Cellular/Molecular

Role of Thalamic Phospholipase Cβ4 Mediated by Metabotropic Glutamate Receptor Type 1 in Inflammatory Pain

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Phospholipase C (PLC) β4, one of the four isoforms of PLCβs, is the sole isoform expressed in the mouse ventral posterolateral thalamic nucleus (VPL), a key station in pain processing. The mouse thalamus also has been shown to express a high level of metabotropic glutamate receptor type 1 (mGluR1), which stimulates PLCβs through activation of Gαq/11 protein. It is therefore expected that the thalamic mGluR1–PLCβ4 cascade may play a functional role in nociceptive transmission. To test this hypothesis, we first studied behavioral responses to various nociceptive stimuli in PLCβ4 knock-out mice. We performed the formalin test and found no difference in the pain behavior in the first phase of the formalin test, which is attributed to acute nociception, between PLCβ4 knock-out and wild-type mice. Consistent with this result, acute pain responses in the hot plate and tail flick tests were also unaffected in the PLCβ4 knock-out mice. However, the nociceptive behavior in the second phase of the formalin test, resulting from the tissue inflammation, was attenuated in PLCβ4 knock-out mice. In the dorsal horn of the spinal cord where PLCβ1 and PLCβ4 mRNAs are expressed, no difference was found between the wild-type and knock-out mice in the number of Fos-like immunoreactive neurons, which represent neuronal activity in the second phase in the formalin test. Thus, it is unlikely that spinal PLCβ4 is involved in the formalin-induced inflammatory pain. Next, we found that pretreatment with PLC inhibitors, mGluR1 antagonists, or both, by either intracerebroventricular or intrathalamic injection, attenuated the formalin-induced pain behavior in the second phase in wild-type mice. Furthermore, activation of mGluR1 at the VPL enhanced pain behavior in the second phase in the wild-type mice. In contrast, PLCβ4 knock-out mice did not show such enhancement, indicating that mGluR1 is connected to PLCβ4 in the VPL. Finally, in parallel with the behavioral results, we showed in an electrophysiological study that the time course of firing discharges in VPL corresponds well to that of pain behavior in the formalin test in both wild-type and PLCβ4 knock-out mice. These findings indicate that the thalamic mGluR1–PLCβ4 cascade is indispensable for the formalin-induced inflammatory pain by regulating the response of VPL neurons.

Key words: **thalamus; phospholipase Cβ4; metabotropic glutamate receptor type 1; formalin test; inflammatory pain; knock-out mouse; electrophysiology**

Introduction

Our previous study demonstrated the characteristic expression patterns for four isoforms of phosphoinositide-specific phospholipase C (PLC) βs in the mouse brain, with PLCβ4 mRNA, one of the major neuronal isoforms, being predominantly expressed in the thalamus, including the ventral posterolateral thalamic nucleus (VPL; Watanabe et al., 1998). PLCβs are activated by interacting with the α subunit of the Gq subclass of GTP-binding proteins (Strathmann and Simon, 1990; Wilkie et al., 1991; Rebecchi and Pentyala, 2000). The Gq–PLCβ cascade is engaged by the activation of hormone or neurotransmitter receptors such as the bradykinin receptor and metabotropic glutamate receptor types 1 and 5 (mGluR1 and mGluR5; the so-called group I mGluRs; Masu et al., 1991; Abe et al., 1992; Haley et al., 2000). PLCβs produce a pair of second messengers, diacylglycerol and inositol 1,4,5-trisphosphate, which activate protein kinase C (PKC) and release Ca^{2+} from intracellular stores, respectively (Exton, 1996). This cascade via mGluR1 underlies various brain functions such as the elimination of climbing fiber → Purkinje cell synapses, cerebellar long-term depression (Kano et al., 1998; Miyata et al., 2001), and motor learning (Ichise et al., 2000; Kishimoto et al., 2001).

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A number of molecular mechanisms that contribute to pain processing have recently been clarified at the peripheral tissue (Bhave et al., 2001; Zhou et al., 2001) and spinal levels (Willis and Coggeshall, 1991). Group I mGluRs have been found to modulate inflammatory pain at the dorsal horn of the spinal cord via activation of their second messenger and protein kinases (Coderre, 1992; Fisher and Coderre, 1996; Young et al., 1997; Karim et al., 2001). Most studies on modulatory molecules in the afferent pain pathway have focused on their involvement at the peripheral and spinal levels, whereas very few studies have implicated the role of glutamate receptor-interacting molecules in pain sensation at the supraspinal level. The NMDA receptor contributes to nociceptive responses and hyperalgesia associated with neurogenic inflammation (Eaton and Salt, 1990; Kolhekar et al., 1997) in the VPL of the thalamus, which receives spinothalamic input (Lund and Webster, 1967; Craig and Burton, 1981; Cliffer et al., 1991), responds to various noxious stimuli (Peschanski et al., 1983; Yokota et al., 1988; Willis and Westlund, 1997), and relays nociceptive information to the cerebral cortex (Willis, 1985). It is reported that metabotropic glutamate receptors play roles in nociceptive responses in the rat VPL (Salt and Eaton, 1994). Forebrain NMDA receptor 2B, one of the subunits of NMDA receptors, is involved in inflammatory pain behavior but not in acute pain behavior in transgenic mice (Wei et al., 2001). The second messenger and downstream kinases from these receptors are presumably linked to pain behavior at supraspinal levels.

Because the mouse VPL expresses a high level of mGluR1 (Shigemoto and Mizuno, 2000), the thalamic mGluR1–PLCβ4 cascade may be involved in modulating pain processing. To clarify this possibility, we studied behavioral and electrophysiological responses to the formalin test in mice lacking the *PLCβ4* gene. We also studied the effects of PLC inhibitors, mGluR1 antagonists, and a group I mGluR agonist on the formalin-induced pain behavior at the supraspinal and thalamic levels. Our results indicate that the mGluR1–PLCβ4 cascade in the mouse thalamus is essential for inflammatory pain processing induced by formalin injection.

Materials and Methods

Animals. Mice (PLCβ4−/− and PLCβ4+/+) of either sex (7–15 weeks old) were used in the study. They were generated with a CJ7 ES cell clone derived from 129sv mice. The generation of PLCβ4 knock-out mice has been described in detail elsewhere (Jiang et al., 1996; Kano et al., 1998). The founder mice were then backcrossed with C57BL/6J mice. Knock-out mice of F4 and later generations were compared with corresponding wild-type littermates used as controls.

Behavioral assays. All animal experiments were conducted in accordance with the guidelines of the National Institute of Mental Health Animal Care and Use Committee.

Formalin test. Thirty microliters of formalin (5%) were administered subcutaneously into the plantar of the left hindpaw. The cumulative duration of licking and lifting of the injected paw was measured every 5 min immediately after the injection. Two hours after the injection, the mouse was deeply anesthetized and perfused with saline followed by 4% paraformaldehyde. The spinal cord was removed and postfixed for c-Fos immunohistochemistry (see below). All statistical analyses were performed using the repetitive ANOVA, with comparison between the experimental groups and the control groups at each time using Dunnett's *post hoc* test.

Thermal pain assay. The hot plate test was performed using an electronically controlled hot plate (MK-350A; Muromachi Instrument, Tokyo, Japan) heated between 42 and 58°C. The latency before the animal licked its hindpaw or jumped on the hot plate was recorded. The cutoff time was 50 sec for 52°C and 30 sec for temperatures of >55°C. The tail flick test was performed using a commercially available apparatus (MK-330A; Muromachi Instrument) consisting of an irradiator for heat stimulation and a photosensor for detection of the tail flick behavior. The latency from the start of irradiation to the tail flick reaction was measured. To prevent the mouse tails from being injured, the cutoff time was determined to be 10 sec. The tail flick reaction was measured two times in one test, and the average was considered as the latency. All statistical analyses were performed using one-way ANOVA.

