memory processes, which include long-term potentiation at the level of dorsal horn and brain, long-lasting genetic and epigenetic changes in DRG neurons (Navarro *et al.* 2007; Zhuo 2007; Uchida *et al.* 2010) and sustained cytokine networks among neurons, astrocytes and microglia (Scholz and Woolf 2007; Milligan and Watkins 2009). Therefore, the complete inhibition of intense nociceptive signal may prevent such memory

processes for the manifestation of neuropathic pain. The pre-emptive morphine analgesia in clinic is based on this concept (Richmond *et al.* 1993). Indeed, we have successfully reproduced similar pre-emptive analgesia in the experimental animal model, in which systemic and central pre-treatments with morphine abolished the nerve injury-induced neuropathic pain (Rashid and Ueda 2005). In this report, the administration of morphine 30 min prior to nerve injury completely abolished neuropathic pain and its underlying mechanisms such as long-lasting PKCγ up-regulation in the dorsal horn. Thus, the present finding showing the central morphine-induced complete blockade of nerve injury-induced elevation of LPC may be related to the concept of pre-emptive analgesia, because LPA converted from LPC plays key roles in the initiation of neuropathic pain (Inoue *et al.* 2004; Ueda 2008). The direct evidence measuring LPA would be the next subject to support this view.

In conclusion, the present study firstly demonstrates the simultaneous MS analysis of several species of LPC molecules, and the nerve injury-induced elevation of LPC, which is likely modified by the *in vivo* conversion to LPA by ATX. In addition, the morphine pre-treatment to block the nociceptive signal abolishes the nerve injury-induced LPC production, underlying the mechanisms for pre-emptive analgesia.

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Current Topics

Lipid Mediators and Pain Signaling

Lysophosphatidic Acid as the Initiator of Neuropathic Pain

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The injury-induced intense stimulation of spinal cord neurons causes lysophosphatidic acid (LPA) biosynthesis. LPA_1 receptor activation causes demyelination and sprouting of dorsal root fibers, leading to an induction of synaptic reorganization underlying allodynia, in which innocuous (tactile) stimuli cause intense pain. The LPA_1 signal also initiates the up-regulation of $Ca_v\alpha2\delta1$ in dorsal root ganglion and PKCγ in the dorsal horn, underlying mechanisms for characteristic neuropathic hyperalgesia in myelinated sensory (A-type) fibers. On the other hand, the LPA_3 receptor mediates microglia activation at the early stage after nerve injury and LPA-induced LPA biosynthesis. Thus, both the LPA_1 and LPA_3 receptors play key roles in the initiation step using a feed-forward system for neuropathic pain.

Key words lysophosphatidic acid; demyelination; feed-forward system; neuropathic pain; microglia

1. INTRODUCTION

Chronic pain should be considered disease pain, though acute or nociceptive pain is sometimes viewed as physiological pain because of its bio-alarming roles. Neuropathic pain is a representative chronic pain, and is caused by damage to peripheral or central neurons in the pain pathway.[1—4] This type of chronic pain commonly occurs as a secondary symptom in diseases such as diabetes, cancer, and herpes zoster infection, or as a side effect of chemotherapeutic treatments.[5—8] Neuropathic pain following partial sciatic nerve injury is often characterized by abnormally hypersensitive sensory perception through A-fibers, called hyperalgesia or allodynia, in which innocuous (tactile) stimuli cause intense pain.[9,10] The abnormal sensory perception also includes C-fiber hypoesthesia,[10—12] which indicates sensory loss.[13,14] A number of studies[10,12,15] demonstrate the altered expression of receptors, neuropeptides and ion-channels in sensory fiber neurons, called dorsal root ganglion (DRG) and in the dorsal horn. According to current studies, on the other hand, the sustained activation of non-neuronal cells such as astrocytes and microglia becomes manifest in the mechanisms underlying neuropathic pain.[9,11] In addition to these mechanisms, the demyelination is supposedly related to chronic pain diseases, and to the physical cross-talk/sprouting and ectopic discharge in sensory fibers, all which may underlie neuropathic hyperalgesia and allodynia.[10,12,16] As neuropathic pain is in general resistant to non-steroidal anti-inflammatory drugs (NSAIDs) or morphine, it has been called intractable pain, though a limited number of medicines are currently available or in progress of development in clinics.[17]

Accumulating findings reveal the important insights into both the basic biology and biomedical importance of signaling initiated by lipid mediator lysophosphatidic acid (1-acyl 2-hydroxyl glycerol 3-phosphate, LPA), LPA, a simple lipid with glycerol, fatty acid and phosphate in its structure. LPA plays roles in many cellular processes including cellular proliferation, cell migration, prevention of apoptosis, cytokine and chemokine secretion and smooth muscle contraction.[18—21] LPA receptors activate multiple signaling pathways and multiple G-proteins,[22,23] but LPA_1 signaling is unique, since it has a downstream coupling to $G\alpha_{12/13}$, as well as $G_{i/o}$ and $G_{q/11}$. Regarding neurobiological actions, LPA causes a growth cone collapse on neurons through its receptor LPA_1 and downstream $G\alpha_{12/13}$-RhoA-Rho kinase (ROCK) system,[24,25] and plays crucial roles in neuronal developmental processes, including neurogenesis, neuronal migration, neuritogenesis and myelination.[26,27] In terms of pain regulation, we first reported that LPA causes an activation of peripheral nociceptor endings directly and indirectly through a release of histamine from peripheral cells, such as mast cells.[28,29] Current studies have revealed that LPA plays a key role in the initiation of neuropathic pain.[10,12,30] As the intrathecal LPA-induced abnormal pain shows quite similar characteristics to those in nerve injury-induced neuropathic pain,[30] LPA could be considered as a good tool for the studies of *in vitro* and *in vivo* mechanisms underlying neuropathic pain.[31—37]

2. LPA_1-MEDIATED INITIATION OF NEUROPATHIC PAIN

Among multiple mechanisms involved in the manifestation of nerve injury-induced neuropathic pain, the enhanced expression of $Ca_v\alpha2\delta1$ expression in DRG, PKCγ expression and microglial activation in dorsal horn seem to be representative mechanisms for hyperalgesia, while the demyelination and following physical cross-talk among sensory fibers may underlie the mechanisms for allodynia. We were the first to find that nerve injury-induced neuropathic pain behaviors were substantially abolished in $lpa_1^{-/-}$ mice.[30] As there was no significant change in the basal nociceptive threshold, it is evident that endogenous levels of LPA do not affect this threshold and that newly produced LPA following nerve injury causes neuropathic pain mechanisms. In order to study the critical time period for LPA_1 receptor-mediated sig-

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naling underlying neuropathic pain and its mechanisms, Ki-16425, a short-lived antagonist of LPA_1 receptor was used.[33] The Ki-16425 blockade of nerve injury-induced neuropathic pain and upregulation of $Ca_v\alpha2\delta1$ expression was maximal as late as 3 h after the injury but not after this critical period. These results suggest that LPA_1 signaling, which underlies the development of neuropathic pain, works at an early stage of the critical period after nerve injury.

3. LPA_1-MEDIATED UPREGULATION OF KEY MOLECULES UNDERLYING HYPERALGESIA

Upregulation of $Ca_v\alpha2\delta1$ expression and subsequent increased pain transmission may underlie the mechanism for hyperalgesia. Gabapentin and pregabalin, which are widely used in clinics for neuropathic pain, are known to inhibit the pain transmission by inhibiting this subunit $Ca_v\alpha2\delta$ activity.[38] The expression of $Ca_v\alpha2\delta1$ is observed only in the small C-fiber neurons of DRG in naïve animals.[8] After partial injury of the sciatic nerve, most of the A-fiber neurons also express this subunit.[30] Pretreatment with Clostridium botulinum C3 exoenzyme (BoNT/C3), an inhibitor of RhoA, abolished this additional expression in A-fiber neurons. Quite similar results of additional $Ca_v\alpha2\delta1$ expression in these neurons and its blockade by BoNT/C3 were observed when LPA was intrathecally injected only once. On the other hand, the nerve injury- or LPA-induced up-regulation of $Ca_v\alpha2\delta1$ was abolished in $lpa_1^{-/-}$ mice.[10,30]

N-Methyl-D-aspartate (NMDA) receptor plays key roles in the transmission of pain in naïve and chronic pain status.[39] Recent studies reported that NMDA receptor is transactivated through EphB signaling initiated by interaction with presynaptic Ephrin B1.[40—42] When the profiling of LPA-induced and BoNT/C3 reversible genes in DRG was performed, *ephrin B1* was found in 82 unique genes.[36] Further characterization revealed that antisense oligonucleotide for *ephrin B1* largely abolished the LPA-induced mechanical allodynia, thermal hyperalgesia and hypersensitivity to electrical stimuli through Aδ and Aβ-fibers. As EphrinB1-Fc caused neuropathic pain-like behaviors in an NMDA receptor antagonist MK-801-reversible manner, LPA-mediated Ephrin B1 upregulation may also contribute to the mechanisms underlying neuropathic hyperalgesia.

As seen in the case with nerve injury, intrathecal injection of LPA causes an up-regulation of PKCγ at the substantia gelatinosa of spinal dorsal horn.[30] This change is known as so-called wind-up facilitation or hyperalgesia observed in neuropathic pain.[43] The up-regulation by nerve injury or LPA was also abolished by BoNT/C3 pretreatment and in $lpa_1^{-/-}$ mice.

