PGE_2 selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons

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Despite the crucial role that prostaglandins (PGs) have in the sensitization of the central nervous system to pain, their cellular and molecular targets leading to increased pain perception have remained elusive. Here we investigated the effects of PGE_2 on fast synaptic transmission onto neurons in the rat spinal cord dorsal horn, the first site of synaptic integration in the pain pathway. We identified the inhibitory (strychnine-sensitive) glycine receptor as a specific target of PGE_2. PGE_2, but not $PGF_{2\alpha}$, PGD_2 or PGI_2, reduced inhibitory glycinergic synaptic transmission in low nanomolar concentrations, whereas $GABA_A$, AMPA and NMDA receptor-mediated transmission remained unaffected. Inhibition of glycine receptors occurred via a postsynaptic mechanism involving the activation of EP2 receptors, cholera-toxin-sensitive G-proteins and cAMP-dependent protein kinase. Via this mechanism, PGE_2 may facilitate the transmission of nociceptive input through the spinal cord dorsal horn to higher brain areas where pain becomes conscious.

Exaggerated pain sensation is one of the cardinal symptoms of inflammation. Two phenomena characterize this sensitization: hyperalgesia, a state of increased sensitivity to stimuli that are already painful under normal conditions (noxious stimuli), and allodynia, which describes the painful sensation of normally innocuous stimuli, such as light touch. The different forms of sensitization can occur as the result of increased excitability of primary nociceptive afferent nerve fibers (peripheral sensitization) or from changes in the central processing of sensory stimuli, in particular, in the spinal cord dorsal horn (central sensitization)[1,2].

Prostaglandins (PGs) have long been recognized as important mediators of pain and inflammation[3]. The rate-limiting step in their biosynthesis is the conversion of arachidonic acid by constitutively expressed cyclooxygenase 1 and its inducible isoform cyclooxygenase 2 into PGG_2 and H_2, which are then further processed by specific PG synthases to give rise to the different biologically active PGs. PGs produced by cyclooxygenase 2 seem to be of particular relevance for inflammation and pain[4]. PGE_2 increases the excitability of the peripheral endings of nociceptive nerve fibers and thereby causes primary sensitization[5]. Accordingly, the analgesic action of cyclooxygenase inhibitors, so-called non-steroidal anti-inflammatory drugs (NSAIDs) or aspirin-like drugs, has long been thought to be primarily due to inhibition of PG synthesis in the (peripheral) inflamed tissue. During the recent years, however, increasing evidence has accumulated indicating that at least part of their analgesic effects arises from blockade of cyclooxygenase 2 in the spinal cord dorsal horn[4].

A large body of behavioral and biochemical evidence supports the involvement of spinal PGs, in particular, of PGE_2 and D_2, in the generation of central pain sensitization (for review, see ref. 6). Peripheral inflammation leads to an increase in cyclooxygenase 2 expression and PGE_2 production not only in the peripheral tissue, but also in neuronal[7] and non-neuronal[8] cells in the spinal cord dorsal horn[9,10]. The pattern of this spinal cyclooxygenase 2 expression is rather diffuse and not strictly limited to the projection area of the inflamed body region[7]. PGs therefore probably serve as volume transmitters[11] rather than as synaptic messengers.

The central sensitization following peripheral inflammation can be mimicked by intrathecal injection of PGs[6]. Specific binding sites for PGE_2[12] and protein or mRNA of all four known subtypes of PGE_2 receptors (EP1 to EP4) have been detected in the spinal cord dorsal horn[13–16].

Despite this evidence, which attributes to PGs a pivotal role in central pain sensitization, the identification of the underlying cellular and molecular substrates has only very recently begun (for example, ref. 17). Of particular interest for their investigation are the superficial laminae of the spinal cord dorsal horn, the first sites of synaptic integration in the pain pathway[18]. We show that PGE_2 specifically suppresses inhibitory glycinergic neurotransmission onto superficial dorsal horn neurons in low nanomolar concentrations via a postsynaptic mechanism involving the activation of an EP2-like PG receptor and protein kinase A (PKA).

Results

Effects of PGE_2 on evoked synaptic responses

We first investigated the effects of PGE_2 on synaptic transmission onto substantia gelatinosa (lamina II) neurons mediated by

