Spinal Prostaglandins Are Involved in the Development But Not the Maintenance of Inflammation-Induced Spinal Hyperexcitability

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Prostaglandins (PGs) are local mediators of several functions in the CNS. Both primary afferent neurons and intrinsic cells in the spinal cord produce PGs, with a marked upregulation during peripheral inflammation. Therefore, the significance of spinal PGs in the neuronal processing of mechanosensory information was herein investigated. In anesthetized rats, the discharges of spinal nociceptive neurons with input from the knee joint were extracellularly recorded. Topical administration of prostaglandin E_2 (PGE_2) to the spinal cord facilitated the discharges and expanded the receptive field of dorsal horn neurons to innocuous and noxious pressure applied to the knee joint, the ankle, and the paw, thus mimicking inflammation-induced central sensitization. Conversely, topical administration of the PG synthesis inhibitor indomethacin to the spinal cord before and during development of knee joint inflammation attenuated the generation of inflammation-induced spinal neuronal hyperexcitability.

However, after development of inflammation, the responses of spinal neurons to mechanical stimuli were only reduced by systemic indomethacin but not by indomethacin applied to the spinal cord. Thus, spinal PG synthesis is important for the induction and initial expression but not for the maintenance of spinal cord hyperexcitability. Spinal PGE_2 application facilitated dorsal horn neuronal firing elicited by ionophoretic delivery of NMDA, suggesting that an interaction of PGs and NMDA receptors may contribute to inflammation-induced central sensitization. However, after development of inflammation, spinal indomethacin failed to reduce responses to ionophoretic delivery of NMDA or AMPA, suggesting that such an interaction is not required for the maintenance of central sensitization.

Key words: central sensitization; glutamate; pain; nociception; NSAID; prostaglandin

Prostaglandins (PGs) are local mediators in a variety of functions in the CNS as well as in peripheral tissues. Considerable information has emerged in recent years regarding the involvement of spinal cord PGs in pain and central sensitization (Vanegas and Schaible, 2001). Indeed, cyclooxygenases 1 and 2 (COX-1 and COX-2), the enzymes that form prostaglandin E_2 (PGE_2) and other PGs from arachidonic acid, are expressed in both dorsal root ganglia (DRGs) and the spinal cord (Willingale et al., 1997; Inoue et al., 1999). Both isoforms of COX are expressed constitutively, but in particular COX-2 is markedly upregulated in the spinal cord during acute and chronic peripheral inflammation (Beiche et al., 1996, 1998a; Hay and de Belleruche, 1997; Hay et al., 1997; Goppelt-Strube and Beiche, 1998; Ebersberger et al., 1999; Samad et al., 2001). In the spinal cord, there is a basal release of PGE_2 as well as an increased release after noxious stimulation, such as electrical pulses (Ramwell et al., 1966), noxious heat (Coderre et al., 1990), subcutaneous formalin (Malmberg and Yaksh, 1995a,b; Hua et al., 1999; Muth-Selbach et al., 1999), and peripheral inflammation (Yang et al., 1996a; Hay et al., 1997; Hay and de Belleruche, 1998; Ebersberger et al., 1999; Gühring et al., 2000). Conversely, intrathecal administration of

PGE_2 causes allodynia and hyperalgesia in awake animals (Taiwo and Levine, 1988; Uda et al., 1990; Minami et al., 1994a, 1996; Malmberg et al., 1995a,b; Nishihara et al., 1995; Ferreira and Lorenzetti, 1996). Finally, antinociceptive nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, inhibit PG synthesis not only in the inflamed peripheral tissues but also in the spinal cord, an effect that may contribute to their antinociceptive action (Vanegas and Schaible, 2001).

Curiously, the effects of PGs on spinal dorsal horn neuronal firing have until now received only scant attention (Vanegas and Schaible, 2001), although this activity is precisely the drive for segmental and suprasegmental reflexes as well as for the thalamocortical transmission that leads to the perception of pain. In the present experiments, we therefore determined the effects of spinally applied PGE_2 on the responses of nociceptive dorsal horn neurons to natural mechanical innocuous and noxious stimulation of the knee and other peripheral tissues. We further investigated whether there is an interaction between PGE_2 and glutamatergic synaptic transmission in the spinal dorsal horn. Finally, we tested whether spinal application of a COX inhibitor influences the development and maintenance of inflammation-induced hyperexcitability of nociceptive neurons to mechanosensory stimuli, because mechanical hyperalgesia (pressure hypersensitivity) is an important pain symptom of joint inflammation.