Tissue preparation and immunohistochemistry. To visualized Fos expression in the dorsal horn of the spinal cord, the animals (wild-type and PLCβ4 knock-out mice) were perfused with 100 ml of normal saline, followed by 100 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Then, the spinal cord was removed and postfixed at 4°C in 0.1 M PB with 30% sucrose overnight for cryoprotection. Coronal sections were cut at 20 μm thickness and collected on silane-coated glass slides. For Fos protein staining, a polyclonal anti-rabbit c-Fos antibody (Ab-5, 1:20,000; Oncogene Product) was used. The ABC method and then DAB staining for visualization were performed as described previously (Imaki et al., 2001). To quantify Fos-like immunoreactive (Fos-LI) neurons, we counted neurons with stained nuclei from laminae I and II, III and IV, and V and VI in the lumbar 4–5 dorsal horn. Statistical analysis of the data were performed by one-way ANOVA for the different groups of animals.

To visualize expression patterns of PLCβ isoforms in the mouse spinal cord, thalamus, and forebrain, fresh-frozen coronal sections of C57BL/6J adult mice were used. Antisense oligonucleotides for mouse PLCβ1–4 mRNAs were synthesized, and *in situ* hybridization histochemistry was performed as reported previously (Watanabe et al., 1998).

Drug injections. Intracerebroventricular injection of 4 μl of a drug was administered under light halothane anesthesia as described by Sanchez-Blazquez et al. (1995) using a 10 μl microsyringe with a 31 gauge needle. Injections were unilateral and performed on the same side. At the end of each experiment, black ink was injected through the same route to ensure that the initial intracerebroventricular injection was successful. For intrathalamic injection, a mouse was initially deeply anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg of body weight) and then placed on a stereotaxic frame. A stainless steel guide cannula (26 gauge blunted needle) was then inserted vertically above the right VPL. The guide cannula was positioned 1.2 mm posterior to the bregma, 1.85 mm lateral to the midline, and 2.7 mm in depth from the surface of the brain and was secured with dental resin. Seven days after the guide cannula was placed, microinjections were performed in awake animals through a stainless steel injection cannula (outer diameter, 0.2 mm; inner diameter, 0.08 mm) inserted into the guide cannula. The cannula was connected to a 1.0 μl microsyringe via polyethylene tubing filled with distilled water. The drugs or vehicles were administered in a volume of 0.2 μl. Before starting a series of experiments, we confirmed the extent of diffusion in the thalamus by injecting 0.2 μl of 10% fast blue solution through the cannula in mice. Diffusion of ~600–700 μm in diameter, including the VPL, was observed, indicating that the drug remained in the ipsilateral thalamus.

The drugs used in the present study were 1-(6-[(17β-methoxyestra-1,3,5 [10]-trien-17-yl) amino] hexyl]-1H-pyrrole-2,5-dione (U73122; Calbiochem, La Jolla, CA), 1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphorylcholine (Et-18-OCH3; Calbiochem), 1-(6-[-([17β]-3-methoxyestra-1,3,5[10]-trien-17yl)amino]hexyl)-25-pyrrolidinedione (U-73343; Sigma-Aldrich), (*RS*)-1 aminoindan-1,5-dicarboxylic acid (AIDA; Tocris, Bristol, UK), 7-(hydroxyimino) cyclopropa [b] chromen-1a-carboxylate ethyl ester (CPCCOEt; Tocris), (*RS*)-3,5-dihydroxyphenylglycine [(RS)-DHPG; Tocris], and 2-methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP hydrochloride; Tocris). They were dissolved in artificial CSF (ACSF), except for U73122, which was dissolved in ACSF with 0.4% DMSO. ACSF contained (in mM): 125 NaCl, 2.5 KCl, 2–2.5 $CaCl_2$, 1–1.3 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 20 glucose. Intracerebroventricular or thalamic injection of ACSF with 0.4% DMSO caused no significant difference in the spontaneous and nociceptive behaviors compared with that with the injection of ACSF alone.

Electrophysiology. Wild-type and PLCβ4 knock-out mice weighing 25–35 gm were anesthetized with urethane (1.2 gm/kg, i.p.). The rectal temperature was maintained between 37 and 38°C with a heating pad.

The depth of anesthesia was monitored by assessing the animals' responsiveness in terms of eye blinks. A mouse was mounted on the stereotaxic holder without prevention of free respiration. The head position was adjusted until the heights of the lambda and bregma skull points were equal. A small window was opened at the dorsal surface of the skull, and a stainless steel electrode (9–12 MΩ) was inserted into the VPL of the thalamus. The coordinates used for recordings in the VPL were 1.2–1.3 mm caudal and 1.8–1.9 mm lateral to the bregma and 2.6–3.5 mm below the cortical surface. Receptive fields of thalamic neurons were first identified by their spike discharges in response to a gentle pinch with forceps and a heating probe (25–58°C; DPS-777J; Dia Medical System, Tokyo, Japan). Activities of thalamic neurons with receptive fields in the contralateral hindpaw were then examined in the formalin test. A 30 gauge needle that was attached to a syringe pump via a silicone tube (10 cm long) was inserted subcutaneously and maintained in place for at least 5 min to eliminate the effect of needle insertion. A bolus (30 μl) of 5% formalin was then injected within 3 sec, and the needle was then immediately removed. Responses of VPL neurons to formalin injection were continuously recorded from 1 min before to 1 hr after the injection. Spike discharges were amplified with a bandpass filter (AVH-11; Nihon Kohden, Tokyo, Japan) at 0.5–10 kHz. The recorded signals were sampled at 10 kHz with an analog-to-digital converter (CED 1401 Plus; Cambridge Electronic Design, Cambridge, UK), and stored in a Pentium personal computer for further off-line analyses (see below).

After each recording session, the recording site was marked by passing a current (10 μA, 20 sec duration) though the electrode. After all recording sessions were completed, the mouse was perfused intracardiacally with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brain sections of 50 μm thickness were prepared. After reaction with 1% potassium ferrocyanide and 1% HCl solution, each marked point was visualized as a small blue spot. The VPL was identified by cytochrome oxidase staining of the same sections. We thereby confirmed that recording sites were located in the VPL.

Off-line analyses. Single-unit discharges were isolated from recorded signals by template-matching procedures using Spike 2 (Cambridge Electronic Design). Firing rate histograms were plotted with a bin width of 1 sec. The average firing rate with its SD 1 min before formalin injection was considered the basal activity. A neuron was regarded as responding to the stimulus when its firing rate after formalin injection exceeded the basal activity. To evaluate the duration of the response in the early phase (0–5 min after the injection) and that in the late phase (15–60 min after the injection), the numbers of bins during the two phases in which the firing rate exceeded the basal activity were counted. All statistical analyses were performed using the unpaired Student's *t* test.