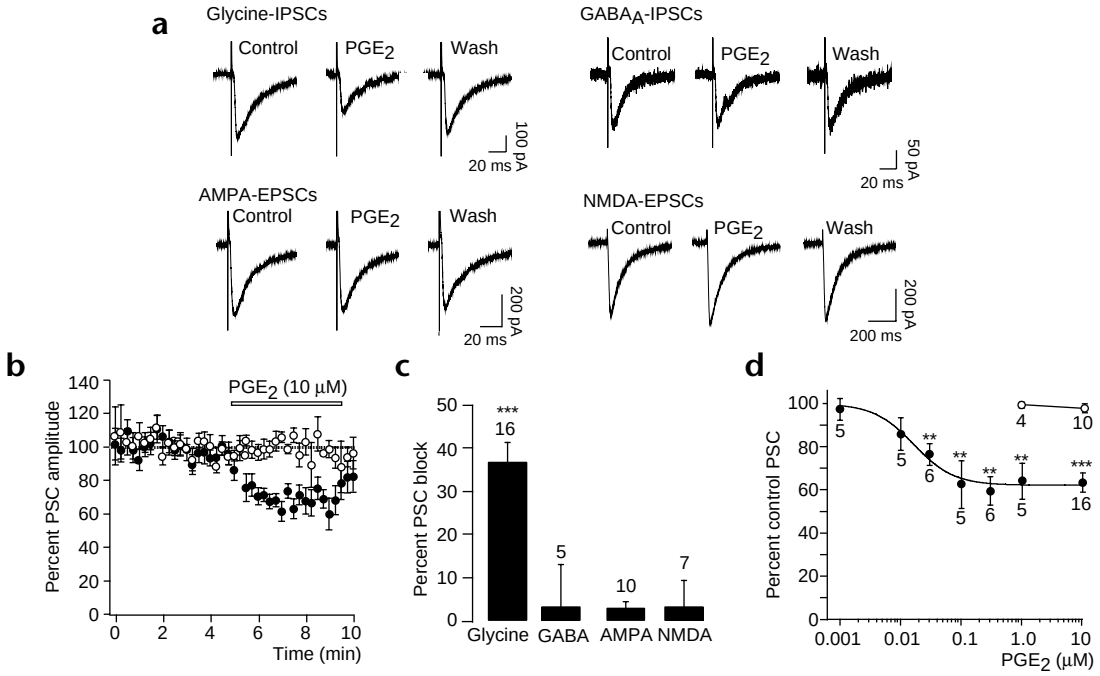

Fig. 1. Effects of PGE_2 on PSCs mediated by glycine, $GABA_A$, AMPA and NMDA receptors. (**a**) Postsynaptic current traces averaged from 10 consecutive stimulations under control conditions, in the presence of PGE_2 (10 μM) and after its removal (wash) mediated by glycine, $GABA_A$, AMPA and NMDA receptors. PSCs were recorded at –80 mV with the exception of NMDA-receptor-mediated EPSCs (NMDA-EPSCs), which were recorded at –30 mV. Note the different time scale of the NMDA-EPSCs. (**b**) Time course of PGE_2-mediated effects on glycinergic IPSCs (black circles) and on AMPA receptor-mediated EPSCs (white circles) averaged from 16 and 10 cells, respectively. (**c**) Statistical analysis of PSC inhibition by PGE_2 (mean ± s.e.m.). (**d**) Concentration response curve of PGE_2-mediated inhibition of glycinergic IPSCs (black circles) and AMPA-receptor-mediated EPSCs (white circles). Percent (mean ± s.e.m.) PSC amplitude remaining in the presence of PGE_2. Details of the curve fitting procedure are given in refs. 39 and 45. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ (ANOVA, followed by Fisher's *post hoc* test). Numbers below or above the data points are numbers of cells tested.

the three fast spinal amino acid transmitters L-glutamate, GABA and glycine (**Fig. 1**). Bath-applied PGE_2 (0.01–10 μM) reversibly reduced the amplitudes of glycine receptor-mediated inhibitory postsynaptic currents (IPSCs) in 38 of 47 neurons recorded in slices obtained from rats 7–14 days old. Inhibition of glycinergic IPSCs occurred in the low nanomolar range with an EC_{50} of 16.4 ± 7.8 nM and a Hill coefficient of 1.32 ± 0.91. At saturating concentrations (≥ 300 nM), inhibition was 38.2 ± 3.4% (**Fig. 1d**). No changes in the time course of glycinergic IPSCs were observed in the presence of PGE_2 (**Table 1**). In contrast to glycine-receptor-mediated neurotransmission, IPSCs mediated by $GABA_A$ receptors and EPSCs mediated by L-glutamate acting on AMPA or NMDA receptors remained unaffected after addition of PGE_2 (10 μM) (**Fig. 1a–c**, **Table 1**).

In a separate set of experiments, we compared the effects of PGE_2 on substantia gelatinosa neurons and neurons in lamina I and lamina V, where most spinal projection neurons are located. Recorded neurons were filled with biocytin for *post hoc* determination of their exact location and morphology (**Fig. 2**). Inhibition was not restricted to a particular cell type but was more prominent in the substantia gelatinosa and lamina I than in lamina V.

To exclude the possibility that block of glycinergic IPSCs by PGE_2 was restricted to young rats (7–14 days old), we performed a limited number of experiments in older rats (26–30 days old). In these experiments, a similar degree of block was observed (48.6 ± 6.5% ($n = 8$) in rats 26–30 days old compared to 38.2 ± 3.4% ($n = 16$) in rats 7–14 days old ($p = 0.15$, unpaired *t*-test)).

Besides PGE_2, other PGs, including PGD_2, $PGF_{2\alpha}$ and PGI_2, have been detected in the spinal cord[6] and have been implicated in the modulation of spinal nociceptive processing. Among these, only PGE_2 significantly interfered with glycinergic neurotransmission, whereas $PGF_{2\alpha}$, PGD_2 and PGI_2 (all 10 μM) showed only modest effects (change in PSC amplitude, 7.5 ± 3.9% ($n = 13$), 0.29 ± 3.7% ($n = 9$) and 2.9 ± 6.2% ($n = 8$), respectively; **Fig. 3**). These results suggest that PGE_2 inhibits glycinergic IPSCs via the activation of PGE_2 specific (EP) receptors, of which at present four different subtypes (EP1 to EP4) are known[19].

