Prostacyclin Regulates Spinal Nociceptive Processing through Cyclic Adenosine Monophosphate–induced Translocation of Glutamate Receptors

Claus Dieter Schuh, Dipl. Biol., Christian Brenneis, Ph.D., Dong Dong Zhang, M. M.Sc., Carlo Angioni, B.Sc. Eng., Yannick Schreiber, C.T.A., Nerea Ferreiros-Bouzas, Ph.D., Sandra Pierre, Ph.D., Marina Henke, B.T.A., Bona Linke, Ph.D., Rolf Nüsing, Ph.D., Klaus Scholich, Ph.D., Gerd Geisslinger, Ph.D., M.D.

ABSTRACT

Background: Prostacyclin (PGI_2) is known to be an important mediator of peripheral pain sensation (nociception) whereas little is known about its role in central sensitization.

Methods: The levels of the stable PGI_2-metabolite 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and of prostaglandin E_2 (PGE_2) were measured in the dorsal horn with the use of mass spectrometry after peripheral inflammation. Expression of the prostanoid receptors was determined by immunohistology. Effects of prostacyclin receptor (IP) activation on spinal neurons were investigated with biochemical assays (cyclic adenosine monophosphate-, glutamate release-measurement, Western blot analysis) in embryonic cultures and adult spinal cord. The specific IP antagonist Cay10441 was applied intrathecally after zymosan-induced mechanical hyperalgesia *in vivo*.

Results: Peripheral inflammation caused a significant increase of the stable PGI_2 metabolite 6-keto-$PGF_{1\alpha}$ in the dorsal horn of wild-type mice (n = 5). IP was located on spinal neurons and did not colocalize with the prostaglandin E_2 receptors EP2 or EP4. The selective IP-agonist cicaprost increased cyclic adenosine monophosphate synthesis in spinal cultures from wild-type but not from IP-deficient mice (n = 5–10). The combination of fluorescence-resonance–energy transfer–based cyclic adenosine monophosphate imaging and calcium imaging showed a cicaprost-induced cyclic adenosine monophosphate synthesis in spinal cord neurons (n = 5–6). Fittingly, IP activation increased glutamate release from acute spinal cord sections of adult mice (n = 13–58). Cicaprost, but not agonists for EP2 and EP4, induced protein kinase A–dependent phosphorylation of the GluR1 subunit and its translocation to the membrane. Accordingly, intrathecal administration of the IP receptor antagonist Cay10441 had an antinociceptive effect (n = 8–11).

Conclusion: Spinal prostacyclin synthesis during early inflammation causes the recruitment of GluR1 receptors to membrane fractions, thereby augmenting the onset of central sensitization. **(ANESTHESIOLOGY 2014; 120:447-58)**

PROSTAGLANDINS are important mediators of inflammatory pain (nociception) and fever.[1,2] They are synthesized *via* two cyclooxygenase-isoforms, cyclooxygenase-1 and cyclooxygenase-2, through conversion of arachidonic acid to the intermediate prostaglandin H_2 which is subsequently isomerized by terminal synthases to the biologically effective prostaglandins (*i.e.*, prostaglandin E_2 [PGE_2] and prostacyclin [PGI_2]).[3] Especially PGE_2 is considered one of the most important mediators of nociception. Inhibition of PGE_2 signaling by nonsteroidal antiinflammatory drugs is of major therapeutical relevance. In the spinal cord the pronociceptive effects of PGE_2 are mediated through the PGE_2 receptor EP2 by blocking the inhibitory input of glycinergic synapses.[4] However, due to the side effects of cyclooxygenase inhibitors, alternative targets for the development of new analgesics are needed. Due to its known pronociceptive role in the peripheral nerve system, the PGI_2 signaling pathway became a potential candidate for the development of new drugs.[3,5]

What We Already Know about This Topic

- Prostaglandins are key pain mediators in the central and peripheral nervous systems. Both prostaglandin E_2 and prostacyclin support nociceptive signaling through interactions with EP and IP receptors, respectively.

What This Article Tells Us That Is New

- Peripheral inflammation increases spinal levels of PGI_2, which in turn stimulates cyclic adenosine monophosphate production and glutamate release. Correspondingly, spinal neuron IP receptor blockade causes analgesia. These results provide a framework for understanding PGI_2 participation in spinal nociceptive signaling.

Data from human clinical trials demonstrate that PGI_2-receptor (IP) agonists cause in humans pain at the infusion site as well as headache.[6] Likewise, IP-receptor agonists elicit writhing behavior in wild-type[7] but not in IP-receptor knockout mice[8] whereas basal pain thresholds were unaltered in these animals.[9]

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The pronociceptive role of prostacyclin in the peripheral nervous system is already well established. In mouse dorsal root ganglias approximately 40% of neurons expressed IP-receptor messenger RNA including unmyelinated C-fibers and thinly myelinated Aδ-fibers[5,10] and PGI_2, like PGE_2, enhances the capsaicin-induced inward current through transient receptor potential vaniloid type 1 channels.[11] Fittingly, IP-receptor agonists cause in C-fibers a greater excitation of active fibers than PGE_2.[12] IP receptors are coupled to the stimulatory G-protein, Gs, and activate through increased cyclic adenosine monophosphate (cAMP) synthesis protein kinase A (PKA) or exchange protein activated by cAMP.[13] The PKA and the exchange protein activated by cAMP activation lead to transient receptor potential vaniloid type 1 sensitization in peripheral sensory neurons and to *N*-methyl-D-aspartate receptor sensitization as well as 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA) receptor translocation in neurons of the central nervous system.[14–16]

In contrast to the role of PGI_2 in primary afferent neurons, to date very little is known about the role of prostacyclin and its receptor in spinal nociceptive processing. Autoradiographic and *in situ* hybridization studies showed a high density of binding sites for the IP-receptor agonist iloprost in the dorsal horn,[17] and intrathecal application of the IP-receptor agonist cicaprost induced mechanical allodynia.[18] However, the mechanisms underlying the enhancement of spinal nociceptive processing by prostacyclin as well as the question whether or not prostacyclin is involved in processes involved in central sensitization during inflammation-induced hyperalgesia have not been investigated adequately.

Here, we studied whether or not prostacyclin fulfills a similar pronociceptive role in spinal cord neurons as reported for the peripheral nervous system. Therefore, we determined the PGI_2 synthesis in the spinal cord after peripheral inflammation and found that spinal prostacyclin synthesis increases during inflammation. Moreover, IP-receptor–mediated cAMP synthesis caused in the spinal cord PKA-mediated GluR1 receptor phosphorylation and translocation. The pronociceptive IP signaling appears to be independent of PGE_2 signaling, because IP and EP receptors were not localized in the same neurons and only IP activation induced GluR1 receptor translocation.

Interestingly, peripheral inflammation induced by different stimuli (*e.g.*, carrageenan, Complete Freund's Adjuvance) causes in the spinal cord the translocation of GluR1 to membrane fractions[19,20] a mechanism described in some brain regions as prostacyclin mediated.[14–16]

Materials and Methods

Animals

C57BL/6NRj mice, pregnant and male adult RjHan:SD rats were supplied by Janvier (Le Genest, France). IP-receptor knockout mice[8] were on C57BL/6NRj background and were compared with age- and sex-matched wild-type mice. Crl:CFW(Sw) mice came from our breeding and were used in the behavioral tests. In all experiments the ethics guidelines for investigations in conscious animals were obeyed and the procedures were approved by the local Ethics Committee (Regierungspräsidium, Darmstadt, Germany).

Reagents

Cicaprost was a gift from Schering (Berlin, Germany). EP-receptor–specific ligands (EP1 agonist ONO-DI-004, EP2 agonist ONO-AE1-259, EP3 agonist ONO-AE-248, and EP4 agonist ONO-AE1-329) were kindly gifted by ONO Pharmaceuticals (Sakurei, Japan).