4. LPA_1-MEDIATED DEMYELINATION UNDERLYING ALLODYNIA

It is known that many demyelinating diseases accompany chronic pain, as seen in the cases with Guillain–Barre syndrome[44] and multiple sclerosis.[45] Demyelination and subsequent physical cross-talk[12,31] and ectopic discharges due to accumulation of sodium channels[46] have been speculated as the mechanisms underlying neuropathic pain. Nerve injury- and intrathecal LPA-induced demyelination of dorsal root fibers through LPA1 receptor activation are evidenced by the down-regulation of myelin proteins, such as myelin basic protein (MBP), myelin protein zero (P0) and myelin-associated glycoprotein (MAG).[31,35,37] The *ex vivo* studies using dorsal root fibers also demonstrated that the addition of LPA causes demyelination within 24 h in scanning and transmission electron microscopy (SEM and TEM) analyses.[31] As well as typical demyelination of A-fibers, there was direct contact between neighboring C-fibers.[31] In co-culture experiments using myelinated fibers built up with isolated DRG neurons and Schwann cells, the addition of LPA also causes a sequential morphological change, in an order of collapse of growth cone at 1 h, sprouting at the nerve endings at 8 h and axon at 18 h and complete spreading of myelinated Schwann cell at 36 h.[47] As the down-regulation of myelin proteins was abolished by the pretreatment with BoNT/C3 or ROCK inhibitor Y-27632, the major pathway is presumably mediated by the LPA_1-$G_{12/13}$-RhoA-ROCK system. Indeed, the LPA-induced down-regulation of myelin protein genes in *in vivo* and *ex vivo* studies using dorsal root fibers was abolished by Y-27632.[30] Most recently, there is a report that c-Jun plays a negative regulator role in the myelin protein gene expression.[48,49] As the RhoA-ROCK system is reported to stimulate c-*jun* expression through JNK activation,[50] it is speculated that the sequential activation of LPA_1-$G_{12/13}$-RhoA-ROCK-JNK, followed by up-regulation of c-*jun*, leads to a negative regulation of myelin protein gene expression. In terms of signal transduction, it is also known that LPA_1 causes the stress-fiber formation and actin rearrangement through $G_{12/13}$-RhoA-ROCK activation.[51,52] Thus, such LPA_1-mediated morphological changes may contribute to rapid mechanisms underlying demyelination without any changes in myelin protein levels.

However, current studies demonstrated a different mechanism independent of the $G_{12/13}$-RhoA-ROCK system.[37] Intrathecal injection of LPA causes a rapid down-regulation of myelin-associated glycoprotein (MAG). By surveying protease inhibitors, the calcium-activated neutral serine protease, calpain was found to play a major role in the down-regulation of MAG. Pretreatment with calpain inhibitors abolished the MAG down-regulation and significantly attenuated the LPA-induced neuropathic pain-like behaviors. Interestingly, calpain activation in dorsal root was only observed by nerve injury and abolished in $lpa_1^{-/-}$ mice, while it was not observed by the pretreatment with inflammatogenic Complete Freund Adjuvant (CFA). Furthermore, calpain inhibitors reversed the nerve injury-induced neuropathic pain, but not CFA-induced pain. Although details remain elusive, it is suggested that the LPA_1-$G_{q/11}$-PLC activation system may play a role in the calcium-mediated protein degradation. Thus, several cellular mechanisms following LPA_1 stimulation may contribute to the demyelination.

In relation to allodynia, the loss of insulation of sensory fibers following LPA1-mediated demyelination may cause a physical cross-talk (or electric synapse/ephapse) among innocuous Aβ fibers and noxious C- or Aδ-fibers,[10,12] which in turn leads to an abnormal pain transmission allodynia. The down regulation of MAG may also cause the sprouting, which is induced by a loss of negative signal through the NOGO/p75 receptor complex,[12] as seen in Fig. 1.

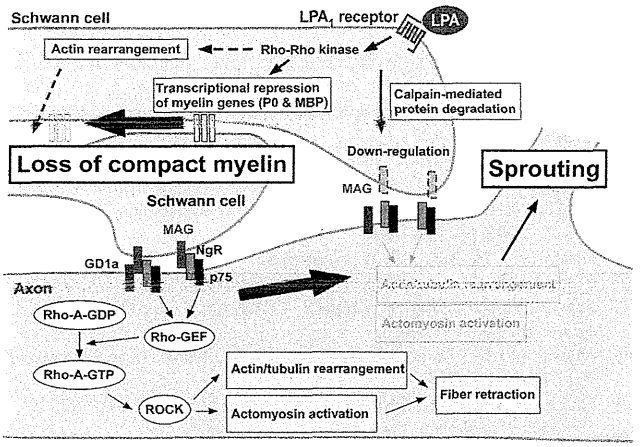

Fig. 1. Schematic Model of LPA_1-Mediated Demyelination and Sprouting

The stimulation of LPA_1 receptor on myelinated Schwann cells causes rapid down-regulation of myelin proteins through calpain-mediated down regulation and gene silencing. The downregulation of compact myelin proteins including myelin binding protein (MBP) and myelin protein zero (P0) leads to a loosening of the myelin sheath. The loss of another myelin protein, myelin associated glycoprotein (MAG), which couples to NOGO/p75 complex (NgR/p75) and RhoA-ROCK system, results in disinhibition of sprouting, possibly through rearrangements of actin and tubulin polymers.

5. LPA_1-MEDIATED SYNAPTIC REORGANIZATION IN PAIN PATHWAY

Extracellular signal-regulated $kinase_{1/2}$ ($ERK_{1/2}$), representing one of the major subfamilies of mitogen-activated protein kinases (MAPKs), is phosphorylated following membrane depolarization and Ca^{2+} influx.[53] It is known that $ERK_{1/2}$ is immediately activated after noxious stimulation in DRG neurons and spinal dorsal horn in a stimulus intensity-dependent manner.[54,55] Therefore, ERK phosphorylation (pERK) could be a biochemical marker of activated neurons, allowing us to visualize the pain-signaling pathways and more objective evidence of neurotransmission. A significant number of neurons at the superficial layer of spinal dorsal horn became pERK-positive following the stimulation of nociceptive C- and Aδ-fibers by use of Neurometer®, while no neuron became pERK-positive by innocuous Aβ stimulation.[56] However, following sciatic nerve injury, Aβ-stimulation showed a significant number of pERK-positive neurons at the superficial layer of dorsal horn, where the innervation with noxious C- or Aδ-fibers is observed. This mechanism seems to explain why the tactile stimuli (through Aβ) cause intense pain. Such nerve injury-induced synaptic reorganization was also abolished in $lpa_1^{-/-}$ mice.[57]

6. LPA_3-MEDIATED LPA BIOSYNTHESIS

The LPA_1-mediated demyelination following partial injury of sciatic nerve was only observed in dorsal root, but not spinal nerve or sciatic nerve.[35] Such dorsal root-specificity was also observed in the down-regulation of myelin-associated glycoprotein (MAG), which plays a key role in the regulation of axonal sprouting.[12] As the addition of LPA causes the demyelination or down-regulation of MAG in all these sensory nerve regions, the dorsal root specificity following sciatic nerve injury seems attributable to the localized LPA production. The most probable source of LPA would be from spinal cord, since the dorsal root as well as spinal cord is within the subarachnoid.

Recent studies demonstrated that the intense stimulation of spinal cord neurons causes synthesis of lysophosphatidyl choline (LPC), which is in turn converted to LPA by lysophospholipase D or autotaxin (ATX).[21,58] In these experiments, the combination of SP and NMDA, both of which cause an activation of representative target receptors for different types of primary afferent neurons, produces LPC.[58] As no significant synthesis of LPC occurs by single application of either compound, it is presumed to require an intense signal caused by nerve injury, but not by regular pain transmission. Recent studies revealed that phosphatidyl choline is converted to LPC by $cPLA_2$ or $iPLA_2$, both of which are regulated by Ca^{2+}-related mechanisms following NK_1 and NMDA receptor activation. Thus produced LPC in the spinal cord is converted to LPA at the spinal cord and dorsal root by an action of ATX leading to demyelination and $Ca_v\alpha 2\delta 1$ up-regulation.[30]

Current studies demonstrated that the intrathecal injection of LPC caused neuropathic pain-like behaviors, and these behaviors were abolished in $lpa_1^{-/-}$ mice or diminished by 50% in $ATX^{+/-}$ mice.[34] The study to examine the biochemical evidence for this LPC-induced LPA production revealed that the amounts of LPA were much higher than that expected from the simple conversion through ATX.[33] Detailed *ex vivo* culture studies using spinal cord slices revealed that LPA-induced amplified production of LPA was abolished in the preparation derived from $lpa_3^{-/-}$ mice. This observation was supported by the behavioral studies, in which nerve injury-induced neuropathic pain was abolished in $lpa_3^{-/-}$ as well as $lpa_1^{-/-}$ mice. Thus, it is suggested that LPA_1 receptor plays direct roles in molecular machineries underlying neuropathic pain, while LPA_3 receptor and ATX play roles in the synthesis of LPA.