Table 1. Changes in PSC amplitudes and decay time constants by PGE_2.

Evoked PSC	Percent PSC block by PGE_2 (10 μM)	τ decay (ms)	
		Control	PGE_2 (10 μM)
AMPA-EPSC	2.72 ± 1.59	11.4 ± 2.5	11.8 ± 2.6 ($n = 10$)
NMDA-EPSC	2.96 ± 6.12	94.3 ± 8.8	99.1 ± 9.6 ($n = 7$)
$GABA_A$-IPSC	3.14 ± 9.86	10.0 ± 1.8	10.1 ± 1.6 ($n = 5$)
Glycine-IPSC	36.9 ± 4.42	21.1 ± 2.4	23.2 ± 2.4 ($n = 16$)

PSC blockade was significant only for glycinergic IPSCs ($p < 0.001$, ANOVA followed by Fisher's *post hoc* test). Changes in decay time constants were insignificant for all PSCs. Values are mean ± s.e.m.

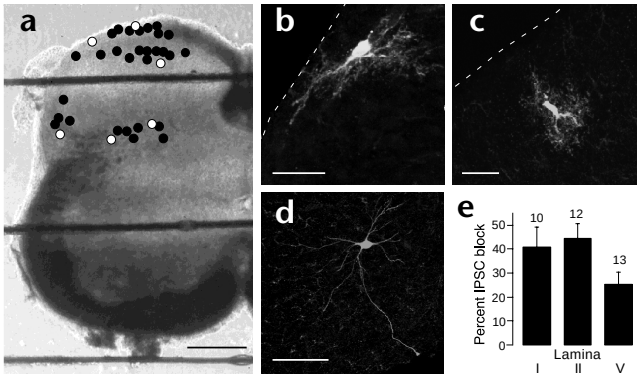

Fig. 2. Localization of prostaglandin (PG)-sensitive and -insensitive neurons in the rat spinal cord dorsal horn. (**a**) Localization of 35 neurons recorded in rat spinal cord slices. Black circles, sensitive neurons; white circles, insensitive neurons. (**b–d**) Biocytin-filled neurons in lamina I (**b**), lamina II (**c**) and lamina V (**d**). IPSCs recorded from neurons in (**b**) and (**c**) were PGE_2-sensitive; neuron (**d**) was PGE_2-insensitive. Scale bars, 300 μm (**a**), 25 μm (**b**, **c**), 100 μm (**d**). (**e**) Average inhibition of glycinergic IPSCs by PGE_2 (10 μM) in 3 different laminae. Degrees of inhibition in lamina II and V (including PGE_2-insensitive cells) were significantly different ($p < 0.05$, ANOVA followed by Fisher's *post hoc* test).

Because no specific antagonists are available for these receptors, we tested four synthetic compounds with preferential agonistic activities at the different EP receptor subtypes[19,20]: 17-phenyl trinor PGE_2 and sulprostone, which are both agonists at EP1 and EP3 receptors; 11-deoxy PGE_1, which acts at EP2, EP3 and EP4 receptors; and butaprost methyl ester, which is a low-potency but rather selective agonist at EP2 receptors. Of these, only butaprost methyl ester and 11-deoxy PGE_1 inhibited glycinergic neurotransmission by 27.2 ± 7.9% ($n = 17$) and by 46.4 ± 8.4% ($n = 7$; both at 10 μM), respectively (**Fig. 4a** and **b**). The concentration-dependencies of both agonists (**Fig. 4d**) with half-maximal effects in the low micromolar versus nanomolar range are in good agreement with their affinities for the rat EP2 receptor21. The EP1 and EP3 receptor agonists 17-phenyl trinor PGE_2 and sulprostone were virtually without effect (**Fig. 4a** and **c**). This pharmacological profile suggests that PGE_2-mediated inhibition of glycinergic IPSCs required the activation of EP2 receptors or at least of an EP2-like receptor.

PGE_2 acts postsynaptically

To distinguish between a pre- and postsynaptic site of PGE_2-mediated inhibition of glycinergic neurotransmission, spontaneously occurring glycinergic miniature IPSCs (mIPSCs) were recorded in the presence of tetrodotoxin (TTX; 1 μM; **Fig. 5a**). In 8 of 11 neurons, PGE_2 (1 μM) significantly reduced median mIPSC amplitudes by 26.6 ± 5.33% ($p \leq 0.05$, paired *t*-test), but had no significant effect on their frequency (change in median inter-event interval, 12.1 ± 6.4%, $n = 11$; **Fig. 5b**) suggesting a mainly postsynaptic site of action. PGE_2 had no effect on the decay kinetics of glycinergic mIPSCs (**Fig. 5b**). Decay time constants were 33.4 ± 6.3 ms and 32.2 ± 5.3 ms ($n = 8$) under control conditions and in the presence of PGE_2, respectively. Further proof for a postsynaptic site came from experiments with GDP-βS (2 mM), an inhibitor of G-protein activation, which abolished the inhibitory effect of PGE_2 when included in the internal recording solution (change in median mIPSC amplitudes, –4.6 ± 8.2%, $p = 0.65$; **Fig. 5c**). Because mIPSC experiments were performed in the presence of TTX, which blocks action-potential-induced transmitter release, they also indicate that the inhibition of glycinergic IPSCs by PGE_2 did not involve a second extracellular mediator, which might be released upon PGE_2-mediated depolarization of spinal cord neurons[17].