Immunohistochemistry

Spinal (L4-L5) tissues were prepared from naive mice that were intracardially perfused with phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde/PBS (pH 7.4). After a 24-h incubation in 30% sucrose/PBS the tissue was cryostat sectioned at 10 µm with the use of the cryotome Leica CM3050S (Leica, Wetzlar, Germany) and stored at −80°C until use. Antibodies against IP (1:1,000) and prostaglandin synthase (PGIS; 1:500) were from Cayman Chemical (Ann Arbor, MI), calcitonin gene-related peptide (1:500) from Abcam (Cambridge, United Kingdom), S100 (1:1,000) from Sigma-Aldrich (St. Louis, MO), NeuN (1:1,000) from Merck (Darmstadt, Germany), glial fibrillary acidic protein (1:100) from Dianova, (Hamburg, Germany) and Isolectin B4-FITC (1:100) from (Sigma). For costaining we used 4′6-diamidino-2-phenylindole (DAPI) (Applichem, Darmstadt, Germany). The tissue was permeabilized in 0.1% Triton X100 in PBS and blocked with 3% bovine serum albumin in PBS for 1 h at room temperature, primary antibodies were incubated for 90 min at room temperature, secondary antibodies Cy3 antirabbit (1:500; Sigma), DyLight550 antigoat (1:500; Bethyl, Montgomery, TX), or CF488 antimouse (1:800; Biotium, Hayward, CA) were incubated for 1 h at room temperature. The stained tissue was analyzed using a ZEISS Observer inverted microscope attached to a monochrome AxioCam Mr (both from Carl Zeiss Microscopy, Göttingen, Germany). Pictures were taken with an EC Plan-Neofluar ×10 and a Plan-Apochromat ×20 objective and analyzed with AxioVision software (Vers. 4.8, all Carl Zeiss Microscopy).

Multiepitope-ligand Cartography

For multiepitope-ligand cartography analysis spinal cord slices cryosections of 10 µm thickness were prepared using the cryotome Leica CM3050S and applied on silane-coated coverslips. The tissue was fixed in 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X100 in PBS, and blocked with 3% bovine serum albumin in PBS for 1 h at room temperature. The multiepitope-ligand cartography technology has been described previously.[21–23]

Liquid Chromatography–Tandem Mass Spectrometry

Liquid chromatography–tandem mass spectrometry analysis of PGE_2 and 6-keto-$PGF_{1\alpha}$ from spinal tissue was performed as described previously with some changes.[24] Briefly, at different time points after intraplantar injection of 20 μl 10 mg/ml zymosan A or 20 mg/ml λ-Carrageenan (both Sigma) into the midplantar surface of the paw the ipsilateral dorsal part of the spinal cord was prepared. Then, the tissue was subsequently weighted, transferred to extraction buffer containing 600 μl ethyl acetate, 100 μl 150 mM EDTA, and 20 μl internal standard solution (25 ng/ml of $[^2H_4]$-PGE_2, $[^2H_4]$-PGD_2, and $[^2H_4]$-TXB_2 and 10 ng/ml of $[^2H_4]$-$PGF_{2\alpha}$ and $[^2H_4]$-6-keto-$PGF_{1\alpha}$ in methanol). The samples were homogenized using Mixer Mill MM400 (Retsch, Haan, Germany), centrifuged at 20,000*g* for 5 min. The extraction was repeated and extraction solvents were removed at 45°C under a gentle stream of nitrogen and stored at −80°C until measurement. The residues were reconstituted with 50 μl of acetonitrile:water:formic acid (20:80:0.0025, v/v, pH 4.0), and injected into the liquid chromatography–tandem mass spectrometry.

Neuron-enriched Spinal Cord Cultures

Mixed spinal cord cell cultures were prepared as described previously with some modifications.[24,25] Briefly, whole spinal cords were prepared from RjHan:SD rats at embryonic day 16. After dissociation, cells were plated onto poly-L-lysine (Sigma)–coated plates and grown for 6 days in neurobasal medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and supplement B27 (all from Invitrogen, Carlsbad, CA). On day 2 the medium was supplemented with 1 μM uridine, 1 μM cytosine-β-D-arabinfuranoside, and 0.1 μM 5-fluoro-3-desoxyuridine (Sigma) to inhibit glia proliferation. Stimulations were performed in neurobasal medium without mitosis inhibitors.

Western Blot Analysis

Neuron-enriched spinal cord cells were washed and stimulated with 1 μM cicaprost or 8-bromo-cAMP in culture medium for 20 min. Then, cells were quickly washed twice with PBS and then harvested in PhosphoSafe extraction reagent (Merck) with 1 mM Pefablock (Carl Roth, Karlsruhe, Germany). The lysate was sonificated and centrifuged at 18,000*g* 10 min at 4°C and the supernatant containing cytosolic and membrane fraction was recentrifuged to enrich membrane fractions by ultracentrifugation at 48,000*g* for 30 min at 4°C. Then, 10–30 μg protein was separated on an 8% sodium dodecyl sulfate-polyacrylamide gel and blotted to a Protran BA 85 nitrocellulose membrane (Whatman, Kent, United Kingdom). Alternatively, 250–300 g RjHan:SD rats were deeply anesthetized with pentobarbital 40 mg/kg and ketamine 100 mg/kg. The spinal canal was exposed at the L6-L7 segments and 10 μl cicaprost (10 μM) or vehicle diluted in artificial cerebrospinal fluid (Harvard Apparatus, Holliston, MA) were injected intrathecally. After 30 min the dorsal part of the spinal cord was dissected and frozen in liquid nitrogen. The tissue was homogenized in PhosphoSafe extraction reagent with 1 mM Pefablock, sonificated, and centrifuged at 18,000*g* 10 min at 4°C. After that the supernatant containing the cytosolic and membrane fractions were recentrifuged to enrich membrane fractions by ultracentrifugation at 48,000*g* for 30 min at 4°C. Then 50 μg protein was separated on an 8% sodium dodecyl sulfate-polyacrylamide gel and blotted to a Protran BA 85 nitrocellulose membrane. AMPA receptors were detected using anti-GluR1 (1:200; Santa Cruz, Santa Cruz, CA), anti–phospho-GluR1 (Ser845) (1:500; Acris, San Diego, CA), and anti-GluR2 (1:1,000; Synaptic Systems, Göttingen, Germany) antibodies. Anti–heat-shock protein 90 (1:1,000; Promega, Madison, WI) or anti-Pan Cadherin (1:500; Sigma) antibodies were used to control for equal loading.

To analyze the spinal expression of IP receptors following zymosan-induced inflammation the mice received an intraplantar injection of 20 μl zymosan (10mg/ml) into one hindpaw. After 0, 1, or 6 h animals were euthanized and the dorsal part of the L4-L5 spinal cord was dissected and frozen in liquid nitrogen. The tissue was homogenized in PhosphoSafe extraction reagent with 1 mM Pefablock, sonificated, and centrifuged at 18,000*g* 10 min at 4°C. Then 40 μg protein was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. The IP receptor was detected with the aforementioned Cayman antibody (1:500) and heat-shock protein 90 was used to control for equal loading. The primary antibodies were detected with horseradish peroxidase–coupled secondary antibodies (all 1:5,000; Sigma), incubated with Pierce ECL (Pierce Biotechnology, Rockford, IL) or Amersham ECL prime (GE Healthcare Biosciences, Pittsburgh, PA) Western blotting reagents, and developed on Cl-xPosure films (Pierce Biotechnology). The film was scanned at an Epson V10 perfection scanner (Epson, Meerbusch, Germany). Densitometric analysis was performed with Image J (version 1.47e; NIH, Bethesda, MD).

cAMP Accumulation

Cyclic adenosine monophosphate amounts in primary spinal cord cells were determined as described previously.[26,27] Briefly, fresh medium containing 100 μM 3-isobutyl-1-methylxanthine (Sigma) was added 5 min before the respective treatment (*e.g.*, cicaprost). Then cells were incubated with 100 μM 3-isobutyl-1-methylxanthine and the respective prostaglandin receptor agonist or 0.5 μM forskolin (Sigma) for 40 min, washed twice with PBS, and the reaction was stopped with 0.1 M HCl containing 1% Triton X100. The cell suspension was harvested, centrifuged for 5 min with 10,000*g* and the cAMP concentrations determined using an enzyme immunoassay kit (Assay Designs, Ann Arbor, MI).