7. MICROGLIA-MEDIATED AMPLIFICATION OF LPA BIOSYNTHESIS

It is now considered that microglia and astrocytes as well as neurons have functional roles in the creation and maintenance of chronic neuropathic pain. Spinal cord glial activation seems to be a common underlying mechanism that leads to chronic pain.[59,60] However, it remains to be learned what signal initiates the glial activation, which is assumed to play a key role in the maintenance of neuropathic pain. An attempt to see the effects of LPA in activating microglia revealed that LPA caused an increase in the expression of brain-derived neurotrophic factor (BDNF) in a primary culture of rat microglia, which express LPA_3, but not LPA_1 or LPA_2 receptors.[61] These actions were mediated by a release of ATP through activation of LPA_3, $G_{q/11}$ and phospholipase C. The released ATP or ectopically converted ADP may in turn cause membrane ruffling (a sign of chemotaxis) *via* $P2Y_{12}$ receptors and $G_{i/o}$ activation, and BDNF expression *via* activation of $P2X_4$ receptors. Current studies using the microglia inhibitor minocycline revealed that LPA-induced microglia activation functions in the early stage development, but not in the late stage maintenance of neuropathic pain.[62] In this study, the early treatment with minocycline abolished

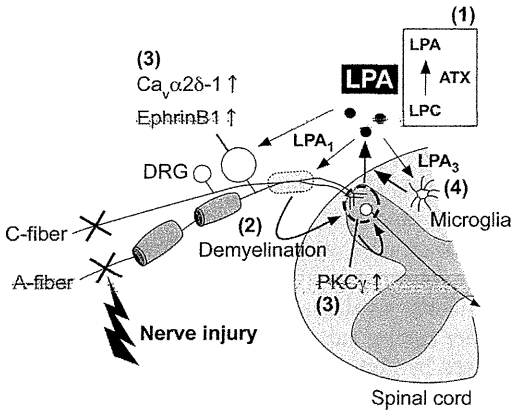

Fig. 2. Working Hypothesis of LPA-Mediated Feed-Forward System Underlying Molecular Mechanisms for Neuropathic Pain

Following (sciatic) nerve injury, intense pain signals activate neurons in spinal dorsal horn and cause a biosynthesis of LPC, which is then converted to LPA by ATX, depicted by (1). Thus produced LPA is transported within 3 h after injury to the ipsilateral side of dorsal root, where it causes demyelination and sprouting, underlying allodynia (2). LPA also causes up-regulation of $Ca_v\alpha2\delta1$ and EphrinB1 in DRG (3), and PKCγ in the spinal dorsal horn, underlying hyperalgesia (3). These mechanisms are believed to cause an activation of spinal neurons, leading to further LPA synthesis, as a feed-forward system. The amplification of LPA biosynthesis also occurs through an activation of LPA_3 receptor and microglia (4). All these mechanisms contribute to the initiation of neuropathic pain.

the LPA-induced and nerve injury-induced neuropathic pain, LPA synthesis and its underlying activation of synthetic enzymes, cytosolic phospholipase A_2 ($cPLA_2$) and calcium-independent PLA_2 ($iPLA_2$). As the post-treatment with minocycline failed to attenuate the established neuropathic pain, microglial activation following LPA_3 signaling seems to take part in the initiation mechanisms for neuropathic pain.

8. CONCLUSION

In the proposed working hypothesis, the LPA-mediated feed-forward system underlying molecular mechanisms for neuropathic pain, the LPA production following intense and mixed pain signals to spinal dorsal horn neurons is the initial mechanism (Fig. 2). Thus produced LPA has two mechanisms: one is related to the actions as an amplifying signal for further LPA production through LPA_3 and microglia activation. The other mechanism is related to the LPA_1-mediated actions as a reverse signal to cause dorsal root demyelination, and upregulation of $Ca_v\alpha2\delta_1$ and Ephrin B_1 in DRG. Demyelination and subsequent sprouting may lead to a pathological pain synapse by Aβ-fibers, underlying allodynia. Up-regulation of key molecules in DRG enhances the pain transmission and may cause subsequent upregulation of PKCγ in the dorsal horn. Enhanced and pathological pain transmission may also contribute to a biosynthesis of LPA through direct and indirect mechanisms. When we consider the drug development to cure neuropathic pain, LPA receptor antagonists or inhibitors of LPA synthesis would be candidates. For this purpose, we need to examine whether this feed-forward system through LPA biosynthesis also occurs in the late phase of neuropathic pain. Assuming that the feed-forward system through LPA synthesis by intense an pain signal (or injury) is true in the central nervous system, the hypothesis may be further extended to central pain induced by various kinds of stress, spinal (brain) injury or stroke.

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MOLECULAR PAIN

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Antinociceptive effect of cyclic phosphatidic acid and its derivative on animal models of acute and chronic pain

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1. Abstract

Background: Cyclic phosphatidic acid (cPA) is a structural analog of lysophosphatidic acid (LPA), but possesses different biological functions, such as the inhibition of autotaxin (ATX), an LPA-synthesizing enzyme. As LPA is a signaling molecule involved in nociception in the peripheral and central systems, cPA is expected to possess analgesic activity. We characterized the effects of cPA and 2-carba-cPA (2ccPA), a chemically stable cPA analog, on acute and chronic pain.

Results: (1) The systemic injection of 2ccPA significantly inhibited somato-cardiac and somato-somatic C-reflexes but not the corresponding A-reflexes in anesthetized rats. (2) 2ccPA reduced sensitivity measured as the paw withdrawal response to electrical stimulation applied to the hind paws of mice through the C-fiber, but not Aδ or Aβ. (3) In mice, pretreatment with 2ccPA dose-dependently inhibited the second phase of formalin-induced licking and biting responses. (4) In mice, pretreatment and repeated post-treatments with 2ccPA significantly attenuated thermal hyperalgesia and mechanical allodynia following partial ligation of the sciatic nerve. (5) In rats, repeated post-treatments with 2ccPA also significantly attenuated thermal hyperalgesia and mechanical allodynia following chronic sciatic nerve constriction.

Conclusions: Our results suggest that cPA and its stable analog 2ccPA inhibit chronic and acute inflammation-induced C-fiber stimulation, and that the central effects of 2ccPA following repeated treatments attenuate neuropathic pain.

2. Background

Cyclic phosphatidic acid (cPA) was originally isolated from myxoamoebae of a true slime mold, *Physarum polycephalum*, in 1992 [1]. The chemical formula of cPA is similar to that of lysophosphatidic acid (LPA), but cPA has a unique structure with a cyclic phosphate ring at *sn*-2 and *sn*-3 of the glycerol backbone [2]. These features provide cPA with distinct/opposing biological functions from those of LPA. For instance, LPA stimulates cell proliferation and cancer cell invasion, while cPA inhibits these activities [3-8]. Interestingly, LPA is enzymatically generated from transphosphatidylation of lysophosphatidylcholine by autotaxin (ATX) [9], but cPA inhibits ATX activity [10]. Thus, cPA could be an endogenous inhibitor of LPA production through ATX.

Exogenous and endogenous LPA cause acute pain through C-fibers and neuropathic pain [11-13]. Recent studies revealed that nerve injury-induced LPA production and neuropathic pain were significantly attenuated in mice with heterozygous ATX deficiency [14,15]. In this study, we characterized the effects of cPA and its chemically stable analog 2ccPA on acute and neuropathic pain.

3. Methods

3.1. Recording the somato-cardiac sympathetic reflex

The experiments were performed using male Wistar rats (n = 11) weighing 270-370 g anesthetized by

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intraperitoneal (i.p.) application of urethane (1.1 g/kg). A femoral vein was catheterized for intravenous (i.v.) administration of supplemental anesthetics and cPA. A femoral artery was catheterized to record arterial blood pressure and heart rate. The animals were immobilized by gallamine triethiodide (20 mg/kg i.v. as required) and artificially ventilated via tracheal cannula. Ventilation was monitored with a gas analyzer (1H26; NEC San-ei, Tokyo) and adjusted to maintain an end-tidal CO_2 level of 3.0%. Body temperature was maintained at 37.5°C using an automatically regulated heating pad and lamp (ATB-1100; Nihon Kohden, Tokyo). Both vagal nerves were cut at the cervical level to prevent vagal contamination of the recorded sympathetic nerve activity.

With the rat placed in the supine position, the right second costal bone was removed. The right inferior cardiac sympathetic nerve was dissected retropleurally, cut as close to the heart as possible, and covered with warm paraffin oil. Cardiac sympathetic efferent nerve activity was recorded from the central segment of the cardiac sympathetic nerve with platinum-iridium wire electrodes using an AC preamplifier (S-0476; Nihon Kohden, Tokyo; time constant set at 0.33 s). Reflex responses elicited by electrical stimulation of a hind limb nerve were averaged (50 trials) by a computer (ATAC 3700; Nihon Kohden, Tokyo or UPO, Unique Medical, Tokyo). The averaged responses were stored as digital signals and recorded on a printer or mini-writer. The size of the reflex response was measured as the area under the evoked response and expressed as the percent of the control size preceding drug injection.

A tibial nerve was dissected from the surrounding tissues and cut. The central cut end segment of the nerve was placed on bipolar platinum-iridium wire electrodes for electrical stimulation. Single square pulse stimuli of 0.5 ms duration were delivered every 3 s by a digital electrical stimulator (SEN-7103; Nihon Kohden, Tokyo).