Results

Behavioral studies in wild-type and PLCβ4 knock-out mice

PLCβ4 knock-out and wild-type mice were subjected to several nociception tests. We first performed the formalin test in the wild-type and knock-out mice. Injection of 5% formalin subcutaneously into the hindpaw of wild-type mice resulted in a typical biphasic nociceptive response (Tjolsen et al., 1992). The first phase, usually lasting within 5 min, occurred immediately after formalin injection and was characterized by intense licking and lifting of the injected paw. The second phase, also characterized by licking and lifting of the injected paw, occurred ~15–20 min after formalin injection and lasted for ~60 min. The first phase of the formalin test is commonly attributed to acute nociception occurring in direct activation of nociceptive fibers (Puig and Sorkin, 1996), whereas the second phase is attributed to tonic nociception resulting from tissue inflammation. There was no difference (Fig. 1) in the duration of the first phase of the pain response to the injection measured within the first 5 min between the knock-out and wild-type mice: 148.8 ± 16.7 sec (mean ± SEM; $n = 10$) and 144.8 ± 18.1 sec ($n = 10$), respectively. In contrast, the mean duration of the pain behavior in the second phase (15–45 min after formalin injection) was significantly attenuated to 41.5% ($p < 0.01$; Fig. 1) in the knock-out mice (334.8 ± 62.1 sec) compared with that in wild-type mice (803.6 ± 46.2 sec). The knock-out mice showed normal edema. There was no significant difference in the mean thickness at the site of the formalin-injected paw between the wild-type mice (3.6 ± 0.11 mm, mean ± SD; $n = 9$) and knock-out mice (3.8 ± 0.12 mm; $n = 9$) 2 hr after the injection, indicating that the inflammatory change at the injected site in the knock-out mice was the same as that in the wild-type mice.

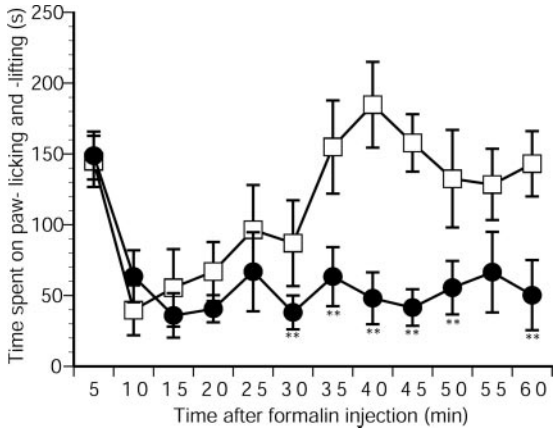

Figure 1. Attenuation of the second phase of formalin-induced nociceptive behavior in PLC β4−/− knock-out mice. Time courses of pain behavior in the formalin test with wild-type mice (open squares; $n = 10$) and PLCβ4 knock-out mice (filled circles; $n = 10$) are shown. Each point represents the mean ± SEM of cumulative durations of paw licking and lifting every 5 min immediately after the formalin injection. $^{**}p < 0.01$ compared with the wild-type mice.

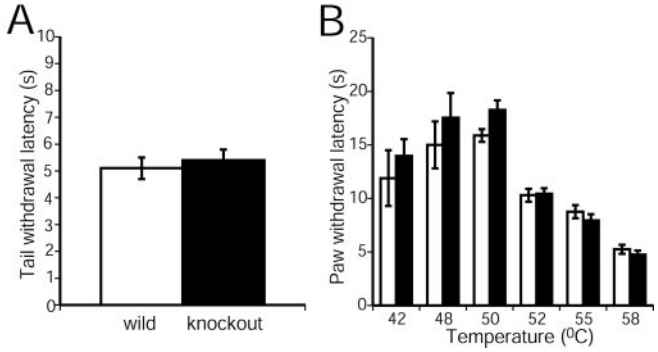

Figure 2. Acute pain responses to thermal stimulus in the tail flick test (*A*) and hot plate test (*B*). No significant differences in the latencies of withdrawal (mean ± SEM) from thermal stimuli were observed between wild-type mice (open bars) and knock-out mice (filled bars) in the tail flick test ($n = 19$) or the hot plate test ($n = 19$).

Because PLCβ4 knock-out mice showed no alternation of pain behavior in the first phase, we performed other acute pain assays. The tail flick test was used to measure spinal pain reflexes, and the hot plate test was applied to measure pain responses involved at the supraspinal level (Chapman et al., 1985). We found no difference in withdrawal responses between PLCβ4 knock-out and wild-type mice in the tail flick test (Fig. 2*A*). We also observed no difference in the withdrawal latencies in the hot plate test at various temperatures (Fig. 2*B*).

These results indicate that lack of PLCβ4 reduces the formalin induced inflammatory pain behavior, whereas it does not influence on the acute pain behavior.

Expression of PLCβ isoforms in the somatosensory system

To identify the site among multiple somatosensory stations involved in the attenuation of pain behavior in the second phase in

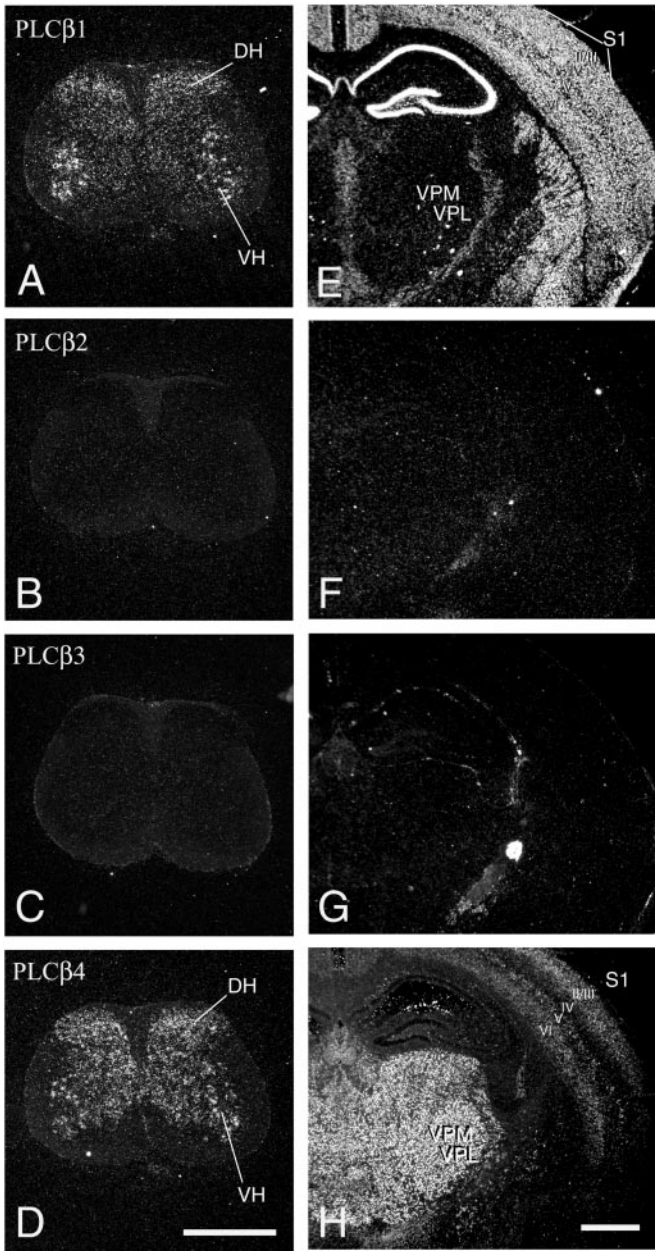

Figure 3. PLCβ isoforms transcribed in the mouse lumbar cord (*A–D*) and forebrain (*E–H*). Note the reciprocal expression of PLCβ1 and PLCβ4 mRNAs in the thalamic VPL and VPM and layer IV of somatosensory cortex S1. PLCβ1 and PLCβ4 mRNAs are, in contrast, both expressed concomitantly in the spinal cord, including the ventral horn (VH) and dorsal horn (DH). Scale bars, 1 mm.