Cyclic adenosine monophosphate amounts in spinal cord slices were determined as followed. Spinal cord slices were incubated with cicaprost (1 μM), forskolin (1 μM), or modified Ringer's solution as control for 30 min at 37°C. After the stimulation, the slice was weighed and frozen in liquid nitrogen. The tissue was homogenized in the sample

diluent provided by the enzyme immunoassay assay kit (here Biotrend, Destin, FL) with 1% Triton X100, sonificated, and centrifuged at 750*g*. The cAMP in the supernatant was determined with the enzyme immunoassay kit

Fluorescence-resonance–Energy-transfer Imaging

Fluorescence-resonance–energy-transfer (FRET) imaging was performed as described previously.[26] Primary spinal cord cell cultures were plated on poly-L-lysine–coated coverslips (Dunn Labortechnik, Asbach, Germany) and transfected with pCDNA-CAT-CFP and pCDNA-RII-L20-YFP constructs[28] with the use of Effectene Transfection Reagent (Qiagen, Monheim, Germany) 4 h after plating. FRET imaging experiments were performed 24–48 h after transfection. Cells were maintained in HEPES-buffered Ringer-modified saline containing 136 mM of NaCl, 5.4 mM of KCl, 0.33 mM of NaH_2PO_4, 1 mM of $MgCl_4$, 10 mM of glucose, 1.8 mM of $CaCl_2$, and 10 mM of HEPES, pH 7.2 at room temperature and imaged using an Axioscope 2 upright microscope (Carl Zeiss Microscopy) with a ×63 Achroplan water immersion objective. Images were acquired and processed using Tillvision software (Till Photonics, Gräfelfing, Germany). FRET changes were measured as changes in the background-subtracted 480/545 nm fluorescence emission intensities on excitation at 430 nm and expressed as R/R_0, where R is the ratio at time t and R_0 is the mean ratio during the first 50 s. Cicaprost was added by bath application dissolved in HEPES-buffered Ringer-modified saline. Cells were included in the responder group when 10 min after cicaprost addition the R/R_0 ratios were greater than 1.01.

Calcium Imaging

Calcium imaging experiments were performed with primary spinal cord neuronal cells plated on poly-L-lysine–coated coverslips for 3–6 days in culture as described previously.[29] Cells were incubated with 10 μM Fura2-acetoxymethyl ester (Fura2-AM) in neurobasal medium for 30 min at 37°C and 5% CO_2 previous to the determination of intracellular calcium levels $[Ca^{2+}]_i$. The cells were washed twice with neurobasal medium and transferred to the perfusion chamber (same setup as described under FRET imaging) and imaged with a ×40 Achroplan water immersion objective (Carl Zeiss Microscopy) at room temperature. $[Ca^{2+}]_i$ was expressed as the ratio of the background-subtracted fluorescence emission at 510 nm (filter type LP 440) after excitation at 340 nm and 380 nm using the Polychrom IV Monochromator (Till Photonics).

Glutamate Release from Acute Spinal Cord Slices

Spinal cords were prepared from adult mice and transferred into ice-cold, HEPES-buffered Ringer's solution (136 mM of NaCl, 5.4 mM of KCl, 0.33 mM of NaH_2PO_4, 10 mM of glucose, 1.8 mM of $CaCl_2$, 10 mM of HEPES, and 1 μM of glycine, pH 7.2). Slices that were 500 μm thick were prepared (Vibratome 1000 Plus; Intracel Ltd., Royston, United Kingdom) and stimulated in carbogen-saturated modified Ringer's solution (136 mM of NaCl, 5.4 mM of KCl, 0.33 mM of NaH_2PO_4, 10 mM of glucose, 1.8 mM of $CaCl_2$, 10 mM of HEPES, and 1 μM of glycine, pH 7.2) under application of different stimuli for 30 min at 37°C. Glutamate concentrations after stimulation of acute spinal cord slices were determined in medium with the use of Amplex Red glutamate/glutamatoxidase Assay Kit (Invitrogen).

Catheter Implantation

IT catheters were implanted as described previously[30] with some modifications. Briefly, mice were anesthetized with isoflurane (Abbott Laboratories, Abbott Park, IL). The skin was incised above the vertebral column from vertebrae Th13 to L3. Muscle tissue around L2–L3 was cleared away. The processus spinosus of L3 was removed, and a laminectomy was done at L2. The catheter was shortened according to the dimensions of the animal and inserted into the peridural space so that the tip reached Th12–Th13 and was fixed with cyanoacrylate glue. The outer catheter was directed subcutaneously to the neck where it exits the skin and was glued with cyanoacrylate glue between the ears. The catheter was closed by a flame till it was used.

Behavioral Tests

The investigator was unaware of the treatments or the genotypes during all behavioral experiments. An injection of 20 μl of a suspension of the yeast glucan Zymosan A (5 mg/ml in PBS) was administered subcutaneously into the plantar side of the left hind paw.[22] Mechanical thresholds of the plantar side of a hind paw were determined using a plantar aesthesiometer (Dynamic Plantar Aesthesiometer; Ugo Basile, Comerio, Italy). Here, a steel rod (2 mm diameter) was pushed against the paw with ascending force (0–5 g over a 10-s period, time resolution 0.1 s) until a strong and immediate withdrawal occurred. The paw withdrawal latency was mean of at least three consecutive trials with at least 20 s in between.[31]

Statistics

Experiments with only two treatment groups were analyzed for statistical significance using Student *t* test. Experiments with more than two groups were analyzed using one- or two-way ANOVA with Newman–Keuls or Bonferroni *post hoc* test. Significance was accepted at a P value of less than 0.05.

Results

Peripheral Inflammation Increases Spinal PGI2 Concentrations

In the first step, we studied whether or not peripheral inflammatory stimuli can modulate spinal PGI_2 concentrations. We found that a single zymosan or carrageenan injection in one hind paw of adult mice caused a significant increase of 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) and PGE_2 in the dorsal horn of the spinal cord (fig. 1). The increase was observed

1 h after injection (fig. 1, A and C) and was comparable with the increase seen for PGE_2 levels (fig. 1, B and D). The early increase of spinal 6-keto-$PGF_{1\alpha}$ concentrations indicates a role of prostacyclin in the early phase of central sensitization.