Parts of this work have been published previously in abstract form (Vasquez et al., 2000, 2001).

MATERIALS AND METHODS

Preparation

Experiments were performed on 75 male Wistar rats (200–350 gm; University of Jena, Jena, Germany) anesthetized with sodium thiopent-

Received May 2, 2001; revised Aug. 28, 2001; accepted Aug. 30, 2001.

This work was supported by Deutsche Forschungsgemeinschaft Grant Scha 404/11-1. E.V. held fellowships from the Deutscher Akademischer Austauschdienst and the Venezuelan Consejo Nacional de Investigaciones Científicas y Técnicas. We thank G. Cuny for skillful assistance with the laboratory work.

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tone (Trapanal; initial dose of 85–115 mg/kg, i.p.; BYK Gulden Ltd., Konstanz, Germany). The trachea was cannulated, and catheters were inserted into the common carotid artery and the external jugular vein. The animals breathed spontaneously, and a gentle jet of oxygen was blown toward the opening of the tracheal cannula. Body temperature was kept at 37°C by means of a feedback-controlled system. Additional intraperitoneal injections of thiopentone (20–50 mg/kg) were given when necessary to achieve a sufficiently deep level of anesthesia as assessed by the absence of corneal or leg withdrawal reflexes. Mean arterial blood pressure was stable at 90–120 mmHg. Spinal cord segments L1–L4 were exposed by laminectomy. The dura mater was opened, and a thin-walled, elliptic rubber ring ($\sim 3 \times 5$ mm) was tightly sealed with silicone gel onto the surface of the cord. This ring thus formed a trough with ~ 30 μ l capacity over the spinal segments in which the recordings were to be performed. This trough was immediately filled with Tyrode's solution. A solution of 3% agar in Tyrode's solution was poured around the trough to seal and stabilize the surgical area.

In 51 rats, an inflammation was induced in the left knee joint, either at the beginning of the experiment before recordings ($n = 33$ rats) or during the recording session while an ipsilateral neuron was being monitored ($n = 18$ rats). With this purpose, a 27 gauge needle was introduced through the patellar ligament, and 70 μ l of a 4% kaolin suspension (Sigma, Deisenhofen, Germany) was slowly injected into the articular cavity. Then, the joint was slowly flexed and extended for 15 min. Thereafter, 70 μ l of a 2% carrageenan solution (Sigma) was injected, and the joint was moved for another 5 min.

Recording and mechanical stimulation

Using glass-insulated carbon filaments for extracellular recording, individual neurons were identified by spike shape and height. The action potentials were continuously monitored on a digital oscilloscope to guarantee that the same neuron was recorded throughout. The signal was also fed into a window discriminator, the output of which was processed by an analog-to-digital interface and a personal computer so that peristimulus time histograms could be constructed. Action potentials were also stored on hard disk by means of the Spike/Spidi software (Forster and Handwerker, 1990). Final spike discrimination was performed off-line according to shape and size. Dorsal horn neurons were chosen for study if they responded to pressure applied to the ipsilateral, left knee but did not respond to brushing or squeezing of the skin over the knee. The size of, and the threshold within, receptive fields were determined using stimulation of the skin (brushing or squeezing of skin folds with forceps) and of the deep tissue (manual compression of joints and muscles). Mechanical test stimuli of two standard intensities were then applied to the knee, the ankle, and the paw. Each test stimulus lasted for 15 sec. A calibrated mechanical device (CORREX; Haag-Streit, Bern, Switzerland) was used for compression of the knee joint in the mediolateral axis; for innocuous intensity, a 1.9 N/40 mm² holding pressure was applied, and for noxious intensity (felt painful when applied to the experimenter's fifth finger), the knee was compressed with 7.8 N/40 mm² (or with 5.9 N/40 mm² when the response was very strong). Two modified crocodile clips with teeth filed away and jaws wrapped in tape were used to apply mediolateral compression of the ankle joint and dorsoventral compression of the middle of the paw (1.1 N/20 mm² for innocuous stimulation, and 5.8 N/20 mm² for noxious stimulation).