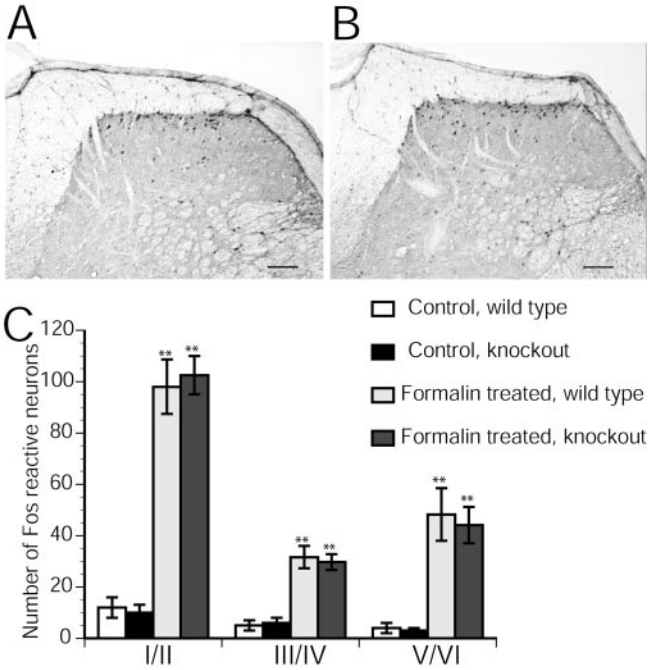

Figure 4. Fos-LI neurons of the dorsal horn of the spinal cord after formalin injection in knock-out and wild-type mice. *A, B*, Fos-LI cells in the dorsal horn of the spinal cord ipsilateral to the formalin injection site in wild-type mice (*A*) and PLCβ4 knock-out mice (*B*). Scale bar, 50 μm. *C*, Numbers of Fos-LI neurons (mean ± SD) in laminae I and II, III and IV, and V and VI of the ipsilateral spinal cord of wild-type and knock-out mice injected with saline (Control: wild type, $n = 9$; knock-out, $n = 6$) or with formalin (Formalin treated: wild type, $n = 9$, knock-out, $n = 6$) into the hindpaw. $^{**}p < 0.01$ compared with the wild-type and knock-out controls, respectively.

the PLCβ4 knock-out mice, we investigated the expression patterns of PLCβ isoforms in the spinal cord, thalamus, and somatosensory cortex (Fig. 3). In the spinal dorsal horn of adult wild-type mice, both PLCβ1 and PLCβ4 mRNAs were expressed (Fig. 3*A–D*). In the VPL of the thalamus, PLCβ4 mRNA was the sole isoform transcribed (Fig. 3*E–H*), consistent with our previous report (Watanabe et al., 1998). In somatosensory cortex S1, PLCβ1 mRNA was expressed at high levels together with PLCβ4 mRNA at a low level (Fig. 3*E,H*). In particular, PLCβ1 mRNA was the sole isoform transcribed in layer IV of the somatosensory cortex, a recipient layer for thalamocortical fibers. PLCβ2 and PLCβ3 mRNAs were almost undetectable in the spinal cord, thalamus, and somatosensory cortex.

On the basis of these results, PLCβ4 is the sole isoform of PLCβs at the thalamic relay station of the VPL, whereas PLCβ1 and PLCβ4 are both expressed in the spinal cord. In the somatosensory cortex, PLCβ1 is the sole isoform in layer IV; both PLCβ1 and PLCβ4 are expressed in the remaining layers.

Fos-LI neurons in the dorsal horn of the spinal cord in the formalin test

Because no difference was found in edema size of the injected paw between the wild-type and knock-out mice, it is reasonable to consider that similar inflammatory inputs were conveyed to the dorsal horn of the spinal cord. The observation that PLCβ4 co-localized with PLCβ1 is expressed in the dorsal horn raises the possibility that lack of spinal PLCβ4 may contribute to the attenuation of pain behavior in the second phase in the knock-out mice. To examine this possibility, we counted the number of Fos-LI neurons in all layers of the ipsilateral dorsal horn 2 hr after the administration of formalin in PLCβ4 knock-out and wild-type mice (Hunt et al., 1987). The number of Fos-LI neurons corresponds to spinal neuronal activity during the second phase (Abbadie et al., 1992, 1997; Todd et al., 1994; Malmberg et al., 1997). The highest number of Fos-LI neurons induced by formalin injection was found in layers I and II of lumbar segment 4–5 in both knock-out (102.5 ± 7.5) and wild-type (98 ± 10.6) mice (Fig. 4*A,B*). Numbers of FOS-LI neurons had no significant differences in all layers of the dorsal horn between the wild-type and knock-out mice (Fig. 4*C*).

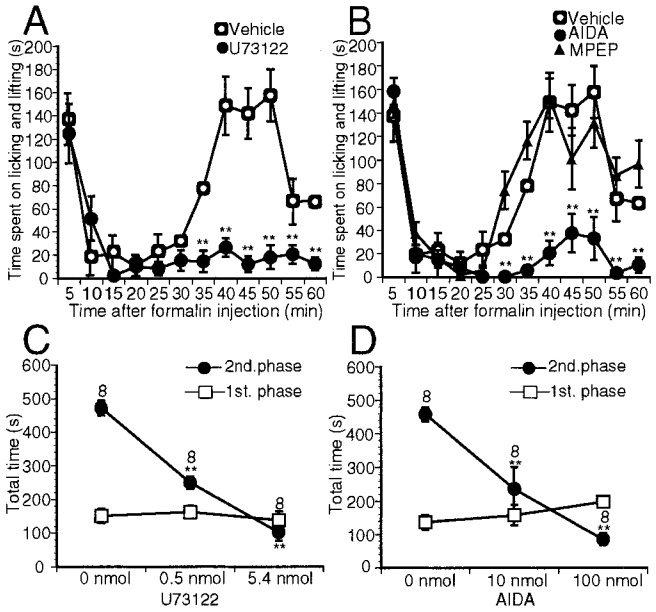

Figure 5. Attenuation of the second phase of the formalin-induced nociceptive behavior in a dose-dependent manner after intracerebroventricular injection of U73122 and AIDA. Time courses after 10 min of pretreatment with 5.4 nmol of U73122 ($n = 8$; *A*), 100 nmol of AIDA ($n = 8$; *B*) and 100 nmol of MPEP ($n = 5$; *B*) compared with those after vehicle injection ($n = 8$) are shown. Each data point represents the mean duration ± SEM. $^{**}p < 0.01$ compared with the vehicle injection. *C*, *D*, Dose dependence curves for the effects of U73122 (*C*) and AIDA (*D*) injection on the first phase (within the first 5 min after formalin injection; open squares) and the second phase (cumulative duration of the pain behavior between 15 and 45 min; filled circles), respectively. Each data point represents the mean duration ± SEM. $^{**}p < 0.01$ compared with the vehicle injection. The number of animals is indicated for each point.

Intracerebroventricular injection of PLC inhibitor in the formalin test

To test that PLCβ4 modulates inflammatory pain processing at the supraspinal level, U73122, a potent inhibitor of PLC (Bleasdale et al., 1990), was injected (0.5 or 5.4 nmol/mouse.) into the lateral cerebral ventricle of wild-type mice, and the formalin test was performed 10 min after the injection. Pain behavior in the first phase was not significantly different between U73122- and vehicle-injected mice regardless of the dose of U73122 (Fig. 5*A*,*C*), with mean durations ± SEM of pain behavior in the first phase of 137.3 ± 28.7 sec for vehicle-injected mice ($n = 8$) and 124.9 ± 25.0 sec for the 5.4 nmol U73122-injected mice ($n = 8$). In contrast, pain behavior in the second phase was attenuated in the U73122-injected mice in a dose-dependent manner compared with the vehicle-injected mice (Fig. 5*A*,*C*). When 5.4 nmol of U73122 was injected, the mean duration of the pain behavior in the second phase decreased to 19.4% (88.2 ± 24.2 sec; $n = 8$; $p < 0.01$) compared with that in the vehicle-injected mice (458.4 ± 40.2 sec; $n = 8$).