Next, we determined the localization of the IP receptor and the PGI_2 synthase (PGIS) in the spinal cord. For the IP receptor a strong immunohistochemical signal in lamina 1 of the dorsal horn was seen, which in part colocalized with the neurotransmitter calcitonin gene-related peptide (fig. 2A). Calcitonin gene-related peptide is a marker for peptidergic C-fibers in incoming peripheral nociceptive fibers and its synaptic endings in dorsal horn neurons. No colocalization of isolectin B4 (IB4), a marker for nonpeptidergic C-fibers, with IP-positive neurons or fibers was observed (fig. 2B). In the inner laminae of the spinal cord a slightly weaker staining for IP was seen in a subset of cells in which it colocalized with the neuronal marker NeuN (fig. 2C). The PGI_2 synthase PGIS was mainly found to be expressed in the outer laminae 1–4 and although it generally did not colocalize with IP, its localization was close to the IP staining (fig. 2D). A colocalization of IP with the glia marker glial fibrillary acidic protein was not observed (Supplemental Digital Content1, fig. 1A, http://links.lww.com/ALN/A972). Notably, PGIS colocalized with S100, a marker for glia cells but not with NeuN (Supplemental Digital Content1, fig. 1B and C, http://links.lww.com/ALN/A972). Notably no change in the expression level of the spinal IP receptor was seen 1 or 6 h after zymosan injection (Supplemental Digital Content1, fig. 2, http://links.lww.com/ALN/A972). Likewise, no significant changes in the localization were seen (data not shown). Thus, to date the data show that prostacyclin is synthesized in the spinal cord in response to a peripheral inflammatory stimulation and that its receptor is localized in sensory dorsal horn neurons, which points toward a potential function of PGI_2 signaling in spinal nociceptive processing.

IP Receptor Activation Induces cAMP Accumulation in Spinal Neurons

The IP receptor is known to be coupled to the stimulatory G-protein, Gs, and to stimulate cellular cAMP synthesis.[13,32] Therefore, we tested whether or not activation of the IP receptor induces cAMP accumulation in spinal cord neurons. Indeed, in embryonic primary spinal cord cultures we observed an increased cAMP accumulation after stimulation with the IP agonist cicaprost (fig. 3A). This stimulated cAMP accumulation was specific for the IP receptor because cicaprost did not elicit cAMP synthesis in spinal cord cultures from IP-deficient mice (fig. 3A). In accordance with previous publications[33] a similar induction of the cAMP synthesis was observed in spinal cord cells when selective agonists for the PGE_2 receptors EP2 and EP4, but not EP1 and EP3, were applied (fig. 3B). Importantly, we observed the cicaprost-induced increase of cAMP also in acute spinal cord slices from adult mice (fig. 3C).

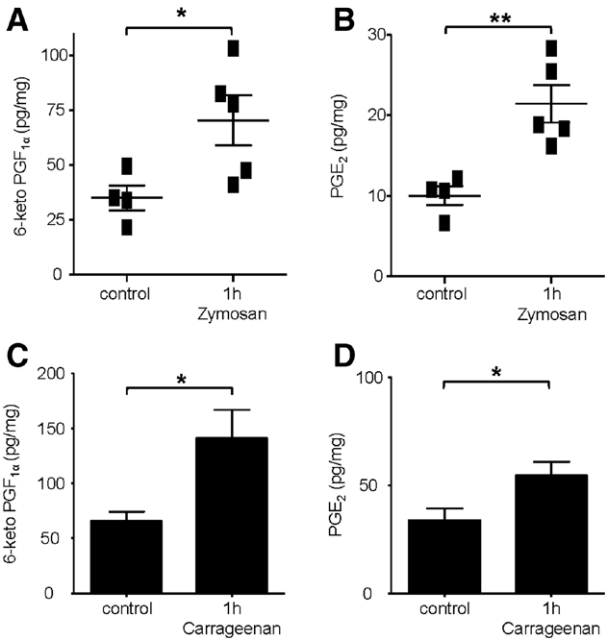

Fig. 1. Spinal prostacyclin synthesis is increased during peripheral inflammation. (*A–D*) After intraplantar injection of either 20 µl zymosan (10 mg/ml; *A* and *B*) or 20 µl Carrageenan (20 mg/ml; *C* and *D*), the dorsal horn of spinal segments L4-L5 were dissected at the indicated times and prostanoid levels were determined by liquid chromatography–tandem mass spectrometry. Levels of the prostacyclin (PGI_2) metabolite 6-keto-$PGF_{1\alpha}$ (*A* and *C*, n = 5) and prostaglandin E_2 (PGE_2; *B* and *D*; n = 5) were determined at the indicated times and compared with those of untreated controls (n = 4 in *A* and *B* and n = 5 in *C* and *D*). Data are shown as mean ± SEM (n = 5). One-way ANOVA with Newman–Keuls multiple comparison test, $^*P < 0.05$; $^{**}P < 0.01$.

To investigate whether or not IP activation increases cAMP accumulation in spinal cord neurons, we combined FRET-based cAMP measurements with subsequent calcium-imaging analyses. First, we determined the effect of cicaprost on intracellular cAMP concentrations by monitoring FRET changes using two constructs (CFP-RIIα and YFP-PKAc) encoding either the regulatory RIIα subunit coupled to the cyan (CFP) or the catalytic C-α subunit of PKA to the yellow (YFP) variants of the green fluorescent protein.[26,28] The constructs were cotransfected in neuron-enriched primary spinal cord cells and FRET changes were recorded in presence of 50 µM 3-isobutyl-1-methylxanthine. The average FRET changes for all cicaprost-treated cells were significantly increased as compared with changes in the vehicle-treated control group (fig. 4, A and B). Of all cells 46% responded to cicaprost 10 min after cicaprost addition with a significant R/R_0 ratio increase against baseline. To characterize the cicaprost-responding spinal cord cells, the cells were loaded with the calcium sensor FURA-2-AM and calcium-imaging analysis was performed by stimulating the cells with *N*-methyl-D-aspartate, which evokes transient calcium increases only in neuronal cells. All cicaprost responders exhibited an *N*-methyl-D-aspartate–evoked calcium increase,