Ionophoretic delivery of glutamatergic agonists

In one set of experiments, we used the ionophoretic administration of AMPA (Research Biochemicals, Deisenhofen, Germany) and NMDA (Research Biochemicals) close to the neurons. A carbon filament, glass-insulated microelectrode was glued onto a multibarrel glass micropipette so that their tips ended together. The carbon microelectrode was used for recording of extracellular unitary action potentials from nociceptive dorsal spinal neurons with input from the knee (see above). The multibarrel micropipette was used for ionophoresis. One barrel was filled with AMPA (10 mM in 155 mM NaCl, pH 7.5, adjusted with NaOH), and another one was filled with NMDA (50 mM in 115 mM NaCl, pH 7.5, adjusted with NaOH). Two other barrels were filled with NaCl; one of them (1 M) served for automatic current balancing, and the other (165 mM) served for control of current application. Ejection currents for NMDA and AMPA were negative, and positive retaining currents of 25 nA were used between ejection periods.

Experimental protocols

Effect of spinally applied PGE₂ on responses to mechanical stimulation. In the first set of experiments (15 rats, one neuron per rat), we tested the effects of PGE₂ on the responses of spinal cord neurons to mechanical stimulation of the left knee, ankle, and paw. Innocuous and noxious test stimuli were applied sequentially to the knee, the ankle, and the paw. This sequence was repeated every 5 min, even when the manipulations described below were being performed. The neuronal baseline responses were established with the vehicle solution (0.07% ethanol in Tyrode's solution) in the spinal trough, and, when the responses were stable, the last 25 min before application of PGE₂ were taken as the predrug control period. After having established the baseline responses, the spinal trough was rinsed and filled with 30 μ l of a solution containing PGE₂ (100 ng/ μ l; Cayman Chemical, Ann Arbor, MI). This dose of PGE₂ was chosen from behavioral experiments using intrathecal application of PGE₂ (Vanegas and Schaible, 2001). The mechanical stimulation of knee, ankle, and paw was continued, and, after 50 min, the PGE₂ solution was replaced by the vehicle solution. The responses to mechanical stimulation were continued for another 25–50 min to observe recovery. In four experiments, the effect of the vehicle alone was tested and found to be nought.

In some experiments, PGE₂ was applied a second time while the same cell (four experiments) or another cell (eight experiments) was being recorded. The same procedure was used with another eight rats, but in these experiments, the knee joint had been inflamed 5–10 (on average 7) hours before the recordings started.

Effect of the application of the COX inhibitor indomethacin. In the second set of experiments, we studied the effect of spinally administered indomethacin on the development and maintenance of inflammation-evoked mechanical hyperexcitability of dorsal horn neurons. To study its effect on development of hyperexcitability, indomethacin was applied before the inflammation was induced. In eight rats (one neuron per rat), the responses of the neurons to mechanical stimulation of knee, ankle, and paw (see above) were monitored for 25 min. Then, indomethacin (Calbiochem, Bad Soden, Germany) was administered at a dose of 8 mM (Vanegas and Schaible, 2001) into the spinal trough, and the responses to mechanical stimulation were tested for another 25 min. Thereafter, the inflammation was induced (see above), and the responses to stimulation of knee, ankle, and paw were followed for another 4 hr still in the presence of indomethacin. Another 10 rats were used to assess the development of hyperexcitability in the absence of indomethacin. In these animals, only the vehicle (10 mM Na₂HPO₄, 17.65 mM NaOH, and 63.4 mM NaCl, pH 7.5, adjusted with HCl) was administered to the spinal cord while the responses to mechanical stimulation of knee, ankle, and paw were monitored before (25 min) and after induction of inflammation (4 hr).

To study the effect of indomethacin on the maintenance of spinal cord hyperexcitability, an inflammation was induced first, and the recordings from neurons with knee input were started 5–10 hr thereafter (eight rats). The responses to mechanical stimulation of the knee, ankle, and paw were assessed for a period of 50 min, with vehicle (see above) on the spinal cord. Then, indomethacin (8 mM) was applied to the spinal cord surface, and the responses to the mechanical test stimuli were monitored for 100 min during which indomethacin was on the cord. Thereafter, indomethacin (4 mg/kg) was given intraperitoneally, and the protocol was continued for another 50 min.