3.2. Recording spinal somato-somatic reflex

Male Wistar rats (n = 4; weighing 330-350 g) were anesthetized using pentobarbital (50 mg/kg i.p.). A jugular vein was catheterized for i.v. administration of cPA. Body temperature was maintained at 37.5°C. The spinal cord was completely transected at the upper thoracic level.

With the rat placed in the supine position, a branch of the right saphenous nerve innervating thigh skin was cut at the thigh level. The central cut end segment of the nerve was stimulated electrically as described above. Electromyogram (EMG) was recorded from the right leg muscles by inserting silver electrodes using the AC preamplifier. With single shocks, post-stimulus time histograms were created by a computer (ATAC 3700; Nihon Kohden, Tokyo) for EMG activity for approximately 50 trials at 3-s intervals. The averaged responses were stored as digital signals and recorded on a mini-writer. The size of the reflex response was measured as the area under the evoked response and expressed as the percent of the control size preceding drug injection.

3.3. Electrical stimulation-induced paw withdrawal (EPW) Test

Male C57BL/6 mice (TEXAM, Japan) weighing 18-22 g were used. The electrical stimulation-induced paw withdrawal (EPW) test conducted using the Neurometer™ CPT/C (Neurotron Inc.) has been reported previously [16]. In brief, electrodes were fastened to the plantar surface and instep of the mice. Transcutaneous nerve stimuli with each of the 3 sine wave pulses (5, 250, and 2000 Hz) were applied using the Neurometer™. The minimum intensity at which each mouse exhibited paw withdrawal was defined as the current stimulus threshold at 10-15 min after i.v. 2ccPA injection.

3.4. Formalin test

According to several preceding works [17,18] and to an economical advantage, we used ICR (CD1) female mice (Charles River, Japan), 6-9 weeks of age for the formalin test. Formalin solution (30 μL, 2% v/v) in saline was subcutaneously (s.c.) injected into the plantar surface of the left hind paw. Immediately after the formalin injection, the animals were placed in a cage and videotaped for 30 min from beneath the transparent floor. The time (in seconds) spent licking and biting the injected paw was counted in 5-min intervals by videotape observation. Two distinct phases of intensive licking and biting activities identified as the early and late phases were defined at 0-10 min and 10-30 min, respectively. At 3 min before formalin was injected, 2ccPA solution (in PBS containing 1% BSA) was i.v. injected. Morphine hydrochloride solution (Takeda Pharmaceutical Company, Osaka, Japan) in saline was i.p. injected at 30 min before formalin injection.

3.5. Neuropathic pain models

In experiments using the C57BL/6 mice, partial ligation of the sciatic nerve was performed under pentobarbital (50 mg/kg) anesthesia, following the methods of Malmberg and Basbaum [19]. 2ccPA was dissolved in artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 3.8 mM KCl, 1.2 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, pH 7.4). The intrathecal injection (i.t.) of 2ccPA was given into the space between spinal L5 and L6 segments according to the method of Hylden and Wilcox [20]. In the thermal paw withdrawal tests, nociception was measured as the latency to paw withdrawal evoked by exposure to a thermal stimulus [21,22]. Unanesthetized animals were placed in Plexiglas cages on the top of a glass sheet and were allowed an adaptation period of 1

h. A thermal stimulator (IITC Inc., Woodland Hills, CA, USA) was positioned under the glass sheet, and the focus of the projection bulb was aimed precisely at the middle of the plantar surface of the animal. A mirror attached to the stimulator permitted plantar surface visualization. The paw pressure test was performed, as described previously [22]. Mice were placed into a Plexiglas chamber on a 6 × 6 mm wire-mesh grid floor and allowed to acclimatize for 1 h. A mechanical stimulus was delivered onto the middle of the plantar surface of the right hind paw using a Transducer Indicator (Model 1601; IITC Inc., Woodland Hills, CA, USA). The pressure needed to induce a flexor response was defined as the pain threshold.

In experiments using rats, the chronic constriction injury was produced according to the procedure of Bennett and Xie [23]. Briefly, rats (182-216 g at day of ligation) were anesthetized with sodium pentobarbital (Nembutal, Dainippon pharmaceutical co., 40 mg/kg i. p.). The left sciatic nerve was exposed at mid-thigh level. Three loose ligatures with 4.0 chromic gut (SG-535; Syneture, USA), about 1-mm spacing, were tied around the sciatic nerve proximal to the trifurcation. Six days after nerve ligation, the development of neuropathy was assessed by measuring paw withdrawal latencies against thermal and mechanical stimuli [24,25]. Briefly, the thermal withdrawal threshold of a hind paw was measured using a beam of radiant infrared heat and a photocell (Planter Test model 7370; Ugo Basil, Milan, Italy). To prevent tissue damage, the cut-off time was set to 20 or 30 sec for rats with or without ligation. The mechanical withdrawal threshold was measured by the von Frey filament test. All rats were administered drug treatment by i.v. injection, and experiments were performed at 10 min and at 2 and 4 hours after drug treatments.

3.6. Drugs

We used chemically synthesized cPA 18:1 and its biologically stable derivative, 2-carba cPA 16:1 (2ccPA) (Figures 1A, B) [6]. These compounds were i.v. or i.t administered after they were dissolved in saline or artificial cerebrospinal fluid (aCSF).

3.7. Statistical analysis

Data were expressed as mean ± S.E. The data were statistically analyzed by Student's t-test, Welch's test, Steel's test, Dunnett's test, Wilcoxon test, or Tukey's multiple comparison tests. A P value < 0.05 was considered statistically significant.

3.8. Ethical approval

All the experiments were performed with an approval of the Animal Care and Use Committee at the Tokyo Metropolitan Institute of Gerontology and Nagasaki University Animal Care Committee at the Nagasaki University Graduate School of Biomedical Sciences (reference number 0706130596).

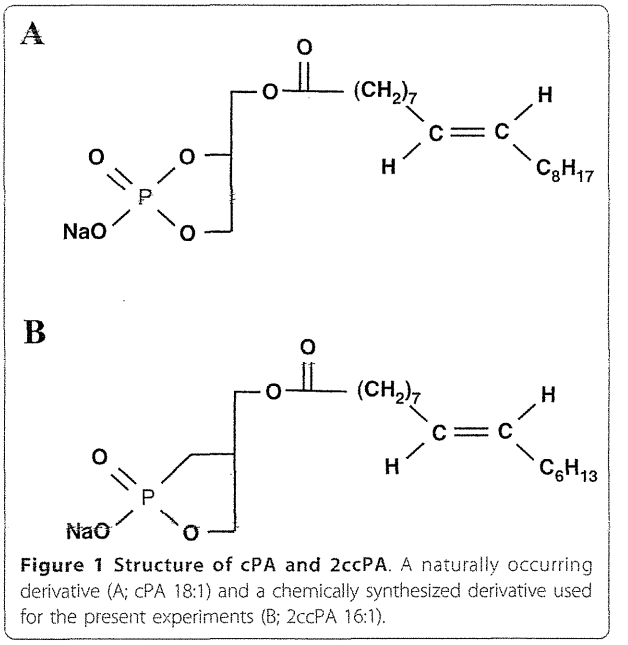

Figure 1 Structure of cPA and 2ccPA. A naturally occurring derivative (A; cPA 18:1) and a chemically synthesized derivative used for the present experiments (B; 2ccPA 16:1).

4. Results

4.1. Effects of cPA and 2ccPA on somato-cardiac sympathetic A- and C-reflexes in anesthetized rats

Single shock stimulation of A- and C-afferent fibers of the tibial nerve (at 10 V with 0.5 ms pulse duration) elicited 2 types of cardiac sympathetic reflex discharges as shown in Figure 2A. These discharges occur in a short latency (about 40 ms) as the A-sympathetic reflex and in a long latency (about 210 ms) as the C-sympathetic reflex, respectively (Figure 2A).

Intravenous injection of 0.1 mg/kg of 2ccPA depressed the C-reflex but not the A-reflex (Figure 2A). In most experiments, the 2ccPA-induced depression of C-reflex components started several minutes after the injection, reached its maximum level in less than 15 min, and then gradually returned to baseline within 40 min. The response was reproducible in successive trials on the same animal. Injection of 2ccPA 18:1 also yielded similar C-reflex suppression (data not shown).

Figure 2B summarizes the effects of i.v. injection of 2ccPA and cPA on the C-reflex in 8 rats. The responses were measured at 10-15 min after i.v. injection of cPA, i.e., after reaching the maximum effect (see above). After injection of 0.2 mg/kg 2ccPA, the C-reflex reached 82 ± 9% of the control. Injection of 2 mg/kg, but not 0.2 mg/kg cPA, significantly depressed the C-reflex.