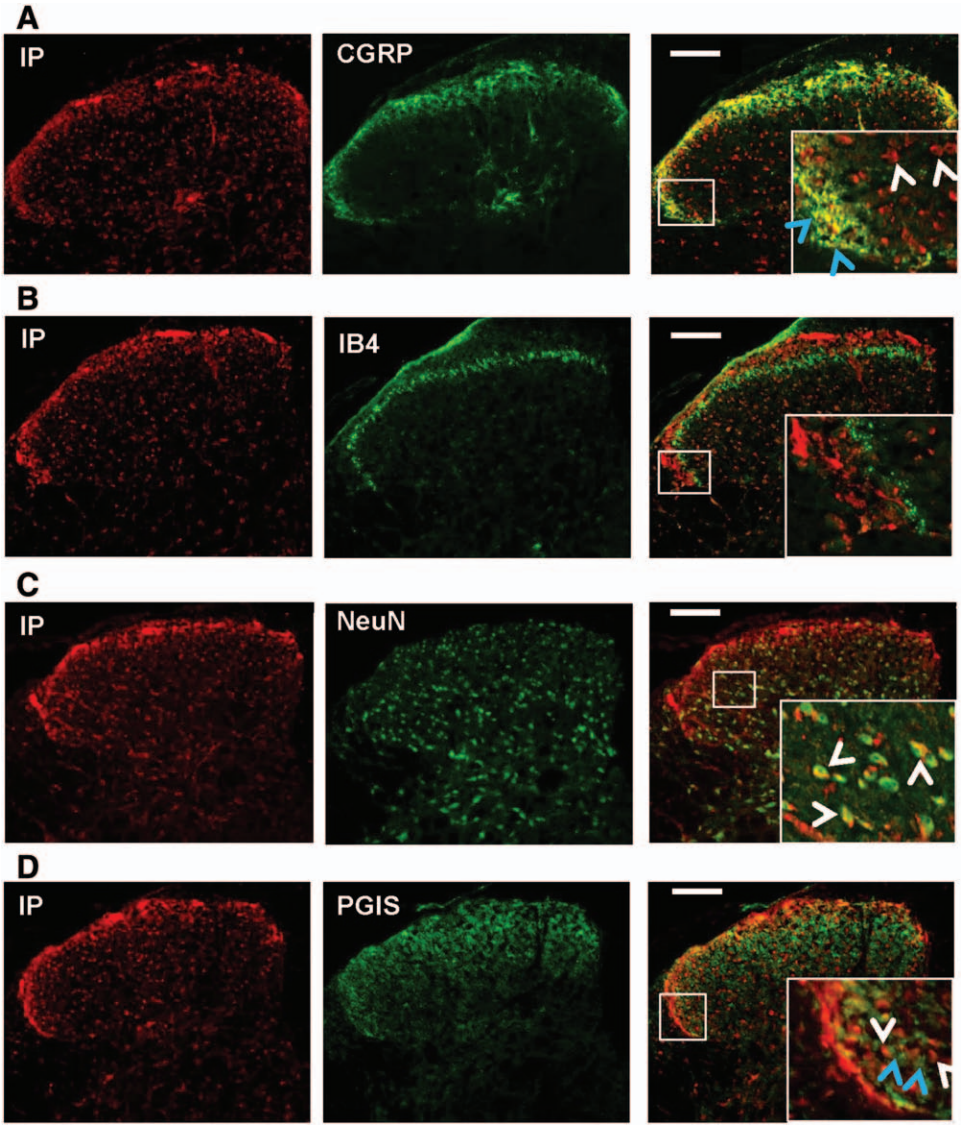

Fig. 2. The IP receptor is expressed in lumbar dorsal horn neurons. (*A*) Immunohistochemical staining showing IP receptor and the peptidergic C-fiber marker calcitonin gene-related peptide (CGRP) in incoming peripheral nociceptive fibers as well as in dorsal horn neurons. (*B*) No colocalization of isolectin B4 (IB4), a marker for nonpeptidergic C-fibers, with IP-positive neurons or fibers. (*C*) IP receptor costaining with the neuron marker NeuN. (*D*) IP-expressing neurons are surrounded by prostacyclin synthase (PGIS) expressing cells in the dorsal horn. *Scale bar* represents 100 μm. The insets are magnifications of the areas shown as *white boxes*.

identifying them as neuronal cells (fig. 4C). Cicaprost itself did not evoke an intracellular calcium increase (Supplemental Digital Content1, fig. 3, http://links.lww.com/ALN/A972).

IP Receptor-mediated Spinal cAMP Synthesis Causes Phosphorylation and Translocation of GluR1

Previously it has been shown that IP activation can induce a cAMP-dependent GluR1 phosphorylation and translocation to synaptic membranes in neurons.[16,34,35] Therefore, we analyzed GluR1 translocation and its effects in cultured neurons and *in vivo* (fig. 5). Fittingly, we found that stimulation of primary spinal cord cells with cicaprost or the stable cAMP analog 8-bromo-cAMP increased the amount of GluR1 in the membrane fraction of these cells (fig. 5A), but did not affect the amount of GluR2 in the membrane (Supplemental Digital Content1, fig. 4, http://links.lww.com/ALN/A972). Interestingly, activation of the two EP receptors (EP2/EP4), which signal through increased cAMP synthesis and increased cAMP accumulation in primary spinal neurons, did not cause GluR1 translocation to the membrane fractions (fig. 5B), suggesting that EP2/4 and IP receptors do not share the same signaling pathway despite their common coupling to cAMP signaling. Importantly, the translocation of GluR1, but not of GluR2, was observed also *in vivo* after adminstration of IT injection of cicaprost in adult animals (fig. 5C). Likewise, injection of zymosan in

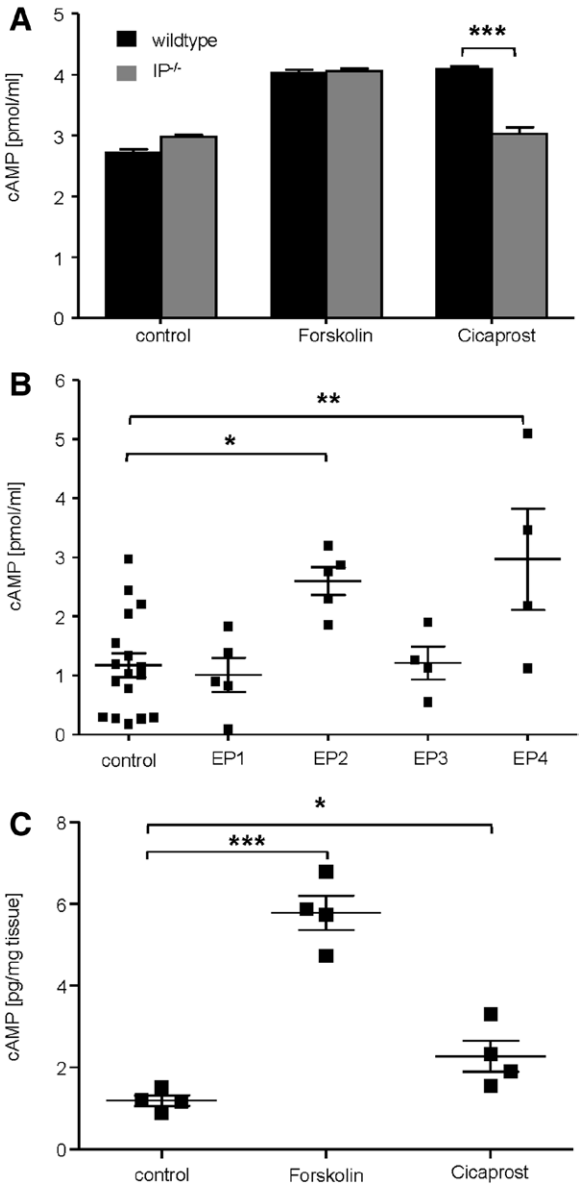

Fig. 3. Cicaprost stimulates cyclic adenosine monophosphate (cAMP) synthesis in spinal cord cells. (*A*) Cicaprost-stimulated (1 μM) cAMP synthesis is mediated by IP receptor because cicaprost does not stimulate cAMP synthesis in spinal cord cultures of IP-deficient mice. Data are shown as mean ± SEM (n = 5 for weight and n = 10 for $IP^{-/-}$). Two-way ANOVA with Bonferoni *post hoc* test, ***$P < 0.001$. (*B*) Stimulation of rat embryonic spinal cord cultures with 0.1 μM of selective EP receptor agonists (ONO-DI-004 [EP1; n = 5], ONO-AE1-259-01 [EP2; n = 5], ONO-AE-248 [EP3; n = 4], and ONO-AE1-329 [EP4; n = 4]) for 40 min. Data are shown as mean ± SEM. One-way ANOVA with Newman–Keuls multiple comparison test, *$P < 0.05$; **$P < 0.01$. (*C*) Cicaprost stimulation (1 μM) of spinal cord slices induced an increase in cAMP synthesis. Data are shown as mean ± SEM (n = 4). One-way ANOVA with Newman–Keuls multiple comparison test, *$P < 0.05$; ***$P < 0.001$.

one hind paw induced in the spinal cord the translocation of GluR1 to the membrane fractions (fig. 5D). The translocated GluR1 was also detected by an antibody recognizing the PKA-dependent phosphorylation of serine 845 (fig. 5D) indicating a PKA-dependent process.

Because the cicaprost-induced GluR1 translocation implies a sensitization of these neurons,[16,34,35] we used acute spinal cord slices to investigate whether or not the IP agonist cicaprost can induce an increased glutamate release. Therefore, we incubated acute spinal cord slices from adult mice with cicaprost in presence and absence of the PKA inhibitor H89. We found that cicaprost increased the glutamate release from the spinal cord slices (fig. 5E). The increased glutamate release was similar to the one induced by the positive control, 50 mM KCl, which causes discharge of all neurons (fig. 5E). The cicaprost-induced glutamate release was blocked by H89, suggesting that the increased glutamate release is mediated by PKA. Spinal cord slices of IP-deficient mice did not respond to cicaprost treatment with an increased glutamate release, demonstrating that this effect is due to a selective IP activation (fig. 5F).