Intracerebroventricular injection of mGluR1 antagonist in the formalin test

PLCβ4 is known to be activated by group I mGluRs (Kim et al., 1997). mGluR1 is highly expressed in the mouse thalamus, whereas mGluR5 is only weakly expressed in this region. First, to examine whether mGluR1 is also involved in the inflammatory pain processing at the supraspinal level, a formalin test was performed 10 min after pretreatment with AIDA (10 or 100 nmol/mouse), an antagonist of mGluR1, injected into the cerebral ventricle in wild-type mice. The doses of AIDA adopted were based on a report by Moroni et al. (1997). The intracerebroventricular injection of AIDA attenuated formalin-induced pain response in the second phase in a dose-dependent manner. When 100 nmol of AIDA was injected, the mean duration of the pain behavior in the second phase decreased to 18.8% (86.2 ± 11.7 sec; $n = 8$; $p < 0.01$; Fig. 5*B*,*D*), compared with vehicle injection (458.4 ± 40.2 sec; $n = 8$). AIDA had no effect on the pain behavior in the first phase, the mean durations of pain behavior in the first phase being 137.2 ± 22.1 sec in the case of vehicle injection ($n = 8$) and 158.9 ± 11.7 sec in the case of 100 nmol AIDA injection ($n = 8$). Second, to test the contribution of mGluR5, MPEP, a specific antagonist (Gasparini et al., 1999), was injected into the cerebral ventricle in the wild-type mice at 10 and 100 nmol [the doses adopted were based on previous reports (Chapman et al., 2000; Berrino et al., 2001; Fisher et al., 2002)]. No significant change in the pain behavior was evident in the first phase (increases in mean duration of only 5 and 8% with 10 and 100 nmol, respectively; $p > 0.05$) or in the second phase (increases in mean duration of only 3 and 2%; $p > 0.05$). The mean duration of pain behavior was 147.8 ± 11.4 sec ($n = 5$) in the first phase and 467 ± 22.5 sec in the second phase in the case of 100 nmol MPEP injection (Fig. 5*B*).

Thalamic injections of PLC inhibitors and mGluR1 antagonists

Because PLCβ4 is the sole isoform in the mouse VPL of the thalamus, we studied whether thalamic PLCβ4 is involved in the formalin-induced pain behavior by injecting 0.2 μl of 5.4 nmol U73122 or 10 nmol Et-18-OCH3, another selective inhibitor of PLC (Powis et al., 1992), into the VPL of wild-type mice via a guided cannula. These doses of the two drugs were based on a report by Narita et al. (2000). The formalin test was performed 10 min thereafter. The intrathalamic injection of U73122 (5.4 nmol/mouse; $n = 7$) decreased the mean durations of the pain behavior in the second phase to 30.5% (208.5 ± 32.1 sec) in comparison with the intrathalamic vehicle injection (683.2 ± 53.5 sec; $n = 12$; Fig. 6*A*). The intrathalamic injection of U73343 (5 nmol; $n = 7$), an inactive analog of U73122 (Bleasdale et al., 1990), did not decrease the duration of pain behavior in the second phase significantly (587.4 ± 72.4 sec; $p > 0.5$), indicating that the effect of U73122 was specific. Similarly, the intrathalamic injection of Et-18-OCH3 (10 nmol/mouse; $n = 7$) 10 min before formalin injection also decreased the mean durations of the pain behavior in the second phase to 42.3% (289.0 ± 32.6 sec). Neither thalamic injection of U73122 nor that of Et-18-OCH3 had a significant effect on the spontaneous behavior and pain behavior in the first phase (170.5 ± 9.6 sec with U73122 and 184.4 ± 8.6 sec with Et-18-OCH3) in comparison with the vehicle (189 ± 8.5 sec) and U73343 (192.6 ± 11.2 sec) injections.

Because PLCβ4 is well known to be activated by mGluR1 (Nakanishi, 1994), which is also expressed in the mouse thalamus, we examined whether thalamic mGluR1 is also involved in the formalin-induced pain behavior by injecting AIDA, an antagonist of mGluR1, into the VPL. Similar to the results obtained from the thalamic injection of PLC inhibitors, thalamic injection of 0.2 μl of AIDA (100 nmol/mouse; $n = 6$), in comparison with the vehicle injection ($n = 8$), had no effect on the pain behavior in the first phase (187.0 ± 11.2 sec) but significantly decreased the mean durations of the pain behavior in the second phase to 61.3% (418.9 ± 37.2 sec; Fig. 6*B*). Furthermore, we performed a similar experiment with thalamic injection of CPCCOEt, an mGluR1-selective noncompetitive antagonist. The dose adopted was 50 nmol based on the report by Maione et al. (2000). Thalamic injection of 0.2 μl of CPCCOEt ($n = 9$) also had no effect

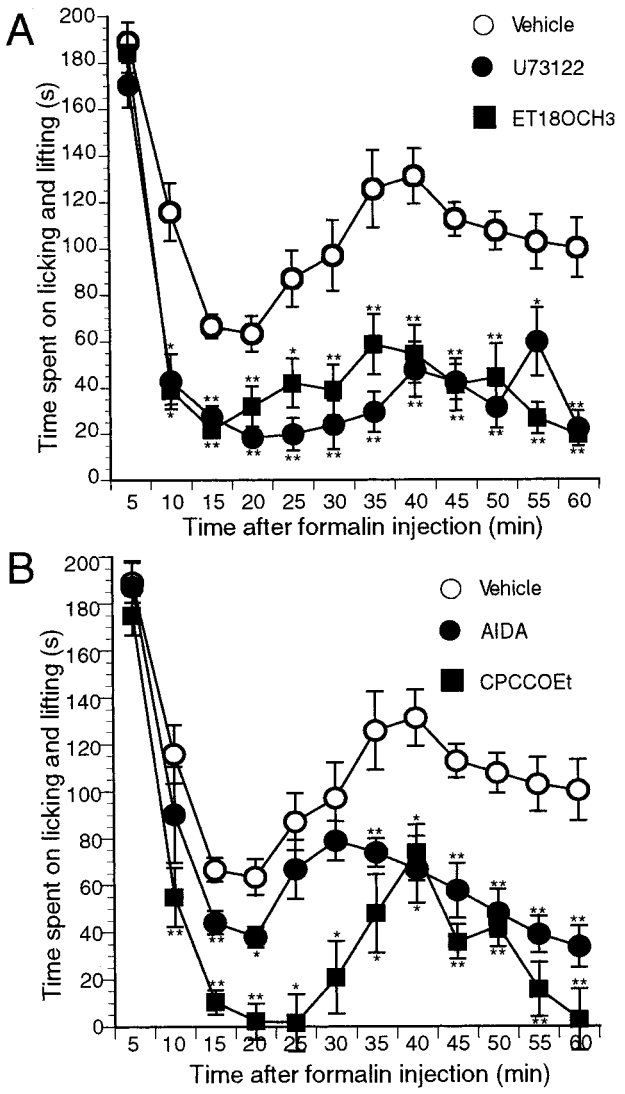

Figure 6. Attenuation of formalin-induced nociceptive behavior in the second phase after thalamic injection with PLC inhibitors (*A*) and mGluR1 antagonists (*B*). *A*, Time course graphs after 10 min of pretreatment with 5.4 nmol of U73122 ($n = 7$) and 10 nmol of Et-18-OCH3 ($n = 7$) compared with that for vehicle injection ($n = 12$). *B*, Time courses after 10 min of pretreatment with 100 nmol of AIDA ($n = 6$) and 50 nmol of CPCCOEt ($n = 9$) compared with that after vehicle injection ($n = 12$). Each data point represents the mean time ± SEM. $^*p < 0.05$; $^{**}p < 0.01$ compared with the vehicle injection.