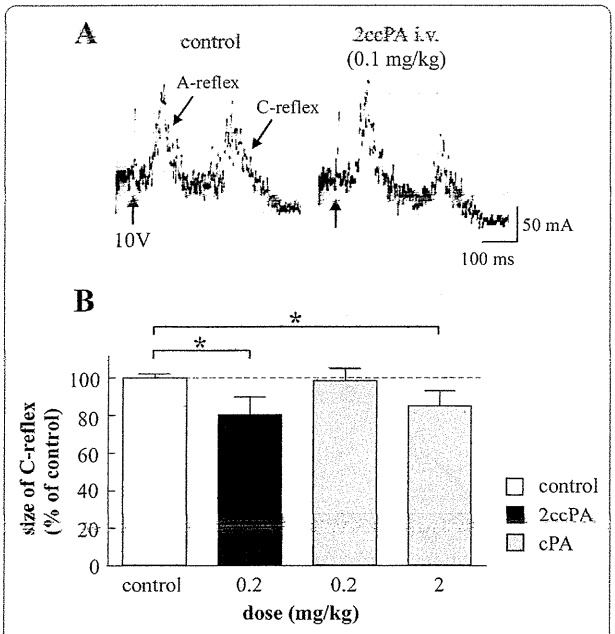

Figure 2 Effects of i.v. cPA and 2ccPA on somato-cardiac sympathetic A- and C-reflexes. A, Specimen records of A- and C-reflexes (averages of 50 trials) elicited by single electrical stimulation of a tibial nerve using 10 V of stimulus strength. Left: prior to 2ccPA application (control). Right: 15 min after i.v. injection of 0.1 mg/kg 2ccPA. B, Graphical summary of the size of the C-reflex at 0-5 min before cPA/2ccPA injection (control; n = 8), at 10-15 min after i.v. injection of 0.2 mg/kg 2ccPA (n = 4), and 0.2 mg/kg (n = 4) and 2 mg/kg (n = 4) cPA. We averaged 1-2 trials for each rat. The mean size (area under the evoked response curve) of the C-reflex at 0-10 min before each injection was expressed as 100%. All subsequent reflexes were expressed as percentages of the control values. Each column and vertical bar represent mean and S.E. *P < 0.05; significantly different from the control response by Student's t-test.

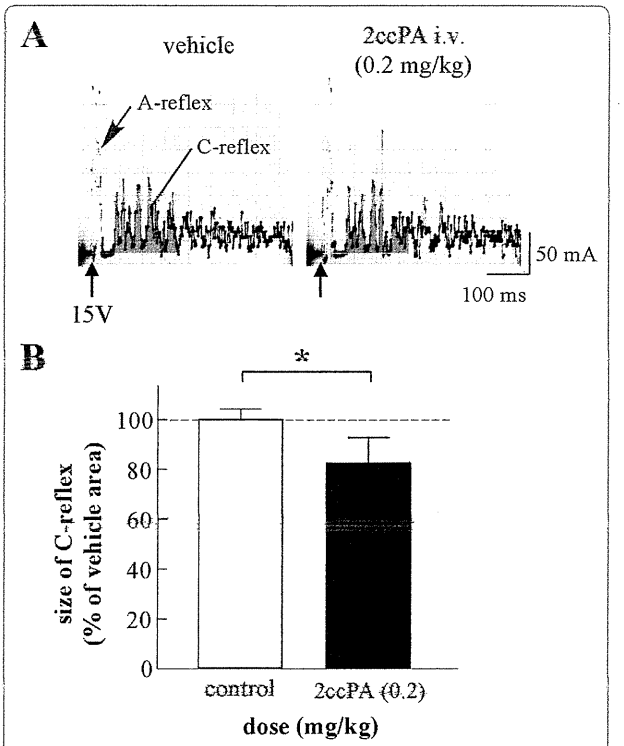

Figure 3 Effects of i.v. 2ccPA on the somato-somatic C-reflex. A, Specimen records of A- and C-reflexes (averages of 50 trials) elicited by single electrical stimulation of a saphenous nerve using 15 V of stimulus strength. Left: prior to 2ccPA application (control). Right: 10 min after i.v. injection of 0.2 mg/kg 2ccPA.B, Graphical summary of the size of the C-reflex at 0-5 min before 2ccPA injection (control; n = 4) and at 10-15 min after i.v. injections of 0.2 mg/kg 2ccPA (n = 4). We averaged 1-2 trials for each rat. The mean size (area under the evoked response curve) of the C-reflex at 0-10 min before each injection was expressed as 100%. All subsequent reflexes were expressed as percentages of the control values. Each column and vertical bar represent mean and S.E. *P < 0.05; significantly different from the control response by Student's t-test.

After injection of 2 mg/kg cPA, the C-reflex reached 86 ± 8% of the control.

4.2. Effects of cPA and 2ccPA on somato-somatic A- and C-reflexes in anesthetized rats

The hypothesis that cPA acts at the primary afferent nerve terminals was tested by recording spinal somato-somatic reflexes in 4 rats (Figure 3). Stimulation of the myelinated A-afferent fibers and unmyelinated C-afferent fibers of the saphenous nerve (at 15 V with 0.5 ms pulse duration) produced 2 distinct A- and C-somatic reflex components in the hind limb EMG, i.e., a short latent (about 10 ms) A-reflex and long latent (about 70 ms) C-reflex. Intravenous application of the same 2ccPA dose (0.2 mg/kg) that depressed the somato-sympathetic C-reflex also depressed the C-somatic reflex component to a similar magnitude. After injection of 0.2 mg/kg 2ccPA, the C-reflex reached 83 ± 5% (n = 4) of the control ($p < 0.05$).

4.3. C-fiber specific analgesic effects of 2ccPA on electrical stimulation-induced paw withdrawal (EPW)

We established the EPW test to distinguish responses mediated by different sensory fibers [16]. The hind paw is given transcutaneous nerve stimuli with sine-wave pulses of 5, 250, or 2000 Hz to stimulate C-, Aδ-, or Aβ-fibers [26,27], and the intensity (μA) required to induce a withdrawal reflex was defined as the threshold. The thresholds of naïve mice for 5, 250, and 2000 Hz stimuli were 64.4 ± 1.9 (C-fibers), 145.0 ± 4.5 (Aδ-fibers), and 401.1 ± 13.6 (Aβ-fibers). When the EPW test was performed at 10-15 min after the 2ccPA (i.v.) injection, the threshold increased in a dose-dependent manner with 5 Hz, but it did not increase with 250- or 2000-Hz stimuli (Figure 4).

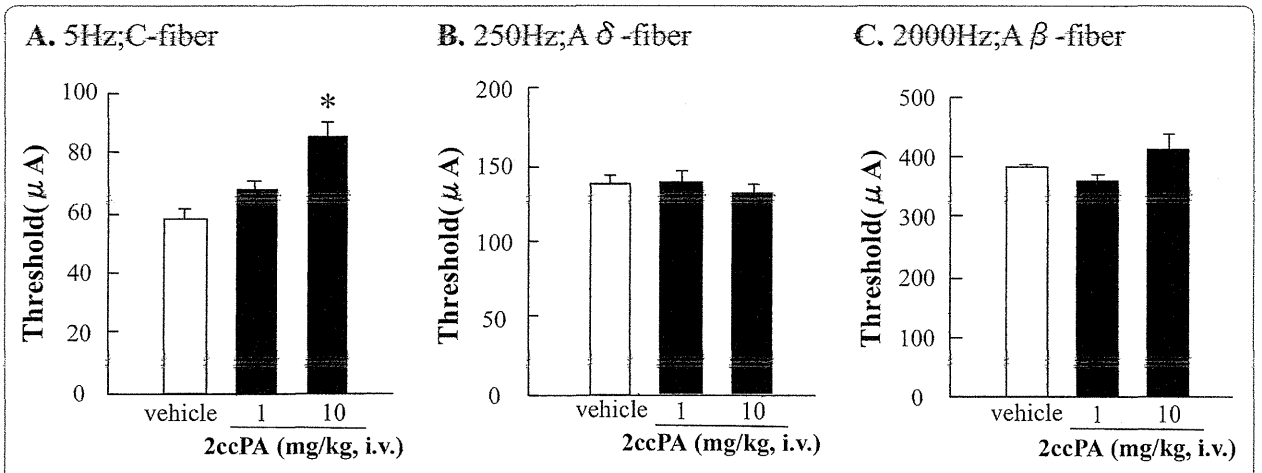

Figure 4 Effects of cPA on the Electrical Stimulation-Induced Paw Withdrawal (EPW) test. The threshold represents the minimum intensity (μA) required to elicit a paw withdrawal response to electrical stimulation with 5 Hz (C-fiber) (A), 250 Hz (Aδ-fiber) (B), and 2000 Hz (Aβ-fiber) (C). The EPW test was performed 10-15 min after 2ccPA (i.v.) injection. All data represent the mean ± S.E. from 3-6 individual mice per group. *P < 0.05; significantly different from the control response by Tukey's multiple comparison.

4.4. Analgesic effects of 2ccPA on mouse formalin-evoked licking and biting behavior

In the formalin-induced nociception tests, ICR (CD1) mice were given an s.c. injection of formalin solution to the hind paw. As shown in Figure 5A, the time course of the total time spent in licking and biting comprised 2 phases. Morphine or i.v. injection of 2ccPA at 30 min prior to formalin injection markedly reduced phase II licking and biting. Quantitative analysis revealed that 2ccPA exerted dose-dependent inhibition of phase II responses, with significant inhibition observed at a 10-mg/kg i.v. dose (Figure 5B). As a reference, significant analgesia was also achieved with 3 mg/kg morphine.