Effect of PGE₂ and indomethacin on glutamatergic responses. In the third set of experiments (nine rats with normal knee and 12 rats with inflamed knee, one neuron per rat), we tested whether the application of PGE₂ to the surface of the spinal cord would alter the responses to ionophoretic administration of AMPA or NMDA. Once a neuron had been characterized, ionophoretic application of AMPA or NMDA was started. After a variable number of seconds, the neuron began to fire, and ionophoretic application was then maintained for 10 sec after the first evoked spike. An ejection current was chosen such that it reproducibly induced clear responses that could be modulated in both directions (increase or decrease). Application of AMPA or NMDA began every 60 sec and was repeated until a stable baseline of 50 min duration was obtained. Then, PGE₂ (100 ng/ μ l) was administered for 50 min, and the application of AMPA or NMDA was performed. Finally, the responses were monitored after washout of PGE₂ for 50 min.

In another five rats with an inflammation in the knee joint, the effect of indomethacin on the responses to AMPA and NMDA was tested. AMPA and NMDA were administered ionophoretically for 50 min as described above, and then indomethacin (8 mM) was administered into the spinal trough and the responses to AMPA and NMDA were contin-

ued for 50 min. Thereafter, indomethacin was replaced by Tyrode's solution, and the responses to AMPA and NMDA were recorded for another 50 min.

Data analysis. The responses to pressure stimuli were calculated by subtracting the ongoing activity in the preceding 15 sec (if any) from the total activity during an innocuous or a noxious test stimulus. To evaluate the effects of PGE₂ on the responses to mechanical stimulation, we averaged all of the test responses to each type of stimulus in the 25 min preceding drug application (baseline), as well as the test responses during the last 25 min of PGE₂ application. Values are given in mean $\pm$ SE. Baseline and PGE₂ values were compared by using the Wilcoxon matched-pairs signed rank test. A similar procedure was used for the evaluation of the effects of PGE₂ on the responses to NMDA and AMPA. The responses in the last 25 min before PGE₂ (baseline) were averaged and normalized to 100%, and the values in the last 25 min of PGE₂ application were expressed as percentage of baseline. A similar procedure was used to analyze effects of indomethacin.

In experiments in which we assessed the effects of indomethacin on the development of inflammation-induced hyperexcitability, the baseline responses before inflammation were averaged (mean $\pm$ SE). After injection of kaolin into the knee, the responses to each stimulus within every hour after kaolin were averaged, and the baseline responses were subtracted. For statistical analysis, we compared the increase of the responses in the nontreated and the indomethacin-treated animals at corresponding intervals of 1 hr using the Mann–Whitney *U* test. In all statistical tests, significance was acknowledged if $p < 0.05$.

RESULTS

All neurons with knee input were located in segments L1–L4 at depths of 700–1200 μ m (mean of 954 μ m), thus mainly in the deep dorsal horn. Typically, the receptive field also included deep tissues of the thigh and lower leg. In 42 rats with normal joints, 29 of 42 neurons were wide dynamic range neurons that responded to innocuous and noxious stimulation of the knee in a graded manner, and 13 of 42 neurons were nociceptive specific and responded only to noxious pressure applied to the knee and other tissues. In 20 of these 42 neurons, the receptive field extended to the ankle and, in eight neurons, further to the paw. Three of these neurons had cutaneous receptive fields located on the thigh and/or the lower leg. Spontaneous activity was usually absent. In the sample of 33 neurons with input from the inflamed knee joint, 26 neurons were wide dynamic range, and seven were nociceptive-specific neurons.

Effect of PGE₂ on the responses of spinal cord neurons

The effects of spinally applied PGE₂ on the responses of dorsal horn neurons to mechanical stimulation of the ipsilateral hind-limb were studied in 15 neurons. Figure 1 shows the responses to innocuous and noxious pressure applied to the knee joint, the ankle, and the paw. During baseline stimulation the spinal cord trough was filled with vehicle solution. When this was replaced by the PGE₂ solution (100 ng/ μ l), the responses to both innocuous and noxious pressure showed a gradual increase. When the PGE₂-containing solution was replaced by the vehicle, the responses declined slowly. However, recovery was incomplete in most of the neurons (this is indicated by the large SE in the post-PGE₂ period).