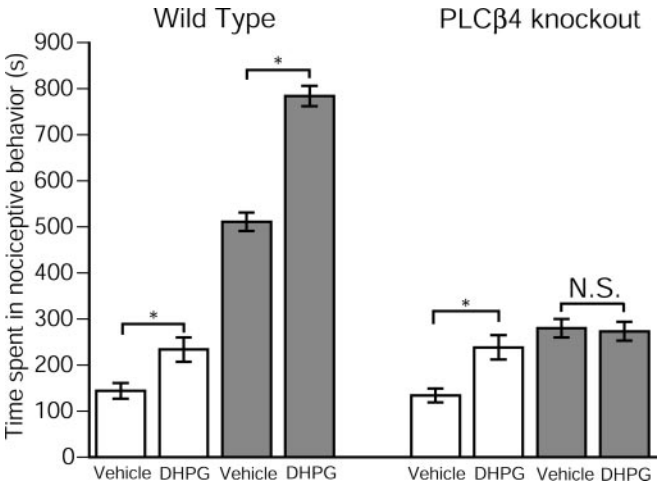

Figure 7. Enhancement of formalin-induced nociceptive behavior in the second phase after thalamic injection with the mGluR1/5 agonist in wild-type mice but not in knock-out mice. The mean duration of pain behavior in the first phase (within the first 5 min after formalin injection; open bars) are shown after the second phase (cumulative duration of the pain behavior between 15 and 45 min; filled bars) in 10 min of pretreatment with vehicle or (*RS*)-DHPG in wild-type mice ($n = 8$) and knock-out mice ($n = 8$). $^*p < 0.01$ compared with the vehicle injection. N.S., Not significant.

on the mean duration of the pain behavior in the first phase (174.2 ± 7.7 sec) but significantly decreased the mean duration of the pain behavior in the second phase to 27.9% (191.0 ± 32.0 sec; Fig. 6*B*). Thalamic injection of either 100 nmol of AIDA or 50 nmol of CPCCOEt alone did not affect the spontaneous behavior of mice.

Thalamic injection of group I mGluR agonist in wild-type and PLCβ4 knock-out mice

Given that pretreatment of the thalamus with either mGluR1 antagonists or PLC inhibitors reduced the pain behavior in the second phase in wild-type mice, it is surely expected that mGluR1 connecting to PLCβ4 in the thalamus enhances the formalin-induced inflammatory pain. To confirm this, intrathalamic injection of the group I mGluR agonist (*RS*)-DHPG (0.2 μl, 100 nmol/mouse) was performed 10 min before the formalin test in the wild-type and knock-out mice. The dose adopted was based on the report by Karim et al. (2001). (*RS*)-DHPG injection in wild-type mice ($n = 8$) significantly increased the mean durations of the pain behavior to 162.1% (233.8 ± 26.3 sec; $p < 0.01$) in the first phase and 151% (784.0 ± 22.3 sec; $p < 0.01$) in the second phase in comparison with intrathalamic vehicle injection (144.1 ± 16.5 sec in the first phase and 511.3 ± 20 sec in the second phase; $n = 8$; Fig. 7). Although intrathecal injection of (*RS*)-DHPG alone was reported to induce spontaneous nociceptive behavior (Karim et al., 2001), we did not observe any such effects with the intrathalamic injection. We performed the same experiment in PLCβ4 knock-out mice. As in the wild-type mice, thalamic injection of (*RS*)-DHPG caused the enhancement of the mean duration of the pain behavior in the first phase to 177% (238.5 ± 26.8 sec; $n = 8$; $p < 0.01$) in comparison with the vehicle injection (134.5 ± 15.0 sec; $n = 8$; Fig. 7). However, there was no enhancement of the mean duration in the second phase (273.3 ± 20.5 sec; $n = 8$) by (*RS*)-DHPG injection in the knock-out mice (decrease of 3% compared with the value of the vehicle injection; 280.2 ± 20.0 sec; $n = 8$; $p > 0.05$; Fig. 7). Co-injection of 0.2 μl of (*RS*)-DHPG (100 nmol) and Et-18-OCH3 (10 nmol) into the thalamus reduced the mean duration of the pain behavior in the second phase to 50.5% in comparison with the (*RS*)-DHPG injection alone in wild-type mice ($n = 5$; data not shown).

Responses of VPL neurons in wild-type and PLCβ4 knock-out mice to formalin injection

PLCβ4 knock-out mice and PLCβ inhibitor-injected wild-type mice exhibited attenuated pain behavior in the second phase in the formalin test, implying that PLCβ4 may modulate responses of VPL neurons to formalin injection. Thus, we performed single-unit recording in VPL and compared neuronal responses between wild-type ($n = 5$) and PLCβ4 knock-out mice ($n = 5$). There was no significant difference ($p > 0.5$) in the basal activity between the wild-type and knock-out mice. The numbers of spikes during 1 min before the formalin injection were 33.1 ± 25.9 (mean ± SD; $n = 10$) in the wild-type mice and 27.5 ± 17.6 ($n = 13$) in the knock-out mice.

Figure 8*A* shows an example of the response of a VPL neuron

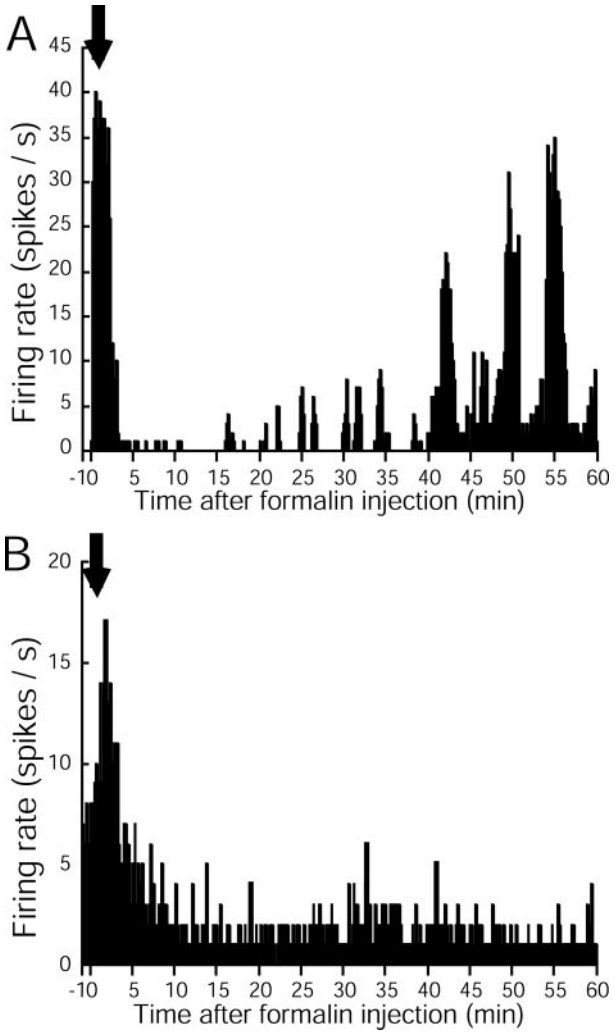

Figure 8. Examples of spike histograms of VPL neurons of wild-type mice (*A*) and PLCβ4 knock-out mice (*B*) before (~1 min) and after (~60 min) formalin injection into the contralateral hindpaw. In wild-type mice, VPL neurons showed biphasic responses to formalin injection, whereas a monophasic response in the knock-out mice was observed. Arrows indicate the time when formalin was injected.