We next tested whether currents elicited by exogenous application of glycine were also inhibited by PGE_2 (**Fig. 6**). Short (1-s) applications of glycine (1 mM) evoked transient inward currents, which were reversibly reduced in amplitude by 34.5 ± 8.4% ($n = 7$) in the presence of PGE_2 (1 μM). Inhibition was again completely abolished by inclusion of GDP-βS (2 mM) in the internal solution (change in current amplitude, –5.20 ± 5.22%; $n = 7$; **Fig. 6a**). In contrast to the currents evoked by glycine, those evoked by GABA (1 mM) were not significantly blocked by PGE_2. Average changes of GABA-evoked current amplitudes were –8.49 ± 9.77% ($n = 6$) and 4.53 ± 9.59% ($n = 7$) in the absence and presence of internal GDP-βS, respectively (**Fig. 6b**). A similar reduction of blockade from 40.0 ± 8.86% ($n = 7$) to 17.6 ± 3.80% ($n = 7$; $p < 0.05$, unpaired *t*-test) was observed for evoked glycinergic IPSCs (data not shown).

Involvement of Gs proteins and protein kinase A

To identify the type of G-protein activated by the EP2 receptor, we next either included the catalytic (A) subunit of pertussis toxin (PTX) or of cholera toxin (ChTX; both 3 μg/ml) in the internal solution (**Fig. 7**). PTX and ChTX mediate ADP ribosylation of inhibitory (Gi/o) and stimulatory (Gs) G-proteins respectively, thereby causing either tonic activation (ChTX) or irreversible inhibition (PTX) of Gi/o or Gs proteins[22,23]. In these experiments, we included 500 μM nicotinamide adenosine dinucleotide (βNAD) in the internal solution, which serves as the donor of ADP ribose in the ADP ribosyltransferase reaction[23]. ChTX itself caused a progressive decrease in the amplitude of glycinergic IPSCs to about 40.4 ± 9.7% ($n = 6$) within

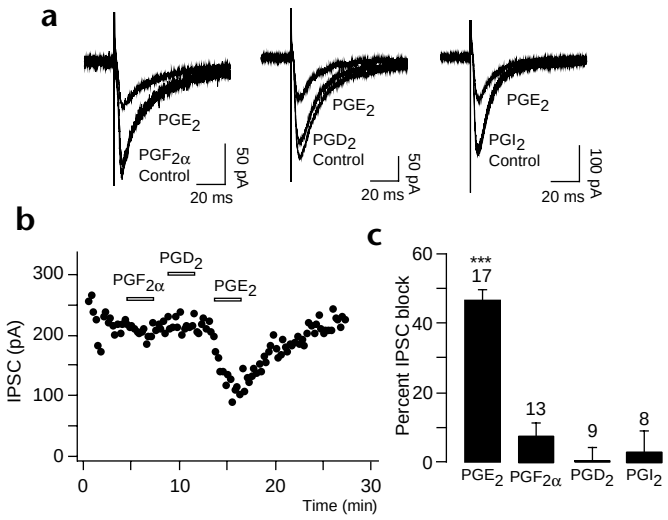

Fig. 3. Effects of different PGs on glycinergic neurotransmission. (**a**) Average IPSCs under control conditions, in the presence of $PGF_{2\alpha}$, PGD_2 or PGI_2, and as a positive control, in the presence of PGE_2 (all 10 μM). (**b**) Time course of an experiment in which $PGF_{2\alpha}$, PGD_2 and PGE_2 were tested. (**c**) Statistical analysis (mean IPSC inhibition ± s.e.m. by the different PGs). Numbers above the bars are numbers of cells tested. ***$p \leq 0.001$; all others, $p > 0.29$ (one sample sign test).