4.5. Pre-injury administration of 2ccPA prevents neuropathic pain development

LPA is produced by ATX in the early phase after nerve injury [13,14]; therefore, we administered 2ccPA (10 nmol, i.t. or 100 nmol, i.t.) at 30 min prior to inducing nerve injury. 2ccPA prevented thermal hyperalgesia and mechanical allodynia in a dose-dependent manner at 5 and 7 days after nerve injury (Figure 6). However, 2ccPA (i.t.) injection in naïve C57BL/6 mice had no significant effect on nociceptive latency at 90 min or 1 or 7 days after injection (additional file 1).

4.6. Repeated administration of 2ccPA induces analgesia against established neuropathic pain in mice

We examined the effects of 2ccPA on established neuropathic pain in C57BL/6 mice. In the thermal withdrawal test, mice with sciatic nerve injury exhibited a decreased threshold at day 7. Under these conditions, a single 2ccPA dose (100 nmol, i.t.) significantly increased nociceptive latency at 30 min on the day 7 after injury (considered as day 1), as shown in Figure 7Ba. When 2ccPA (i.t.) was administered daily to the injured mice, the basal latency (before the i.t. injection) time-dependently increased from day 1 to 7, though no significant change was observed on the 7th day (Figures 7Ba-d). A significant increase was observed at 90 min after the 2ccPA injection on the day 4 and at all time points until 120 min on the 7th day (Figure 7Bc). The reason for more pronounced 2ccPA analgesia on the 7th day may be attributed to the fact that there is some, but not significant increase in the basal latency on the 7th day. A single 2ccPA injection (10 mg/kg i.v.) had no effect on thermal hyperalgesia (Figure 7Ca). There was significant analgesia by 2ccPA (i.v.) on the seventh day following daily injections (i.v.), with no change in basal latency throughout the 7 days (Figures 7Ca-d).

In the paw-pressure test, repeated post-injection (i.t. or i.v.) of 2ccPA yielded similar effects on day 8 (Figure 8). Following repeated i.t. injection, there was an increasing trend in basal latency on day8, while there was no change with i.v. injection. Significant analgesia was also observed at several time points after the 2ccPA i.t. injection but not after the i.v. injection. When the area under the curve (AUC) was evaluated, there was significant analgesia with both i.t. and i.v. injections on day 8 (Figure 8D).

4.7. Repeated 2ccPA administration induces analgesia against established neuropathic pain in rats

Similar studies to examine the analgesic effects of 2ccPA against established chronic pain were performed using a different chronic-pain model in rats. In the chronic

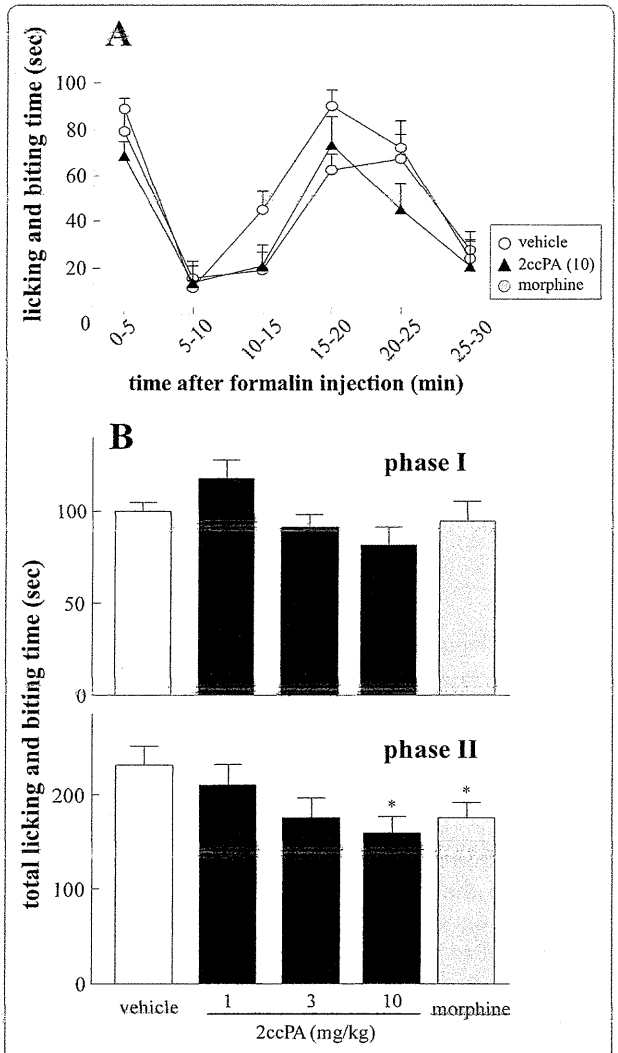

Figure 5 Effects of i.v. 2ccPA on licking and biting time. A, Time variations of licking and biting time of mice s.c. injected with 30 μL of 2% formalin in the mouse hind paw immediately after i.v. injection with 10 mg/kg (closed triangle) 2ccPA or vehicle (open circle). Morphine (3 mg/kg i.p.) was applied 30 min prior to formalin injection as a control. Each data point represents the average licking and biting time for 8-13 mice. B, Graphical summary of the total phase I and II licking and biting times of mice at 1 mg/kg, 3 mg/kg, or 10 mg/kg 2ccPA. All data represent the mean ± S.E. *P < 0.05; significantly different from the control response by Student's t-test.

constrictive sciatic nerve injury (CCI) model, the same experimental schedule was performed, as described above (Figure 9). In the thermal withdrawal test, there was significant analgesia at 2 h after i.v. injection of 2ccPA (10 mg/kg) on the seventh day following repeated injections, with no significant change in the basal latency at time 0 min (Figure 9B). There was weak but not significant analgesia at 2 h with 3 mg/kg (i.v.) on the seventh day (Figure 9C), while significant analgesia was observed on the fourth day with 10 mg/kg (Figure 9D). The 2ccPA-induced analgesia was slightly weaker, but comparable to the analgesic effects of gabapentin (90 mg/kg i.v.). Similar results were observed with the paw-pressure test (Figures 9E-9G). In this case, weak but significant analgesia against mechanical allodynia was observed only with 10 mg/kg at 4 h on the seventh day. However, the analgesic effect was much lower than that with gabapentin.

5. Discussion

We designed and chemically synthesized the metabolically stabilized derivatives of cPA, to avoid cPA hydrolysis in animals [10]. In 2ccPA, the phosphate oxygen of cPA is replaced with a methylene group at *sn*-2 (Figure 1). This study showed that the effective dose of 2ccPA was almost 10-fold less than that of natural cPA, consistent with other studies [6,7,10]. Differences in chemical stability and/or structural traits might account for the difference in the effective dose of natural cPA and 2ccPA required to achieve analgesia. The specificity of these compounds has been extensively reported [7,10].

Our initial examination demonstrated that intragastral administration of 1 mg/kg of 2ccPA resulted in a remarkable 40% reduction of the somato-cardiac sympathetic C-reflex (additional file 2), suggesting practical stability against gastric digestion as well as rapid gastric absorption of 2ccPA. We examined another carba-cPA, 3ccPA, in which the phosphate oxygen of cPA is replaced with a methylene group at *sn*-3, and found that effective doses of 3ccPA 16:1 were similar to those of 2ccPA for suppression of somato-cardiac sympathetic reflexes (additional file 3). In this report, we demonstrated that cPA and 2ccPA suppressed the supraspinal sympathetic and spinal kinetic reflexes, specifically the C-fiber, but not the A-fiber reflex, in anesthetized animals. This result was consistent with the experiments with mice, in which 2ccPA increased the nociceptive threshold only for Neurometer™ electrical stimulus with 5 Hz, which is supposed to stimulate C-fibers, but not with 250 or 2000 Hz, which are supposed to stimulate Aδ and Aβ-fibers [16]. These results suggest that both cPA and 2ccPA suppress nociceptive responses by primary afferent C-fibers. Both compounds are reported to possess selective and potent ATX inhibitory activities [10]. Endogenous LPA in the peripheral tissues or plasma exerts tonic stimulation of C-fibers, as evidenced by previous findings that LPA injected into the hind paw of mice caused nociceptive flexor responses, partially via substance P release from the nociceptor endings of C-fibers [11,28]. As there was no significant analgesia with i.t.-injected 2ccPA (additional file 1), the inhibitory responses of these compounds are unlikely to be mediated by inhibition of LPA synthesis in naïve

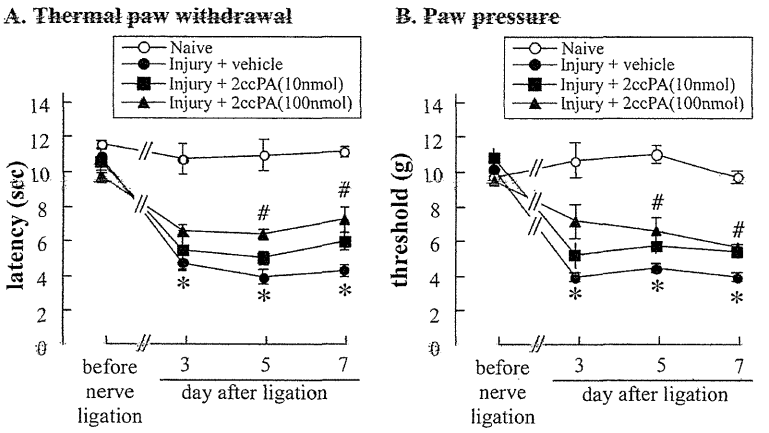

Figure 6 Pre-injury administration of 2ccPA (i.t.) prevents development of neuropathic pain. Neuropathic pain was induced by partial sciatic nerve injury in mice. 2ccPA (10 or 100 nmol/5 μL i.t.) was injected at 30 min prior to nerve injury. The threshold was measured on days 3, 5, and 7 after nerve injury, using the thermal withdrawal (A) and paw-pressure (B) tests. All data represent the mean ± S.E. from 3-6 individual mice per group. *P < 0.05; significantly different from the control response by Tukey's multiple comparison tests.