IP-receptor–Mediated Nociception Does Not Share the Pronociceptive Mechanism of EP2 Signaling

Previously, it has been shown that the EP2 receptor mediates the pronociceptive effects of PGE_2 in the spinal cord.[4] Because IP, but not EP2 activation, induced the translocation of GluR1 in spinal cord neurons, we compared the localization of the PGE_2 receptor EP2 with the IP receptor in spinal cords from adult mice (fig. 6).[21–23] In support of the notion that EP2 and IP do not share the same signaling pathway, we found that IP and EP2 do not colocalize in the spinal cord of adult mice (fig. 6C). Likewise, GlyRα3, the receptor that is inhibited by EP2 activation and mediates the spinal pronociceptive EP2 effects, colocalized only partially with IP (fig. 6A) whereas there was a strong colocalization of EP2 and GlyRα3 (fig. 6B). In accordance with previous studies EP4 was not detected in spinal cords of adult mice.

Importantly, intrathecal administration of the IP-receptor antagonist Cay10441 increased significantly mechanical pain thresholds 6 h after injection of zymosan in one hind paw (fig. 7A). IT application of compounds has the potential to affect processes in the spinal cord as well as dorsal root ganglia. However, 6 h after zymosan injection the 6-keto-$PGF_{1\alpha}$ concentrations were significantly increased in the spinal cord (fig. 7B) but not in the dorsal root ganglias (Supplemental Digital Content1, fig. 5, http://links.lww.com/ALN/A972).

Discussion

In contrast to the well-documented pronociceptive role of the prostacyclin receptor (IP) in peripheral sensitization processes little is known about its participation in spinal nociceptive processes and is debated with opposing views.[36,37] Some studies question the role of prostacyclin in spinal nociceptive processing. However, in these studies prostacyclin itself was used to investigate its physiological effects despite its instability.[38–40] Doi *et al.*[18] showed that IT application of the IP-receptor agonist cicaprost induces mechanical allodynia. Fittingly, we show that

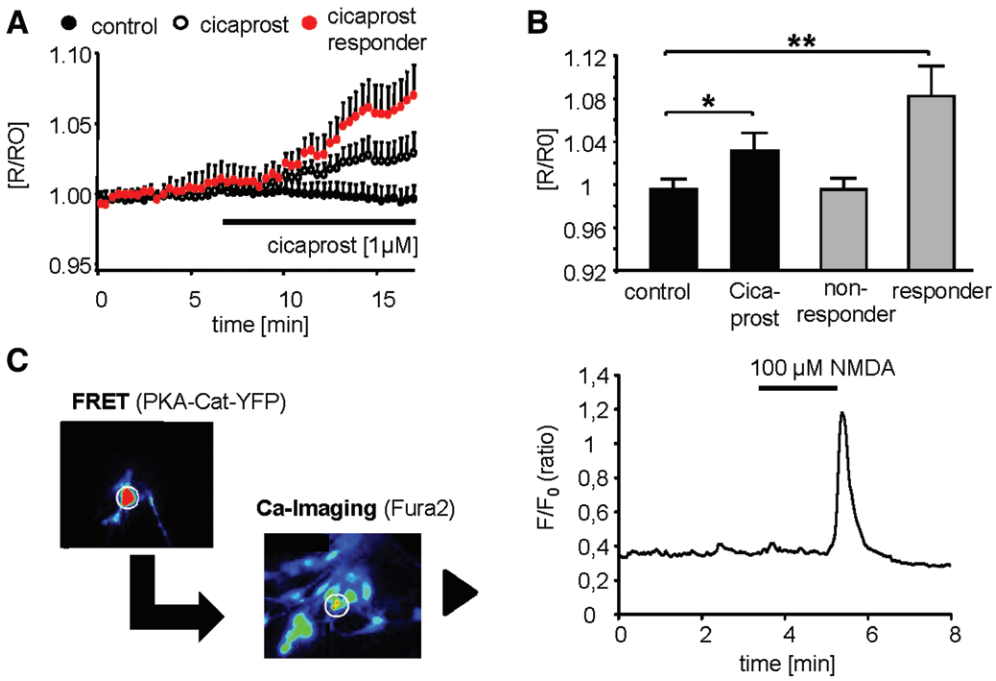

Fig. 4. Cicaprost induces cyclic adenosine monophosphate (cAMP) synthesis in embryonic spinal cord neurons. (*A*) Fluorescence-resonance–energy-transfer (FRET)–based cAMP imaging of cultured embryonic spinal cord cells after cicaprost (1 µM) stimulation. Results are shown as median of all unstimulated cells, all cicaprost-stimulated cells, and cicaprost-responding cells with R/R0 as ratio at time *t* (R) divided by the mean ratio of the first 50 s (R0). (*B*) Summary of all experiments performed in the same conditions as in *A*. The mean of 15 independent experiments ± SEM are shown (*black bars*). Cicaprost-treated cells are also shown as responder (n = 6) and nonresponding cells (n = 5; *gray bars*). Two-tailed Student *t* test: $*P < 0.05$, $**P < 0.01$ as compared with untreated cells. (*C*) All cicaprost-responding cells that were identified by FRET-based cAMP imaging (the signal of the YFP-labeled catalytic subunit of protein kinase A, PKA-Cat-YFP, is shown) were loaded afterward with the Ca^{2+} indicator FURA-2-AM and Ca^{2+} imaging was performed using *N*-methyl-D-aspartate stimulation to identify neurons. A representative experiment is shown. NMDA = N-methyl-D-aspartate; PKA = protein kinase A; YFP = yellow fluorescent protein.

prostacyclin levels in the dorsal horn are significantly increased after peripheral inflammation and that inhibition of spinal IP receptors decreases zymosan-induced mechanical hyperalgesia, thus, establishing prostacyclin as a mediator of spinal pronociceptive processing (central sensitization). Moreover, the increase in spinal prostacyclin concentrations was higher than for PGE_2 and taken together with the strong antinociceptive effect of IT-administered IP antagonists the data suggest a major role of prostacyclin in spinal nociceptive processing. Notably, the increased prostacyclin concentrations are seen 1 and 6 h after initiation of peripheral inflammation, indicating a role for spinal IP signaling in the early as well as the late phase of central sensitization.

The IP receptor was predominantly expressed in the spinal cord in lamina 1 and colocalized with calcitonin gene-related peptide, a marker for peptidergic C-fibers in incoming peripheral nociceptive fibers and at its synapses in dorsal horn neurons. It is known that IP receptors are expressed in peripheral sensory neurons that terminate in lamina 1 and 2 of the dorsal horn.[10] However, in this study we show that the IP receptor is also expressed in spinal cord neurons and, therefore, can potentially contribute to the central sensitization processes by pre- and postsynaptic mechanisms.

Toward the underlying mechanisms we found that activation of the IP receptor caused the translocation of the AMPA subunit GluR1 but not of GluR2 to membrane fractions. This translocation of the GluR1 receptor to membrane fractions is indicative for its insertion in synaptic membranes. In the spinal cord GluR1 and GluR2 are the mainly expressed AMPA receptor subunits.[41] Both receptors differ significantly in their calcium permeability,[42] and their ratio is crucial for mediating long-term potentiation in the spinal cord.[43,44] Also in different pain models a shift in the balance of GluR1 and GluR2 expression at synapses in the spinal cord has been shown to be involved in synaptic activities.[45–47] However, electrophysiological studies should be carried out in the future to study the effects on long-term potentiation.