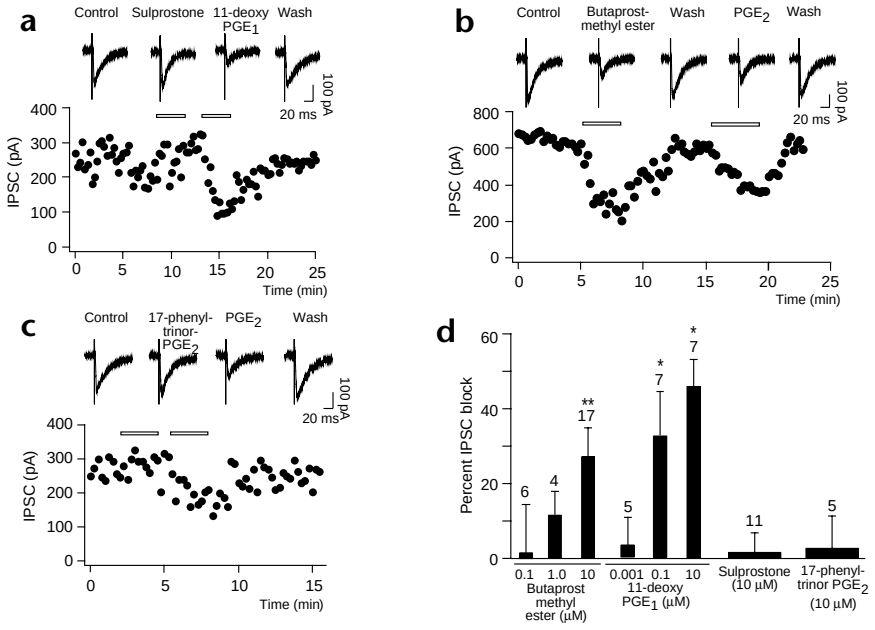

Fig. 4. Effects of several synthetic EP receptor agonists with preferential activity at EP1–4 receptor subtypes on glycinergic neurotransmission. (**a–c**) Averaged IPCSs traces in the presence of various prostanoid receptor agonists (top) and time course of changes in IPSC amplitudes (bottom). (**a**) Sulprostone (10 μM), a mixed EP1 and EP3 agonist, and 11-deoxy PGE_1 (10 μM), a mixed agonist at EP2, EP3 and EP4 receptors. (**b**) Butaprost methyl ester (10 μM), a weak EP2 receptor-specific agonist, and PGE_2 (10 μM) as a positive control. (**c**) 17-phenyl trinor PGE_2 (10 μM), a mixed agonist at EP1 and EP3 receptors, and PGE_2 (10 μM). (**d**) Concentration-dependencies of butaprost methyl ester and 11-deoxy PGE_1 and statistical analysis. $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ (one sample sign test). Numbers above bars are numbers of cells tested.

approximately 10 minutes (**Fig. 7a** and **c**). Inhibition by subsequently applied PGE_2 was reduced to 12.7 ± 4.3% and became statistically insignificant ($p = 0.22$). In contrast to ChTX, PTX neither affected IPSC amplitudes nor prevented blockade by PGE_2 (**Fig. 7b–d**). After 10 minutes of whole-cell recording, IPSC amplitudes remained close to the initial value (+3.3 ± 3.1%, $n = 8$) and inhibition by PGE_2 was virtually identical to that observed in the absence of PTX (34.9 ± 7.1%) or when ChTX was heat-inactivated (–42.6 ± 9.7%, $n = 8$).

The different EP receptors can lead to activation of PKA and protein kinase C (PKC), and both kinases have been shown to regulate glycine receptor function[24]. We therefore tested for a possible involvement of these protein kinases. Inclusion of PKA but not of PKC inhibitor peptide led to a statistically significant reduction of the inhibitory effect of PGE_2 on glycinergic IPSCs (**Fig. 8**). IPSC reduction was 10.4 ± 5.79% ($n = 11$) versus 47.3 ± 2.61 ($n = 7$), when PKA or PKC inhibitory peptide was included, respectively. A similar difference (0.64 ± 12.6%, $n = 7$, PKA inhibitor peptide, versus 54.9 ± 12.7%, $n = 7$, PKC inhibitor peptide) was found for currents elicited by exogenously applied glycine.

Discussion

Here we showed that PGE_2 specifically reduces glycinergic synaptic input onto neurons in the superficial dorsal horn. Although other PGs, in particular, PGD_2 and $PGF_{2\alpha}$, have been implicated in central pain sensitization[25,26], only PGE_2 (not PGD_2, PGI_2 or $PGF_{2\alpha}$) inhibited glycinergic IPSCs. This corresponds well to *in vivo* data, which have revealed that PGE_2 concentrations in the CNS increase dramatically upon peripheral inflammation[7]. Several studies, which used spinal microdialysis to quantify spinal inflammation-induced PGE_2 formation, have found concentrations in range of about 30–150 nM[27,28], which closely matches the concentration range required for inhibition of glycinergic IPSCs in our study (10–100 nM).

Inhibition of glycinergic IPSCs occurred via EP2 receptors and involved activation of ChTX-sensitive Gs protein. mRNA encoding for the EP2 receptor has been detected in the rat spinal cord dorsal horn[13]. These receptors specifically couple to Gs proteins, which increase intracellular cAMP formation and, as a consequence, activate PKA[19]. ChTX irreversibly activates the alpha subunit of Gs proteins, and thereby occludes the effects of activators of Gs-coupled receptors[23]. Inhibition of glycinergic IPSCs further depended on PKA activation. Phosphorylation of strychnine-sensitive glycine receptors by PKA has clearly been demonstrated[29], but its functional consequences remain controversial (for reviews, see refs. 24, 30). It seems possible that in addition to glycine receptors, PKA phosphorylates some intermediate effector, which in turn regulates glycine receptor function.

Several lines of evidence indicate that block of glycine receptors by PGE_2 indeed provides a cellular and molecular basis for the pain-sensitizing action of PGE_2 in the spinal cord dorsal horn. Reduction of glycinergic neurotransmission by means different from PGE_2 application also induces states of increased pain sensitivity, which are primarily characterized by a hypersensitivity to normally innocuous tactile and thermal stimuli[18,31–33]. Treatment of mice[25] and accidental poisoning of humans[34] with the glycine receptor blocker strychnine not only disturbs motor function, but also increases the sensitivity to tactile stimuli[35,36]. In the spinal cord dorsal horn, iontophoretically applied strychnine enhances and glycine reduces discharge of sensory neurons[37]. A reduction in the synaptic release of glycine (and GABA) by nocistatin, a recently identified neuropeptide[38], increases nociceptive behavior after intrathecal injection of nanomolar doses in the rat formalin test[39]. Conversely, some forms of allodynia (such as those that are seen after intrathecal injection of low doses of nociceptin/orphanin FQ) can be antagonized by intrathecal injection of glycine[40].