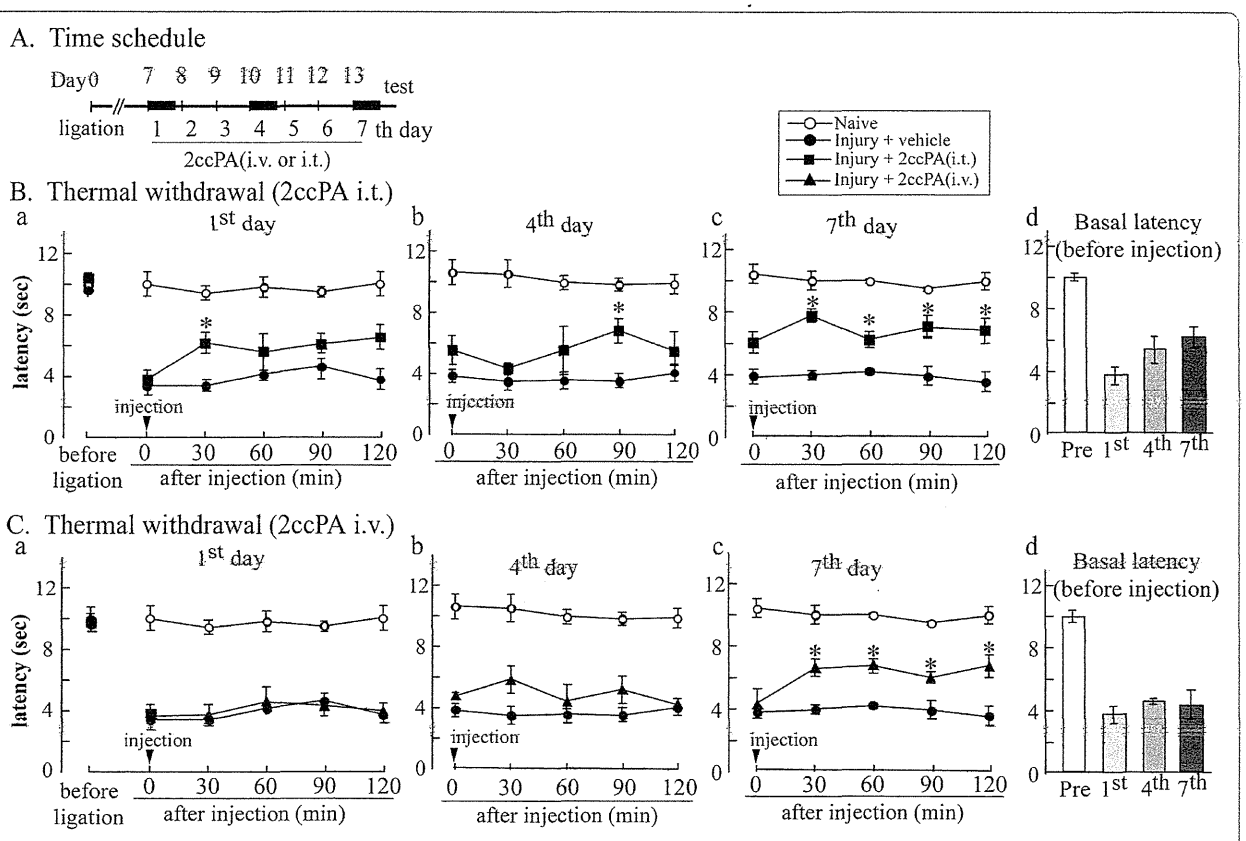

Figure 7 Repeated administrations of 2ccPA (i.v. or i.t.) induces analgesia against established thermal hyperalgesia in mice with neuropathic pain. A, Neuropathic pain in mice was induced by partial sciatic nerve injury. Vehicle or 2ccPA (i.v. or i.t.) was injected daily for 7 days, starting 7 days after injury. B, Vehicle or 2ccPA (100 nmol/5 μL 1-3 days and 10 nmol/5 μL 4-7 days, respectively, i.t.) was daily injected for 7 days, starting 7 days after injury. The withdrawal latency was measured at 30, 60, 90, and 120 min after the injection of 2ccPA (i.t.) on the first (a), fourth (b), or seventh day (c). The basal latency was measured before injection of 2ccPA (i.t.) (d). (C) Vehicle or 2ccPA (10 mg/kg, i.v.) was daily injected for 7 days, starting 7 days after injury. The withdrawal latency was measured at 30, 60, 90, and 120 min after the injection of 2ccPA (i.v.) on the first (a), fourth (b), or seventh day (c). The basal latency was measured before injection of 2ccPA (i.t.) (d). All data represent the mean ± S.E. from 3-6 individual mice per group. *P < 0.05 indicates significant difference from the vehicle response by Tukey's multiple comparison test.

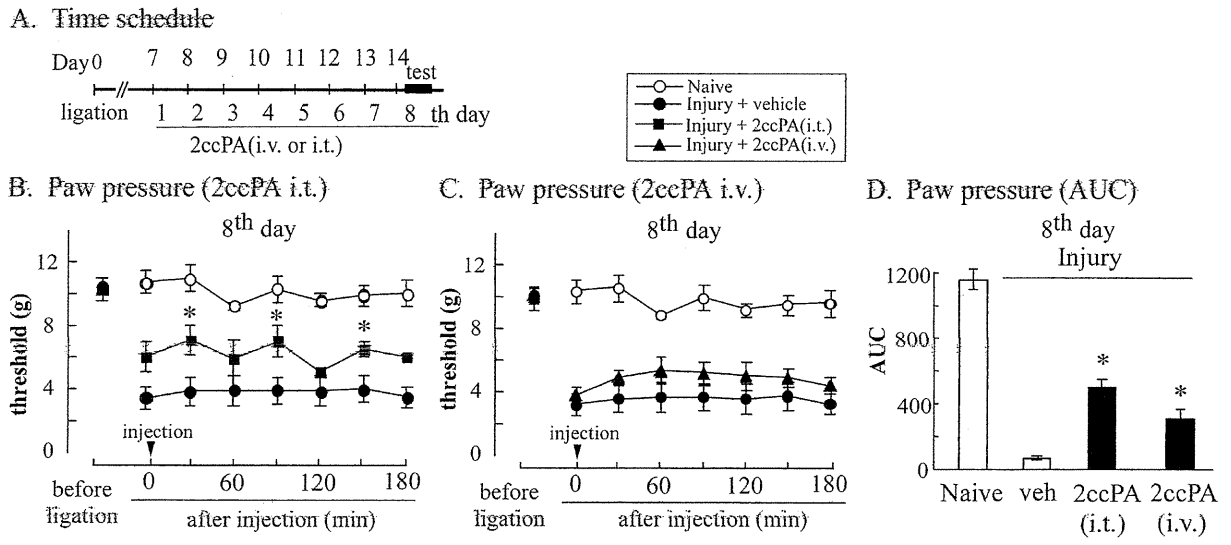

Figure 8 Repeated administration of 2ccPA (i.v. or i.t.) induces analgesia against established mechanical allodynia in neuropathic pain mice. Vehicle or 2ccPA (10 mg/kg i.v.) was daily injected for 8 days, starting 7 days after injury (A). The threshold in the paw pressure test was measured at 30, 60, 90, 120, 150, and 180 minutes after 2ccPA application (i.t. or i.v.) on the eighth day (B and C). Quantitative analyses with 2ccPA (i.t. or i.v.) were performed with the area under the curve (AUC) (D). AUC data represent increased thresholds by subtracting the thresholds before injection in vehicle-treated mice. All data represent the mean ± S.E. from 3-6 individual mice per group. *P < 0.05 indicates significant difference from the vehicle response by Tukey's multiple comparison test.

mice. However, we cannot exclude the possibility that cPA and/or 2ccPA may have inverse agonist actions on C-fiber nociceptor endings, since they have some weak actions on LPA receptors [7,10].

We also found that 2ccPA exerted anti-nociceptive effects in the formalin test. Formalin-induced characteristic behaviors in phase I are the result of direct C-fiber-evoked excitation, whereas the behaviors in phase II are evoked by repetitive C-fiber stimulation [29,30]. 2ccPA reduced both phase responses, but the inhibition of phase II responses was significant. We speculate that repetitive C-fiber stimulation may cause the ATX-catalyzed production of LPA in the periphery and stimulate C-fibers in an autocrine manner.