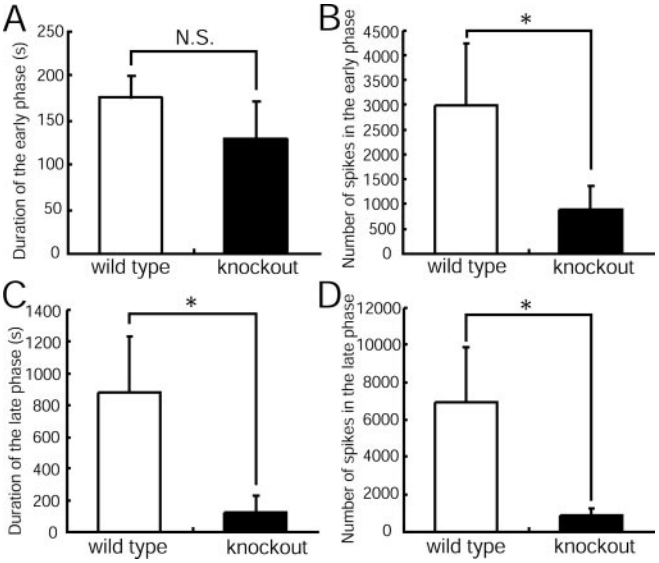

Figure 9. Comparison of the response of VPL neurons to formalin injection between wild-type mice ($n = 5$) and PLCβ4 knock-out mice ($n = 5$). *A*, Total duration of the period in which the firing rate exceeded the basal activity level (see Materials and Methods) in the early phase (0–5 min) after formalin injection. *B*, Total number of spikes in the early phase (0–5 min) after formalin injection. *C*, Total duration of the period in which the firing rate exceeded the basal activity in the late phase (15–60 min). *D*, Total number of spikes in the late phase after formalin injection. Each data point represents the mean ± SD. *$p < 0.01$. N.S., Not significant.

of a wild-type mouse. After the formalin injection, all VPL neurons in the wild-type mice showed a biphasic response profile similar to those previously reported for the peripheral C fiber and dorsal horn of the spinal cord (Dickenson and Sullivan 1987a,b; Haley et al., 1990; McCall et al., 1996; Puig and Sorkin, 1996). Immediately after the injection, the firing rate of VPL neurons increased drastically and remained elevated for ~5 min. The time course of the response during this early phase (0–5 min) corresponded to that in the first phase of pain behavior in the formalin test (Fig. 1, wild-type). The firing rate then dropped abruptly to the basal activity, followed by a quiescent period lasting for ~10 min. Fifteen minutes after the injection, the firing rate increased again: the increase was progressive but often interrupted by prolonged quiescent periods. This late phase (from 15 min after the injection) continued until the end of the recording period. The saline injection was conducted as a control experiment. The firing response increased transiently but lasted only 17.6 ± 7.6 sec ($n = 3$) after the saline injection.

In contrast to wild-type mice, the initial drastic increase in the firing rate of a VPL neuron induced by formalin injection in PLCβ4 knock-out mice was limited to the first 5 min (early phase), as shown in Figure 8*B*. The firing rate then decreased to approximately the basal activity, which was maintained during the rest of the recording period. To compare the time courses of the response of VPL neurons and that of the pain behavior in the early phase in the formalin test, we first measured the duration of the period in which the firing rate exceeded the basal activity (see Materials and Methods). In the wild-type mice, the response of VPL neurons continued for 178.2 ± 21.4 sec (mean ± SD), whereas the response duration was 129.0 ± 43.7 sec in PLCβ4 knock-out mice. These two values were not significantly different ($p > 0.5$; Fig. 9*A*), consistent with the observation that there was no difference in the pain behavior in the first phase in the formalin test between the wild-type and knock-out mice (Fig. 1). However, the number of spikes in the early phase was much smaller in the knock-out mice than in the wild-type mice (Fig. 8*A*,*B*). Figure 9*B* shows the total number of spikes in the early phase after formalin injection. The number of spikes of VPL neurons in wild-type mice was 3038.2 ± 1208.7 (mean ± SD), whereas that in the knock-out mice was only 901.8 ± 478.5 in the initial 5 min after the injection. These two values were significantly different ($p < 0.01$).

To evaluate the response in the late phase, we then measured the total duration of the response and the number of spikes in the late phase (15–60 min). The total duration in the knock-out mice (124.4 ± 108.6 sec) was significantly reduced compared with that in the wild-type mice (890.2 ± 350.0 sec; $p < 0.01$; Fig. 9*C*). In the late phase, the number of spikes of VPL neurons in the knock-out mice (911.2 ± 343.1) was also significantly lower than that in the wild-type mice (7010.3 ± 2859.1; Fig. 9*D*), indicating a decreased activity of VPL neurons in the knock-out mice in the late phase. This result is consistent with the observation that the pain behavior in the second phase in the formalin test was attenuated in the knock-out mice (Fig. 1), therefore demonstrating a close temporal relationship between the pain behavior

in the formalin test and responses of VPL neurons in both wild-type and knock-out mice.

Discussion

PLCβ4 is crucial for the formalin-induced inflammatory pain but not acute pain

We have studied mice genetically lacking PLCβ4 in the formalin test, which is commonly used to assess the acute pain and subsequent inflammatory pain induced by tissue injury. We found no alteration in pain behavior in the first phase, which represents acute pain response, in PLCβ4 knock-out mice (Fig. 1). Consistent with this result, acute pain responses examined in the hot plate and tail flick tests were unaffected in PLCβ4 knock-out mice (Fig. 2*A*,*B*). However, the nociceptive behavior in the second phase, which represents the inflammatory pain response, was evidently attenuated (Fig. 1). These results indicate that PLCβ4 is a crucial element for the formalin-induced inflammatory pain, whereas it does not contribute to acute pain.

Thalamic PLCβ4 is responsible for the inflammatory pain

PLCβ4, together with PLCβ1, is a major neuronal isoform with distinct regional expression in the brain (Tanaka and Kondo, 1994; Watanabe et al., 1998). In the lateral pain pathway, we found that PLCβ4 mRNA was the sole isoform of PLCβs expressed in the VPL of the thalamus, whereas a large amount of PLCβ1 mRNA was co-expressed with PLCβ4 mRNA in the spinal cord (Fig. 3). In the somatosensory cortex, PLCβ1 was the sole isoform of PLCβs in layer IV, which is the primary recipient of thalamocortical nociceptive input (Fig. 3). Thus, it is highly likely that PLCβ4 plays a crucial role in nociceptive processing in the VPL.

We provided several lines of evidence that thalamic PLCβ4 contributes to the formalin-induced inflammatory pain. First, the intracerebroventricular injection of a potent inhibitor of PLC attenuated nociceptive behavior in the second phase in a dose-dependent manner in wild-type mice (Fig. 5), implying that PLCβ4 contributes to the inflammatory pain processing at the supraspinal level. Second, the injection of PLC inhibitors into the VPL also resulted in the reduction in the nociceptive behavior in the second phase in the formalin test (Fig. 6). Because mRNA expression of other types of PLC, such as PLCγ and PLCδ, is very low in the thalamus (Ross et al., 1989), the primary target of PLC inhibitors in the mouse VPL is presumably PLCβ4. Thus, our results indicate that the thalamic PLCβ4 is involved in the formalin-induced inflammatory pain in the VPL.