For statistical analysis, we compared the average responses in the last 25 min of PGE₂ application versus the average baseline responses by using the Wilcoxon matched-pairs signed rank test. The increase of the responses was significant for innocuous ($p < 0.005$) and noxious ($p < 0.001$) pressure applied to the knee ($n = 15$ neurons), for innocuous ($p < 0.005$) and noxious ($p < 0.01$) pressure applied to the ankle ($n = 12$ neurons), and for innocuous ($p < 0.05$) and noxious ($p < 0.05$) pressure applied to the paw ($n = 7$ neurons). The number of neurons for knee was greater

Figure 1. Effect of PGE₂ on the responses of dorsal horn neurons to mechanical stimulation of the ipsilateral knee joint, ankle, and paw. Each symbol shows the mean $\pm$ SE of the responses. *nox.*, Noxious pressure; *innoc.*, innocuous pressure. During the first 25 min, only vehicle solution was in the spinal cord trough. This was replaced for 50 min (shaded area) by a solution containing PGE₂ (100 ng/ μ l).

Figure 2. Effect of PGE₂ (shaded area) when spinally applied after a previous application of PGE₂. The graph displays the responses of 12 spinal cord neurons to noxious (*nox.*) and innocuous (*innoc.*) pressure onto the knee joint. Same type of display as in Figure 1.

than for ankle or paw because their receptive fields did not always extend to these latter regions. Interestingly, however, PGE₂ application caused an expansion of the receptive field in some neurons. Thus, under PGE₂, three neurons that did not respond to ankle stimulation initially became responsive to noxious compression of the ankle, and three neurons whose receptive field did not include the paw initially became responsive to noxious stimulation of the paw. After PGE₂, the responses increased in both nociceptive-specific and wide dynamic range neurons.

In four experiments, a second application of PGE₂ was made during recording from the same neuron, 2 hr 15 min after the first application. In eight additional experiments, a second neuron was identified $\sim$ 2 hr after the protocol with the first neuron had been completed, and the same protocol was performed with the new neuron. In contrast to the first application of PGE₂, the second administration did not change the responses to mechanical stimulation ($n = 12$) (Fig. 2).

During inflammation of peripheral tissues, the milieu in the spinal cord is modified such that excitatory synaptic transmission becomes more efficient and spinal nociceptive neurons become hyperexcitable. Furthermore, endogenous release of PGE₂ is increased (see introductory remarks). In view of the above results with a second PGE₂ application, in eight neurons we tested the effects of PGE₂ on the responses to mechanical stimulation when the knee joint had been inflamed for 5–10 (on average 7) hours. When applied during inflammation, PGE₂ increased the neuronal

Figure 3. PGE₂-induced changes in the responses to mechanical stimulation of knee, ankle, and paw in rats with non-inflamed knees and in rats with inflammation of the stimulated knee joint. The columns show the mean $\pm$ SE increase of the responses above baseline in the last 25 min of the PGE₂ application. * $p < 0.05$, significant difference between increase above baseline in rats with normal knees and rats with inflamed knees.

Figure 4. Effect of spinal application of indomethacin on the development of inflammation-induced hyperexcitability of spinal cord neurons. The graphs show the changes of the responses to noxious (nox.) and innocuous (innoc.) pressure ipsilaterally applied to the knee and to the ankle in the 4 hr after induction of inflammation in the knee by intra-articular injection of K/C. When the spinal trough was filled with vehicle solution (open squares) or with indomethacin (filled squares), the baseline (BL) values before K/C were set to zero, and the values after K/C injection show the mean $\pm$ SE change of all of the responses within each hourly interval compared with baseline. For stimulation of the knee joint, each value shows at least the averaged responses from seven animals and for stimulation of the ankle at least from four animals.

responses to mechanical stimuli, but the effects were smaller than in non-inflamed animals (Fig. 3). However, this comparison reached statistical significance only for knee stimulation ($p < 0.05$; one-sided; Mann–Whitney U test).