Additional evidence comes from experiments done with mice lacking the neuron-specific isoform of the type I regulatory subunit (RIβ) of PKA[41]. Because blockade of glycine receptors by PGE_2 required the activation of PKA in our experiments, this pathway should not be functional in PKA-deficient mice. Indeed, mice lacking neuronal PKA exhibit much less thermal hyperalgesia and pain-related behavior in the formalin test in response to intrathecal injection of PGE_2 than wild-type mice[42]. Our results attribute to glycine and its postsynaptic receptors an involvement in central nociceptive processing. Inhibitory glycine receptors seem to be important targets of PGE_2, an endogenous key mediator of central pain sensitization. After binding to EP2 receptors, PGE_2 acti-

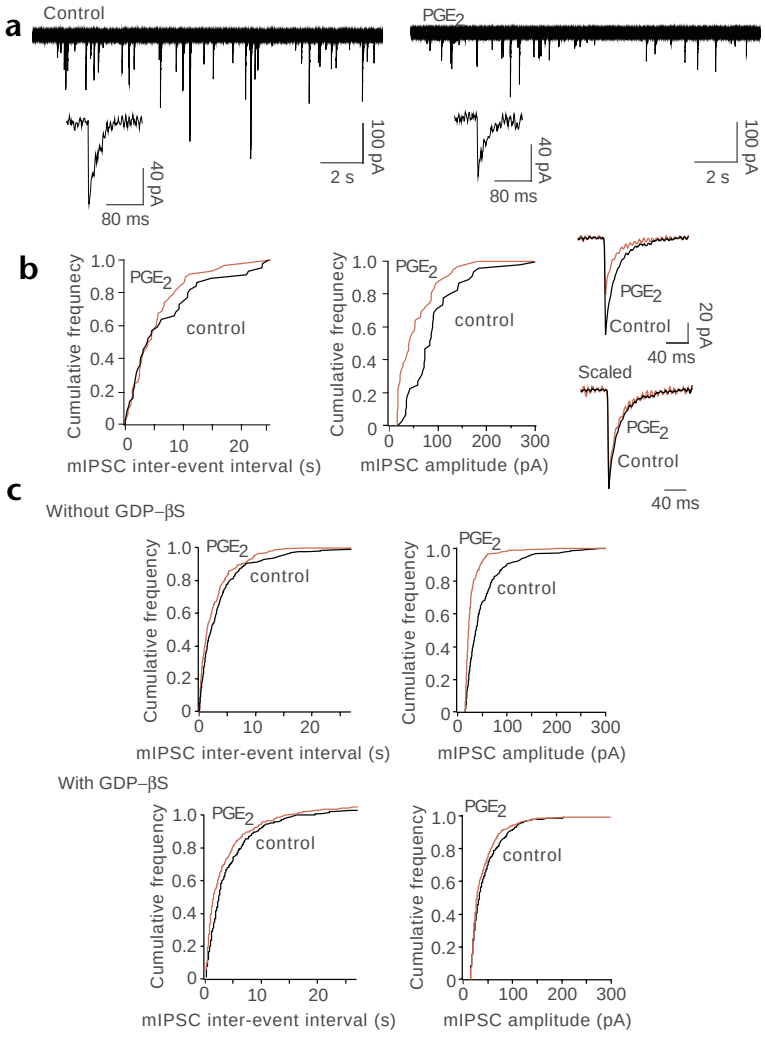

Fig. 5. PGE_2 reduced glycinergic mIPSCs in amplitude, but not in frequency. (**a**) mIPSCs recorded from a substantia gelatinosa neuron under control conditions and in the presence of PGE_2 (1 μM). Top, superposition of 10 current traces each 16 s long. Bottom, individual mIPSC in a higher time resolution. (**b**) Cumulative interval histograms of mIPSC inter-event intervals (left) and amplitudes (middle) of mIPSCs recorded from one cell under control conditions (black) and in the presence of PGE_2 (1 μM; red). Right, top, average mIPSC recorded under control conditions (black) and in the presence of PGE_2 (red). Right, bottom, same mIPSCs with scaled amplitudes. Note that the time course of activation and inactivation of the averaged mIPSC remained unchanged. (**c**) Top, cumulative interval histograms of mIPSC inter-event intervals and amplitudes pooled from 8 cells under control conditions and in the presence of PGE_2 (1 μM). A total of 1508 mIPSCs were analyzed. Bottom, same as top, but with GDP-βS (2 mM) included in the internal solution, pooled from 5 cells (1052 mIPSCs).

vates PKA, inhibits glycinergic neurotransmission onto superficial dorsal horn neurons and thereby probably facilitates the transmission of nociceptive input through the spinal cord dorsal horn to higher brain areas. Inhibitors of the cyclooxygenases, especially of cyclooxygenase 2, probably exert part of their analgesic action by preventing this process through blockade of spinal PGE_2 formation.