The reduction in the mean duration of pain behavior in the second phase after the intrathalamic injection of drugs was smaller than that after the intracerebroventricular injection. The drugs through the intracerebroventricular injection, presumably, were more diffused, not only in the VPL but also in wider brain regions, including the medial thalamus. Because both PLCβ1 and PLCβ4 mRNAs are expressed in the medial thalamic nuclei that mediate the motivational-affective component of the pain, it is possible that the diffusion after the intracerebroventricular injection resulted in the larger reduction in the mean duration of the formalin-induced inflammatory pain.

mGluR1 connecting to PLCβ4 in the VPL contributes to the inflammatory pain

In situ hybridization and immunohistochemical studies showed the adult mouse VPL to express a predominantly high level of mGluR1 but a very weak level of mGluR5 (Masu et al., 1991; Shigemoto et al., 1992; Tanabe et al., 1993; Shigemoto and Mizuno, 2000). Both mGluR1 and mGluR5 are known to stimulate PLCβs through activation of heterotrimeric G-proteins of the Gαq/11 family (Nakanishi, 1994; Pin and Duvoisin, 1995). From the results of the injection of AIDA (Fig. 6*B*), MPEP, or both, thalamic mGluR1 but not mGluR5 is mainly involved in the modulation of inflammatory pain behavior in the formalin test. Furthermore, we found that thalamic injection of a group I mGluR agonist enhanced pain behavior in the second phase in the wild-type mice, but this enhancement was impaired in PLCβ4 knock-out mice (Fig. 7). Co-injection of PLC inhibitor with the group I mGluR agonist into the thalamus of the wild-type mice also impaired the enhancement of the pain behavior in the second phase. These results indicate that mGluR1 connects to PLCβ4 in the VPL and that this cascade is responsible for the formalin-induced inflammatory pain. The thalamic mGluR1 activation somewhat enhanced the pain behavior in the first phase in both wild-type and PLCβ4 knock-out mice (Fig. 7), suggesting that the signal cascade other than mGluR1–PLCβ4 signaling may be involved in the acute pain response.

PLCβ4 regulates the response of VPL neurons to the inflammatory pain

It is generally proposed that neuronal responses in the VPL to nociceptive input account for pain behavior (Guilbaud et al., 1990), much like in the dorsal root ganglion and spinal dorsal horn (Hylden et al., 1989; Young et al., 1997). Guilbaud et al. (1986, 1987a,b) reported that responses of rat VPL neurons are enhanced after peripheral inflammation. In the present study, the time course of the response of VPL neurons in wild-type mice corresponded well to that of the biphasic pain behavior in the formalin test (Figs. 1, 8*A*). The response of VPL neurons in knock-out mice peaked only in the early phase and was significantly attenuated in the late phase (Fig. 8*B*), which also correlated well with the pain behavior. The major ascending sensory afferents to the VPL and descending corticothalamic pathways to the VPL almost certainly use L-glutamate as the transmitter (DeBiasi and Rustioni, 1990; Broman and Ottersen, 1992; DeBiasi et al., 1994). This glutamate, acting at thalamic NMDA receptors and mGluRs, mediates nociceptive input to thalamic neurons (Salt and Eaton, 1994, 1996; Eaton and Salt, 1996; Kolhekar et al., 1997). Among mGluRs, we found that thalamic mGluR1, which connects to PLCβ4, is involved in the inflammatory pain. Both mGluR1 (McCormick and von Krosigk, 1992; Golshani et al., 1998) and PLCβ (Takano et al., 1996; Lee and Boden, 1997; Lee et al., 1999) have excitatory or depolarizing effects in various brain tissues. Interestingly, thalamic mGluR1 shows feature localization that exists at postsynaptic sites of the corticothalamic synapses but not at the spinothalamic synapses (Liu et al., 1998). On the basis of these results, we speculate that the thalamic mGluR1–PLCβ4 signaling cascade at the corticothalamic synapses provided the feedback excitation for the VPL. The feedback mechanism via this cascade may greatly contribute to the coding for the continued pain of formalin-induced inflammation. Reduction in the firing rate in knock-out mice presumably resulted from blockade of the feedback excitation via this mGluR1–PLCβ4 cascade.

In the first phase of pain behavior in the formalin test, the durations of paw licking and lifting were not significantly different between the knock-out and wild-type mice. However, spikes of VPL neurons in the early phase in knock-out mice decreased compared with those in the wild-type mice but still significantly exceeded the basal activity (Figs. 8, 9). Because most nociceptive neurons in the VPL encode stimulus intensity by their firing rate

(Guilbaud et al., 1980; Peschanski et al., 1980), there seems to be a discrepancy between the pain behavior and the response of VPL neurons in the knock-out mice. The exact reason for this discrepancy is as yet unknown. This, however, may be attributable to the method by which the pain behavior was evaluated; the total time that mice spent in licking and lifting their paws in our study is an appropriate score for quantifying the time course of pain behavior but is not sufficient for evaluating the magnitude of pain. Another possibility is that the activity of other pain-related brain regions, such as the medial thalamus, may also be involved in the formalin-induced pain behavior, which is voluntary pain behavior.

Spinal PLCβ4 in the inflammatory pain

The activity of populations of spinal cord nociceptive neurons stimulated by the hindpaw formalin injection has been assessed by expression of c-Fos protein (Williams et al., 1989, Todd et al., 1994). Because of a lack of a specific inhibitor of PLCβ, we counted the number Fos-LI neurons in the dorsal horn of the wild-type and knock-out mice to estimate the contribution of spinal PLCβ4 during the second phase. Fos expression, which is detected 2 hr after the formalin injection, is maintained by neuronal activity of the dorsal horn of the spinal cord during the second phase in the formalin test (Abbadie et al., 1997; Malmberg et al., 1997), and the activity in the two phases of the formalin test contributes to the pattern of Fos expression in the laminae of the dorsal horn in the spinal cord differently (Abbadie et al., 1997). In our study, the numbers of Fos-LI neurons in all laminae of the dorsal horn of the spinal cord demonstrated no difference between PLCβ4 knock-out and wild-type mice (Fig. 4). Thus, the contribution of spinal PLCβ4 is relatively minor, if any, in the second phase of the formalin-induced pain behavior. Coderre (1992) reported that the intrathecal pretreatment of a PLC inhibitor reduces the formalin-induced pain behavior in the second phase in the rodent, suggesting that other types of spinal PLCs, such as PLCγ, may contribute to the inflammatory pain sensation at the spinal level. Alternatively, spinal PLCβ1, which is a counterpart of PLCβ4, may compensate for the lack of PLCβ4 function in the dorsal horn in knock-out mice. Yashpal et al. (2001) reported that intrathecal treatment with an mGluR1/5 antagonist does not influence formalin-induced activation of spinal PKC, suggesting that the spinal group I mGluR–PLCβ–PKC cascade is not crucial for the formalin-induced nociception. Furthermore, a recent study revealed the existence of extracellular signal-related kinase, which is downstream of spinal group I mGluRs and active in modulation of nociceptive transmission of formalin-induced inflammatory pain at the spinal level (Karim et al., 2001). The signal cascade in the dorsal horn of the spinal cord could therefore be expected to be different from the thalamic mGluR1–PLCβ4 cascade, even if spinal PLC contributes to formalin-induced inflammatory pain behavior.

In conclusion, our results demonstrate that the thalamic mGluR1–PLCβ4 cascade is crucial for processing formalin-induced inflammatory pain. This cascade is therefore a possible target for novel treatments of chronic inflammatory pain at the thalamic level.

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