Effect of indomethacin on the development and maintenance of inflammation-induced hyperexcitability

In these experiments, we addressed the role of endogenous spinal PGs in the induction and maintenance of inflammation-induced hyperexcitability for mechanical stimuli. The PG synthesis inhibitor indomethacin that reduces stimulus-evoked PGE₂ release from spinal cord (Malmberg and Yaksh, 1994) was applied into the spinal trough. Figure 4 illustrates the experiments in which indomethacin was given before induction and during development of inflammation. First, we recorded the baseline responses, and then we induced the knee inflammation by injection of kaolin and carrageenan (K/C) and monitored the neurons for another 4 hr. Indomethacin was given 25 min before kaolin was injected and had no immediate effect on neuronal responses. Indeed, in five neurons, the average response to noxious pressure onto the knee joint before indomethacin was 273 ± 89 impulses/15 sec and after

Figure 5. Effects of indomethacin on neuronal responses after development of hyperexcitability. The recordings started 6–11 hr after induction of inflammation in the ipsilateral knee joint. Each symbol represents the mean $\pm$ SE of the responses of spinal cord neurons to innocuous (innoc.) and noxious (nox.) pressure onto the knee joint under spinal application and subsequent intraperitoneal administration of indomethacin.

indomethacin was 260 ± 93 impulses/15 sec. However, indomethacin attenuated the inflammation-induced changes. Figure 4 shows the responses to noxious and innocuous pressure applied to the knee and ankle in nontreated control animals and in animals that received indomethacin in the spinal trough. In all graphs, the responses in the baseline period were set to zero, and the values after K/C express the increase of the responses above baseline. In the untreated rats, we observed a pronounced increase of the responses to mechanical stimulation of the injected knee (showing peripheral sensitization and ensuing spinal cord activation and sensitization) and of the non-inflamed ankle (showing central sensitization) within the 4 hr of knee inflammation (Fig. 4, open squares). The circumference of the injected knees increased on average from 43 to 50 mm. In contrast, when indomethacin was present in the spinal trough before and during development of knee inflammation, there was no increase in the responses to innocuous or noxious stimulation of the non-inflamed ankle or to innocuous stimulation of the injected knee. The responses to noxious pressure on the knee showed a small but significant increase. For statistical analysis, we compared for all stimuli the values obtained in each of the 4 hr after induction of inflammation (Mann–Whitney U test). In neurons from rats without indomethacin, the responses to mechanical stimulation of the inflamed knee and of the non-inflamed ankle were significantly larger than in rats with indomethacin treatment (Fig. 4). In rats with spinal indomethacin, the circumference of the injected knees increased on average from 44 to 50 mm (same as in control experiments).

The inflammation induced by K/C and the resulting hyperexcitability of spinal cord neurons reach a plateau after 5–6 hr, i.e., the responses usually do not further increase after this time (Neugebauer et al., 1993). In additional experiments, we tested the effect of indomethacin when the compound was given after development of inflammation (Fig. 5). Indomethacin (8 mM) was administered to the spinal cord 6–11 hr after induction of inflammation. Although indomethacin was applied to the spinal cord for 100 min, no reduction of the responses to knee stimulation was noted, nor were the responses to stimulation of the ankle and paw reduced. These data therefore suggest that inhibition of spinal PG synthesis after development of inflammation does not influence central sensitization. When indomethacin was given intraperitoneally at a later time, the responses to pressure onto the knee showed a progressive reduction. When the last three values after

Figure 6. Effects of PGE_2 on the responses of spinal cord neurons to the ionophoretic application of AMPA or NMDA. Neurons in the *top panels* were in animals with normal joints, and neurons in the *bottom panels* were in rats with inflammation of the ipsilateral knee joint. The *line graphs* below the column diagrams show the responses to each test application of AMPA or NMDA, averaged in the total sample of neurons. For each neuron, responses are expressed as percentage of the average of all its responses during baseline. Each *square* represents the mean $\pm$ SE of the response of all neurons to one AMPA application (*left*) or one NMDA application (*right*). The *column diagrams* display the average response in the 25 min before PGE_2 , the average responses in the last 25 min of PGE_2 application (*shaded area*), and the average responses after washout of PGE_2 , expressed as percentage of baseline. * $p < 0.05$ versus baseline.

intraperitoneal injection of indomethacin were compared with the last three values before intraperitoneal injection of indomethacin, the reduction of the responses to noxious pressure was statistically significant for noxious pressure ($p < 0.05$; Wilcoxon matched-pairs signed rank test; $n = 6$ neurons). This is probably attributable to extraspinal effects of indomethacin.