Other processes may contribute to PGE_2-mediated central pain sensitization. PGE_2 selectively depolarizes deep (lamina V) dorsal horn neurons via a EP2 receptor-dependent activation of a cation channel[17]. This effect requires significantly higher PGE_2 concentrations than inhibition of glycinergic IPSCs. It may therefore be particularly relevant to central pain sensitization under conditions of excessive PG production.

It has been a long-standing hypothesis that PGE_2 causes its pro-nociceptive effects via a facilitation of the spinal release of the excitatory neurotransmitter L-glutamate. *In vitro* experiments using isolated spinal cord tissue have indeed shown that glutamate release increased after PGE_2 application[43,44]. Our results and those of others[17] do not support a direct effect of PGE_2 on glutamate release. On the other hand, the reduction in glycinergic neurotransmission described here is likely to cause a disinhibition of excitatory glutamatergic neurons and may thereby indirectly facilitate the release of glutamate.

In summary, our results provide a molecular mechanism for the central pain sensitizing effect of PGE_2. Identification of the molecular and cellular elements involved in central pain sensitization may help develop new analgesics possibly devoid of many of the unwanted effects of classical aspirin-like drugs.

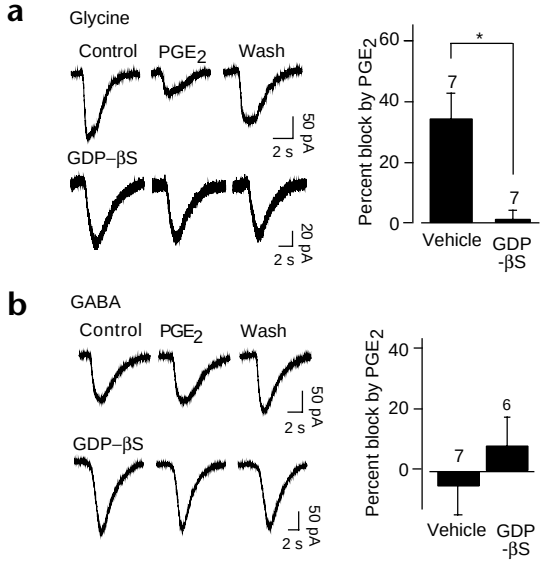

Fig. 6. Inhibition by PGE_2 of whole-cell currents evoked by exogenous application of glycine and GABA. Left, current responses evoked by exogenous application of glycine (1 mM) (**a**) and GABA (1 mM) (**b**) before application of PGE_2 (control), during application (PGE_2, 10 μM) and after its removal (wash), with and without GDP-βS included in the internal solution. Right, statistical analysis (mean ± s.e.m.). *$p \leq 0.05$ (unpaired *t*-test). Numbers above the data points are numbers of cells tested.

Methods

Slice preparation and electrophysiological recordings. Sprague–Dawley rats (7–14 and 26–30 days old) of either sex were killed under ether narcosis by decapitation. The killing of the animals was performed in accordance

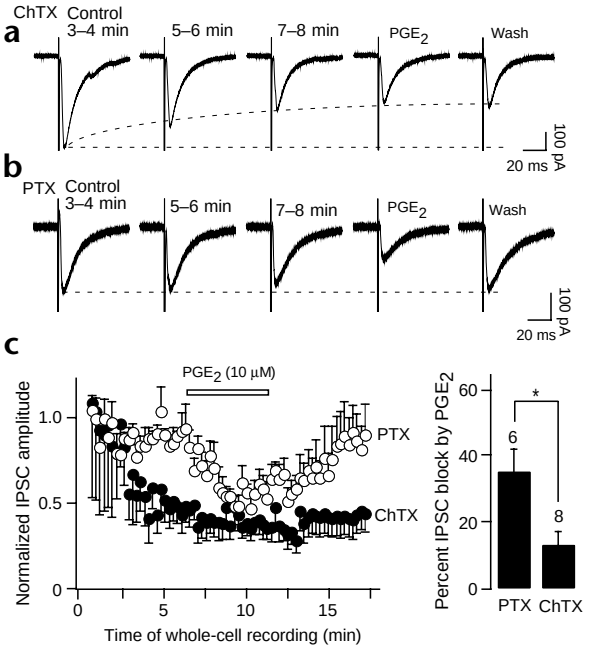

Fig. 7. Inhibition of glycinergic IPSCs by PGE_2 required the activation of a ChTX-sensitive G-protein. (**a**, **b**) Average glycinergic IPSCs recorded at different time intervals after reaching the whole-cell configuration under control conditions, in the presence of PGE_2 and after its removal. (**a**) With catalytic A subunit of ChTX; (**b**) with catalytic A subunit of PTX included in the intracellular recording solution. (**c**) Time course of average IPSC amplitudes obtained from 6 (ChTX, black circles) and 8 (PTX, white circles) cells versus time of whole-cell recording. (**d**) Statistical analysis (mean ± s.e.m.). $^*p \leq 0.05$ (unpaired *t*-test).