It should be noted that 2ccPA attenuated neuropathic pain possibly via the central nervous system. Our initial study revealed that i.t. injection of 2ccPA prevented nerve injury-induced neuropathic pain in mice. This finding is consistent with a series of studies by Ueda and colleagues, in which nerve injury induces LPA production by ATX in the spinal cord and causes neuropathic pain through the LPA_1 receptor [12,13,31]. The most striking evidence is that repeated administration of 2ccPA through i.t. and i.v. routes produced significant analgesia against established neuropathic pain in mice and rats. The i.t. injection of 2ccPA on day 7 after injury produced weak analgesia against thermal hyperalgesia. More pronounced analgesia was observed when it was given daily by the seventh i.t. injection on day 13 after injury. As there is some, but not significant, recovery of the basal threshold before the seventh injection of 2ccPA, LPA production may occur in the late phase to maintain the neuropathic pain status, as well as at the early phase to trigger the initiating mechanisms [12]. Similar analgesic effects were observed with i.v. injection by the seventh injection on day 13 after injury, though there was no tendency to recover the basal threshold. The difference of basal latency following repeated injections between i.t. and i.v. routes may be related to the fact that there are some residual increases at as late as 120 min in the case with i.t., but not i.v. injections at the 1st and 4th day. Although the lack of elevation in the basal threshold cannot be explained at this time, it seems to occur after repeated i.v. treatments: i.v.-injected 2ccPA-induced analgesia was equivalent to that yielded by the first i.t. injection. We previously reported that cPA and carba derivatives penetrate into the central nervous system through the blood-brain barrier [32]. In the present study, the effective dosage of 2ccPA for mice were about five times higher than that for rats, both for i.v. and i.p. injection, possibly because these two animals exhibit different sensitivities against administered drugs depending on their chemical species [33].

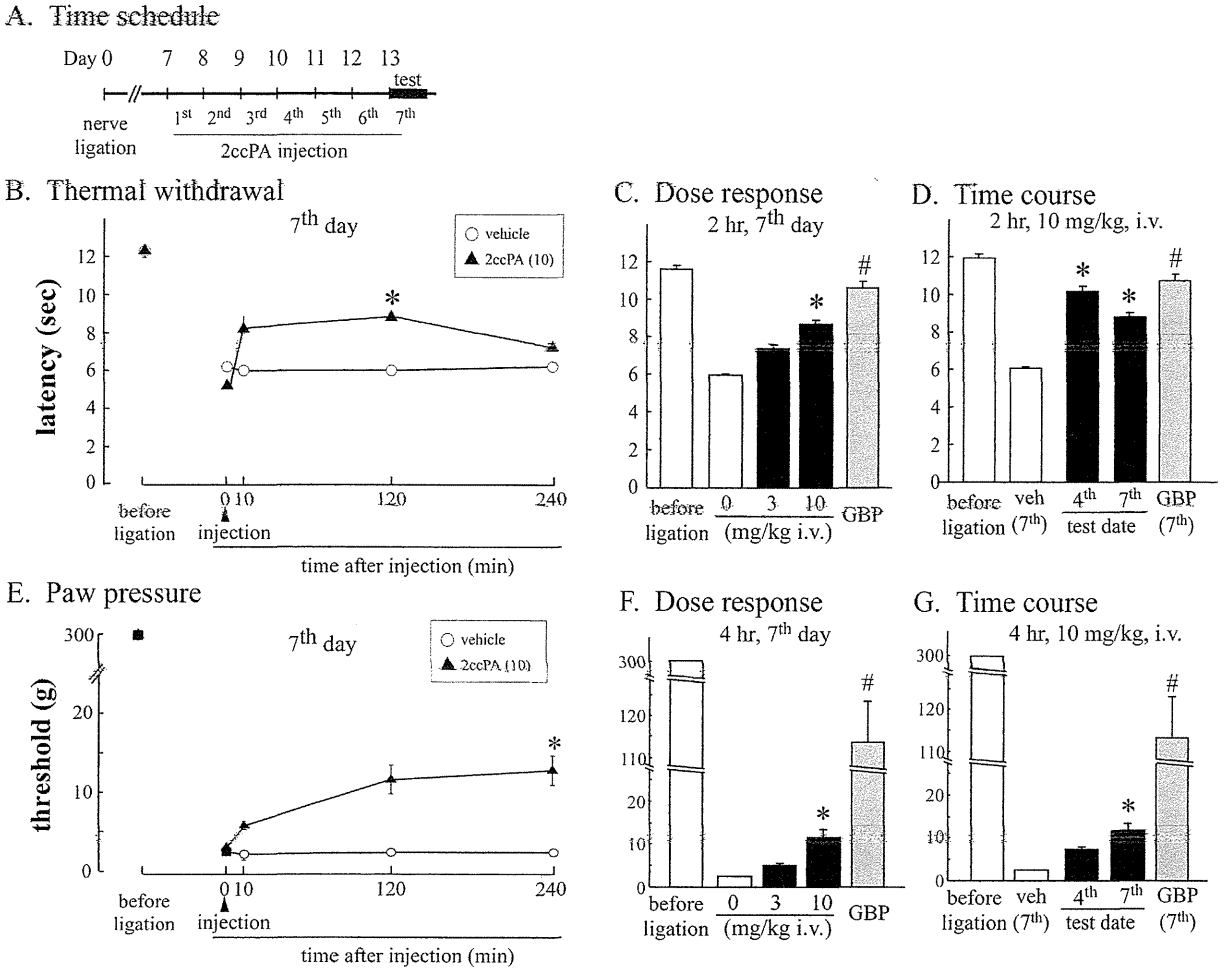

Figure 9 Effects of i.v. 2ccPA on thermal paw-withdrawal and mechanical allodynia of nerve-ligated rats. A, The rat sciatic nerve was ligated 7 days prior to i.v. injection of 2ccPA, applied once daily for 7 consecutive days. B, A hind paw was irradiated with infrared light to evoke thermal withdrawal, and the time variation of the withdrawal latency was measured prior to and at 10 min, 2 h, or 4 h after the application of 2ccPA on the seventh day. C, Dose response; 2-h injection of 2ccPA at 3 or 10 mg/kg applied once daily for 7 consecutive days. D, Time course; 2-h injection of 2ccPA (10 mg/kg) applied once daily for 4 or 7 consecutive days. Gabapentin (90 mg/kg i.v.) was applied as a control. *P < 0.05 and #P < 0.05 indicates significant difference from the vehicle response by Dunnett's test and by Student's t-test or Welch's test, respectively. E, The responses of rats to tactile stimulation was tested with 5 von Frey filaments, and threshold filament sizes to evoke paw withdrawal were measured prior to and at 10 min, 2 h, or 4 h after application of 2ccPA on the seventh day. F, Dose response; 4-h injection of 2ccPA at 1, 3 or 10 mg/kg applied once daily for 7 consecutive days. G, Time course; 4-h injection of 2ccPA (10 mg/kg) applied once daily for 4 or 7 consecutive days. Gabapentin (90 mg/kg i.v.) was applied as a control. *P < 0.05 and #P < 0.05 indicates significant difference from the vehicle response by Steel's test and by Wilcoxon test, respectively.

Recently, we demonstrated LPA-induced LPA production; i.t. injection of LPA or the addition of LPA to spinal cord slices markedly increased the LPA level in a time-dependent manner with the peak occurring at 3 h [14,34]. This finding indicates the presence of feed-forward amplification of LPA production in initiating neuropathic pain. As LPA production declines, however, there may be end-product inhibition. Although it is a fascinating mechanism that cPA is a natural ATX inhibitor [10] produced by ATX, it remains to be seen whether the amounts of cPA produced are sufficient to exert this effect.

6. Conclusion

Our results indicate that cPA and 2ccPA are potent inhibitors of nociceptive transmission by C-primary-afferents and reverse inflammatory and neuropathic pain. These chemicals may be good candidates for use in clinical pain management.

Additional material

Additional file 1: No effect of 2ccPA (i.t.) injection in naïve mice. The thresholds were measured at 90 min (A) and on days 1 and 7 (B) after 2ccPA injection, using the thermal withdrawal test. All data represent the mean ± S.E. from 3-6 individual mice per group.

Additional file 2: Time variation of relative C-reflex level after oral administration of 2ccPA (16:1). Relative C-reflex levels were measured after oral administration of 2ccPA (16:1) at 1 mg/kg and plotted over time until 120 min. Each data point represents the average four independent measurements, and vertical bar represents S.E. $^{**}P < 0.01$ and $^{*}P < 0.05$; significantly different from time 0 by one-way ANOVA, Dunnett's multiple comparison test.

Additional file 3: Effects of i.v. 3ccPA on the somato-carcdiac C-reflex. Relative C-reflex levels of the somato-cardiac response were measured after i.v. injection of 3ccPA (18:1) at 100 μg/kg (n = 3). Vertical bar represents S.E. $^{**}P < 0.01$; significantly different from the vehicle by Student's t-test.

List of Abbreviations Used

aCSF: artificial cerebrospinal fluid; ATX: autotoxin; ccPA: carba-cyclic phosphatidic acid; cPA: cyclic phosphatidic acid; i.p.: intraperitoneal; i.v.: intravenous; LPA: lysophosphatidic acid; PBS: phosphate-buffered saline; s.c.: subcutaneously

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Authors' contributions

YK and JN participated in the experimental designing, collection and analyses of data, and drafted the manuscript in equal contribution. MG performed the statistical analyses and drafted the manuscript. HH participated in the designing of the study, carried out surgical manipulation, data collection, and drafted the manuscript. HM conceived of the study, and participated in its design. TO participated in EPW assay. HU and KM conceived of the study, participated in its design and coordination. All authors read and approved the final manuscript.

Conflicts of interests

The authors declare that they have no competing interests.

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