After carrageenan- or Complete Freund's Adjuvance–induced peripheral inflammation, PKA phosphorylates the GluR1 subunit at serine 845,[19] which causes its recruitment to the synaptic membrane.[35,48] The increased insertion of the GluR1 enhances the calcium permeability and, therefore, the excitability of the neurons.[49] Accordingly, in GluR1 knockout mice the inflammatory hyperalgesia is markedly decreased, whereas in GluR2 knockout mice it is increased.[41] It is long known that the IP receptor couples with Gs and activates adenylyl cyclases. We show that in spinal cord neurons IP activation causes the induction of cAMP synthesis and PKA-dependent phosphorylation

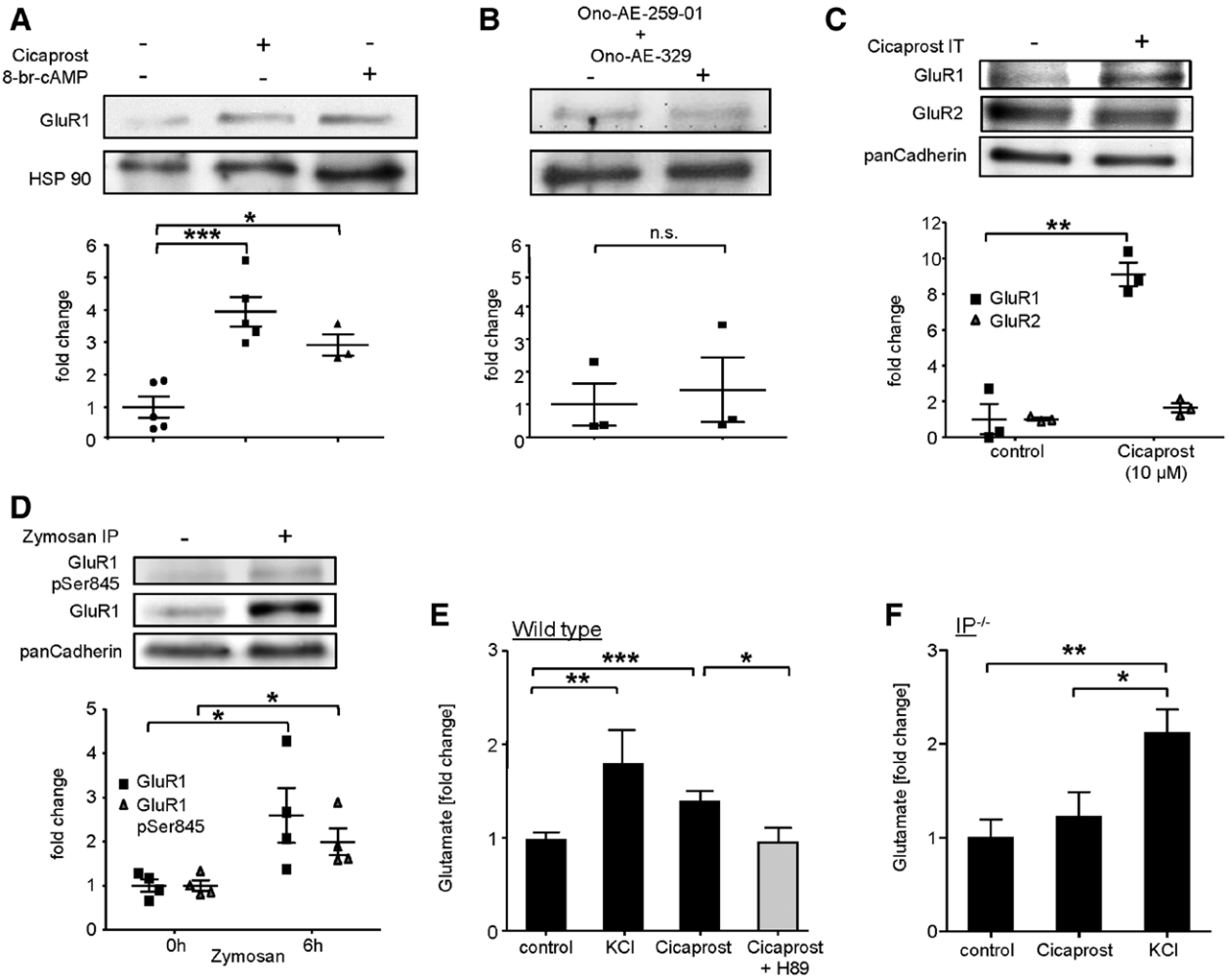

Fig. 5. Cicaprost induces a protein kinase A–dependent GluR1 channel phosphorylation. (*A* and *B*) Rat embryonic spinal cord cultures were treated with 1 µM cicaprost (n = 5), 20 µM 8-bromo-cyclic adenosine monophosphate (n = 3; *A*), or 0.1 µM EP2/EP4 agonists (Ono-AE-259-01 and Ono-AE-329; n = 3; *B*) for 20 min. Western blot analysis showing GluR1 protein level after stimulation in membrane fractions. Densitometry of the GluR1 signals corrected for heat-shock protein (HSP) 90 loading and normed to the control is shown in the lower panel. Data are shown as mean ± SEM (n = 3–5). One-way ANOVA with Newman–Keuls multiple comparison test, **P* < 0.05, ****P* < 0.001. (*C*) Intrathecal (IT) injection of cicaprost (10 µM) in rats resulted in translocation of GluR1 but not GluR2 subunits to the membrane shown by Western blot analysis of membrane fractions. Densitometry of the GluR1 and GluR2 signals corrected for pan Cadherin loading and normed to the control is shown in the lower panel. Data shown as mean ± SEM (n = 3). Two-way ANOVA with Bonferoni *post hoc* test, *** *P* < 0.001. (*D*) Rats were injected with 100 µl zymosan (12.5 mg/ml) in the hind paw (intraperitoneal) and the L4-L5 spinal dorsal horn was extracted after 6 h. Western blot analysis shows GluR1 phosphorylation at Serine 845 (pSer845) and a translocation of GluR1 to the membrane. The densitometric analysis is shown in the panel below. Data are shown as mean ± SEM (n = 4). Two-tailed Student *t* test: **P* < 0.05. (*E*) Spinal cords of adult mice were dissected, sliced to 500 µm at a vibratome, and stimulated to measure the released glutamate of the cells in the supernatant. Cicaprost 1 µM (n = 58 slices) and 50 mM KCl (n = 13) increased glutamate release. This effect was blocked by the selective PKA inhibitor H89 (10 µM, n = 18). Data shown as mean ± SEM. One-way ANOVA with Newman–Keuls multiple comparison test, **P* < 0.05, ***P* < 0.01, *** *P* < 0.001. (*F*) Same as *C* except that spinal cord slices from IP knockout were used (n = 10). Data are shown as mean ± SEM. One-way ANOVA with Newman–Keuls multiple comparison test, **P* < 0.05, ***P* < 0.01.

as well as translocation of the AMPA receptor GluR1 to membranes. Importantly, IP receptor activation had no effect on the amount of GluR2 in the membrane, therefore shifting the GluR1/GluR2 ratio. Fittingly we found that the cicaprost-induced glutamate release from spinal cord slices could be blocked by a PKA inhibitor. Taken together, the data strongly suggest that in spinal cord neurons cAMP-dependent signaling of IP receptors is substantially involved in the transduction of the pronociceptive effects of prostacyclin.