with the institutional guidelines of the University of Erlangen-Nürnberg. Transverse slices (250 μm thick) of the lumbar spinal cord were prepared as described previously[45]. Whole-cell patch-clamp recordings were performed from neurons identified under visual control using the infrared gradient contrast technique coupled to a video microscopy system[46]. Slices were completely submerged and continuously superfused with external solution, which contained 125 mM NaCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose (pH 7.30, 315 mOsm/l) bubbled with 95% O_2, 5% CO_2. Patch pipettes (4–5 MΩ) were filled with internal solution containing 130 mM K-gluconate, 20 mM KCl, 2 mM $MgCl_2$, 0.05 mM EGTA, 3 mM Na-ATP, 0.1 mM Na-GTP and 10 mM Na-HEPES (pH 7.30). QX-314 (5 mM) was added to the internal solution to block voltage-activated sodium currents. Postsynaptic current responses were elicited at a frequency of 1/15 s by extracellular electrical stimulation (100 μs, 3–10 V) using a glass electrode filled with standard extracellular solution and placed about 50 μm from the recorded neuron. PSCs were recorded at room temperature at a holding potential of –80 mV, except for NMDA receptor-mediated EPSCs, which were recorded at –30 mV to partially remove the Mg^{2+} block. CNQX (10 μM), D-APV (50 μM), bicuculline (10 μM) and strychnine (300 nM) were used to block postsynaptic current responses mediated by AMPA, NMDA, $GABA_A$ and glycine receptors, respectively. Combinations of these blockers were used to isolate PSCs mediated by the respective neurotransmitter receptor. Short hyperpolarizing voltage steps to –90 mV were applied every minute to monitor input and access resistance. Drug containing solutions were applied by bath perfusion at a rate of 1–2 ml/min. To study membrane currents elicited by exogenous glycine or GABA, both transmitters (at a concentration of 1 mM) were applied from glass pipettes to the soma of the recorded neuron by short (1-s) manually controlled pressure pulses.

Percent inhibition of current responses was determined from the average amplitude of 10 consecutive currents evoked immediately before application of the PGs and when a steady state of inhibition was reached, usually about 3 min after application.

Spontaneously occurring mIPSCs were recorded in the extracellular presence of TTX (1 μM) and with internal K-gluconate substituted by equimolar CsCl to reduce baseline membrane conductance. Amplitude and frequency distributions were analyzed using a custom made Igor macro[45].

Biocytin labeling. To analyze the morphological characteristics, neurons were filled during whole-cell recording (≥ 15 min) with standard internal solution containing biocytin (5 mg/ml). After removal of the pipette, slices were kept in the recording chamber for an other 30 min to allow diffusion and transport of biocytin into fine neuronal processes[47]. Slices were then cryoprotected in buffered sucrose and serially cryostat sectioned at 40 μm. Biocytin was detected by incubating the sections on slide in a 1:1000 solution of Cy3 tagged streptavidin in tris buffered saline (TBS) at pH 7.4 for 2 h at room temperature. After coverslipping in glycerol-containing TBS, confocal laser scanning microscopy was performed with a Biorad MRC 1000 (Biorad, München, Germany) attached to a Nikon Diaphot (Nikon, Düsseldorf, Germany). Images were generated by superposing up to 27 confocal optical sections taken at *z* intervals of 1 μm in the extended focus mode.

Chemicals. PGE_2, PGD_2, $PGF_{2\alpha}$ and PGI_2 (all from Calbiochem-Novabiochem, Bad Soden, Germany) and butaprost methyl ester, sulprostone, 11-deoxy PGE_1 and 17-phenyl trinor PGE_2 (all from Cayman Chemicals, Ann Arbor, Michigan) were dissolved in DMSO (final DMSO concentration ≤ 0.1%). Biocytin, βNAD, TTX, GDP-βS, PKC inhibitor fragment 19-36 and PKA inhibitor fragment 6-22 amide were from Sigma (Deisenhofen, Germany). Catalytic A subunits of PTX and of ChTX were from Calbiochem-Novabiochem.

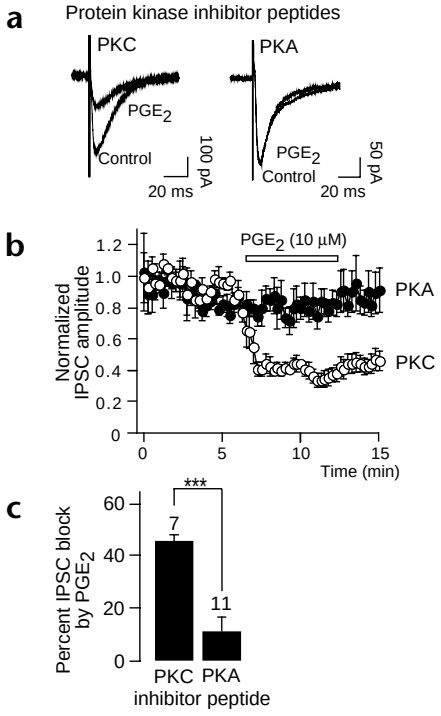

Fig. 8. Involvement of protein kinase A, but not of protein kinase C. (**a**) Averaged glycinergic IPSCs recorded under control conditions (control) and in the presence of PGE_2 (10 μM; PGE_2) with PKC or PKA inhibitor peptide. (**b**) Time course of average IPSC amplitudes obtained from 11 (PKA, black circles) and 7 (PKC, white circles) neurons plotted versus time of whole-cell recording. (**c**) Statistical analysis (mean ± s.e.m.). $^{***}p \leq 0.001$ (ANOVA, followed by Fisher's *post hoc* test). Numbers above the data points are numbers of cells tested.

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Competing interests statement

The authors declare that they have no competing financial interests.

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