This sensitizing and therefore pronociceptive mechanism distinguishes itself from the reported pronociceptive effects shown for PGE_2, which is mediated by EP2 and is based on inhibition of glycinergic signaling.[4,37] The IP receptor, as EP2 and EP4, activates in spinal cord neurons the cAMP signal transduction pathway.[32] However, in contrast to IP

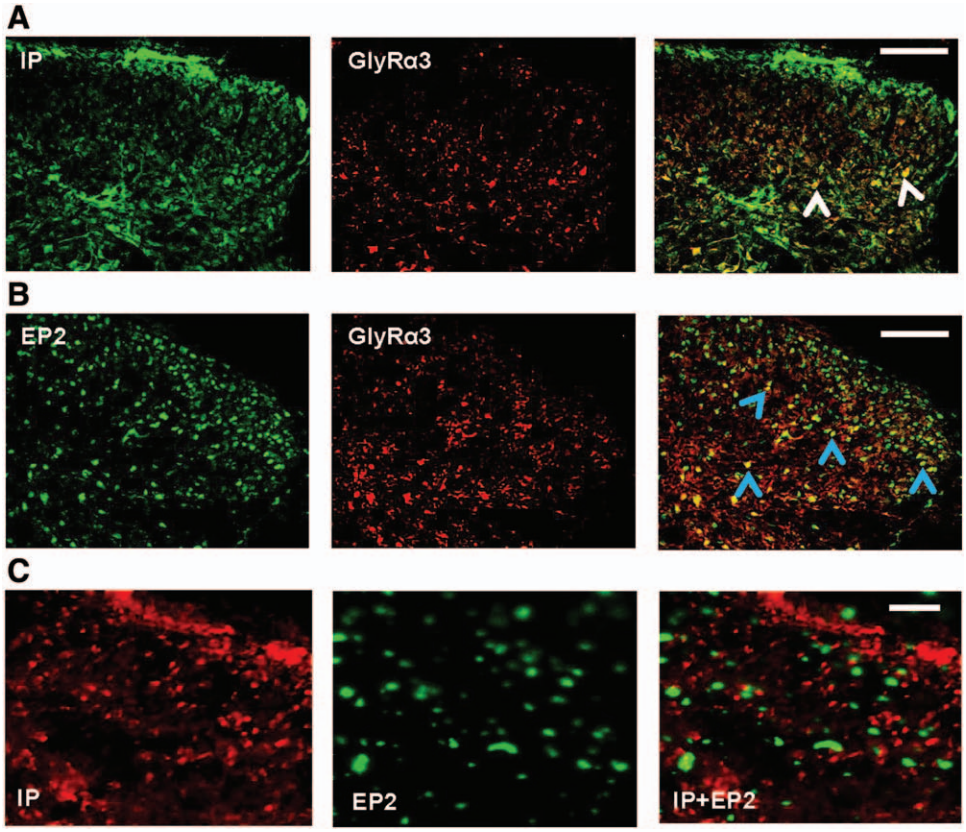

Fig. 6. The prostaglandin receptors IP and EP2 are not colocalized in the lumbar dorsal horn. (*A* and *B*) Immunohistochemical staining shows glycine receptor α3 (GlyRα3) with IP receptor (*A*) or EP2 receptor (*B*) in the dorsal horn. (*C*) The lower panels show a representative multiepitope ligand cartography analysis for IP and EP2 receptor. *Scale bar* represents 100 μm.

the activation of both receptors did not induce the translocation of GluR1 in spinal cord neurons, and the localization of IP differed clearly from the localization observed for the Gs-coupled EP2 receptor. EP4 is not expressed in the spinal cord of adult mice. Moreover, although it has been shown that EP2 activation is able to induce the translocation of GluR1 to membrane fractions in neurons from the preoptic area,[50] this mechanism has been ruled out earlier for spinal EP2-mediated signaling.[39] Finally, the notion that IP and EP2 do not share the same signaling mechanism is further underlined by the fact that both receptors are not expressed in the same neuronal populations and that the IP receptor does not colocalize with GlyRα3, which mediates the pronociceptive effects of EP2. Thus, because prostacyclin and PGE_2 signaling pathways are targeting different nociceptive mechanisms, they provide alternative ways to decrease nociception. Also, combined inhibition of both pathways may lead to additive or synergistic antinociceptive effects. If continuous IP receptor activation is necessary to maintain central sensitization, as indicated by the antinociceptive effect of intrathecally delivered Cay10441, it may be possible to initiate a successful pain therapy using intrathecally administered IP-receptor antagonists, which would also limit potential side effects as compared with systemically applied IP antagonists.

Taken together, we show here that peripheral inflammation increases spinal prostacyclin synthesis and that this increase induces a cAMP-dependent translocation of GluR1 receptors to membrane fractions. The up-regulation of the prostacyclin synthesis in the spinal cord is seen as early as 1 h after induction of peripheral inflammation, suggesting a functional role during the onset of central sensitization. The early role of prostacyclin during central sensitization is supported by the translocation of GluR1 as one mechanism through which prostacyclin enhances spinal nociceptive processing. Generally, the recruitment of GluR1 to synaptic membranes after nociceptive stimulation is regarded as an element of the first phase of activity-dependent central sensitization, allowing fast augmentation of excitatory glutamergic synapses.[51] Thus, due to its important role in peripheral and central sensitization processes, the prostacyclin signaling pathway is becoming an interesting target for the development of new analgesic drugs. However, it seems reasonable to speculate that besides the cAMP/PKA-dependent GluR1 recruitment other signaling events, such as the cAMP-mediated regulation of gene expression, can also be triggered by increased spinal prostacyclin level. Thus, it can be expected that future studies will be able to identify additional mechanisms that mediate effects of prostacyclin on central sensitization processes.

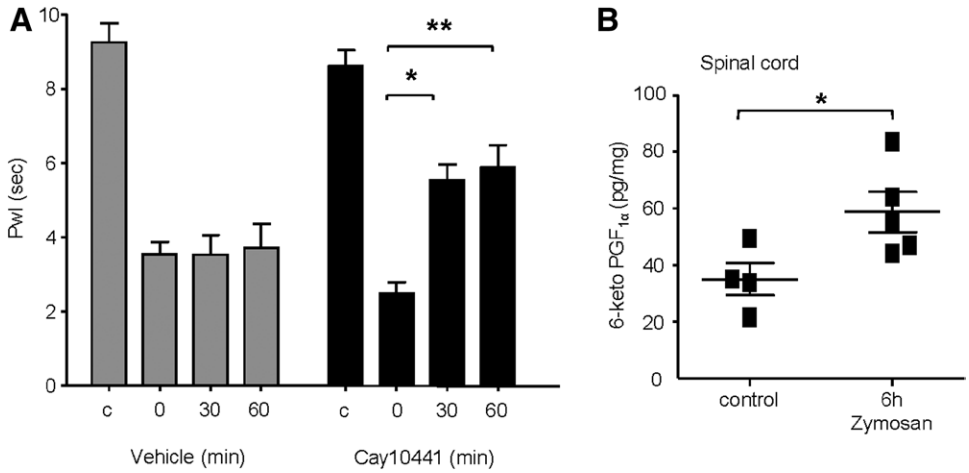

Fig. 7. The IP antagonist Cay10441 alleviates zymosan-induced mechanical hyperalgesia. (*A*) Swiss Webster mice were implanted with an intrathecal catheter and received a zymosan injection (20 µl, 5 mg/ml) in one hind paw. Mechanical pain thresholds were determined at the indicated times. Six hours after zymosan injection, 1 µM Cay10441 (n = 11) or vehicle (n = 8) was administered intrathecally. The intrathecal injection was defined as time point 0 min in the graph. Data are shown as mean ± SEM. Two-way ANOVA with Bonferoni *post hoc* test, $*P < 0.05$, $**P < 0.01$. (*B*) The levels of the stable prostacyclin-metabolite 6-keto-Prostaglandin $F_{1\alpha}$ were still increased 6 h after intraplantar zymosan injection (n = 5) compared with levels in control (n = 4). Data are shown as mean ± SEM. Two-tailed Student *t* test, $* P < 0.05$.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Geisslinger: pharmazentrum frankfurt, ZAFES, Institute of Clinical Pharmacology, Klinikum der Goethe-Universität Frankfurt, Theodor Stern Kai 7, 60590 Frankfurt, Germany. geisslinger@em.uni-frankfurt.de.

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