Effect of PGE_2 on the responses to AMPA and NMDA

Behavioral experiments have shown that the hyperalgesia evoked by PGE_2 can be blocked by antagonists at NMDA and non-NMDA receptors (Minami et al., 1994b; Nishihara et al., 1995a). Furthermore, NMDA receptors are thought to be key players in the generation and maintenance of inflammation-induced hyperexcitability (Woolf and Thompson, 1991; Neugebauer et al., 1993). Thus, an interaction between PGE_2 and glutamatergic synaptic transmission is of particular interest. We therefore studied whether the application of PGE_2 would influence responses of spinal cord neurons to the application of AMPA and NMDA, the agonists at ionotropic glutamate receptors. Neurons that responded to mechanical stimulation of the knee joint were stimulated by the ionophoretic administration of AMPA or NMDA. The knee joint was either normal or inflamed. Figure 6 shows the

Figure 7. Effects of indomethacin on responses to AMPA or NMDA after development of hyperexcitability. The recordings started 6–11 hr after induction of inflammation in the ipsilateral knee joint. *Graphs* illustrate the effects of topical administration of indomethacin to the spinal cord on the responses of dorsal horn neurons to ionophoretically administered AMPA or NMDA. Each *symbol* displays one neuron.

effects of PGE_2 on the responses of neurons to AMPA or NMDA. The *line graphs* show the average responses to each test application of AMPA or NMDA. The *columns* display the average responses in the 25 min before PGE_2 , the responses in the last 25 min of PGE_2 application, and the responses after washout of PGE_2 , expressed as percentage of baseline. The baseline responses were obtained while Tyrode's solution was in the spinal cord trough. In animals with normal knees (Fig. 6, *top*), only the responses to NMDA showed a significant increase ($p < 0.05$; Wilcoxon matched-pairs signed rank test) during application of PGE_2 . However, when the knee was inflamed, both the responses to AMPA and to NMDA (Fig. 6, *bottom*) showed marked progressive increases (for AMPA, $p < 0.005$; for NMDA, $p < 0.005$; Wilcoxon matched-pairs signed rank test).

To test whether a reduction of endogenous spinal PGs would alter the activation of glutamate receptors after inflammation is established, we tested the effect of spinal application of indomethacin on the responses to AMPA and NMDA. As with mechanical stimulation, indomethacin (8 mM) was administered 6–11 hr after induction of inflammation. Figure 7 shows that neither the responses to AMPA nor the responses to NMDA were significantly changed in the presence of indomethacin.

DISCUSSION

In the present study, topical application of PGE_2 to the spinal cord enhanced the responses of dorsal horn neurons to mechanical stimuli and expanded their receptive fields. These effects mimic the central sensitization that develops during knee joint inflammation. Spinal application of indomethacin before and during development of knee joint inflammation did not prevent the inflammation in the knee but markedly attenuated the generation of hyperexcitability in spinal cord neurons. Administration of indomethacin 6–11 hr after development of inflammation, however, did not reduce the enhanced responses. Thus, endogenous PG synthesis in primary afferent neurons and/or spinal cord seems necessary and sufficient for the induction of central sensitization but not for its maintenance. Topical application of PGE_2

to the spinal cord enhanced the responses of nociceptive neurons to the ionophoretic application of NMDA, thus suggesting that PGE₂ interaction with glutamatergic synaptic transmission contributes to the PG role in central sensitization. However, application of indomethacin after development of inflammation did not reduce responses to ionophoretically administered NMDA and AMPA, again suggesting that after establishment of inflammation spinal PG synthesis is not essential for glutamatergic sensitivity.

Prostaglandins and the increase in responsiveness of spinal cord neurons

The effects of PGE₂ on neurons *in vitro* as well as on animal behavior have been studied extensively (Vanegas and Schaible, 2001). However, we have herein investigated the effects of PGE₂ on nociceptive neuronal discharges, i.e., messages that may lead to perception and behavioral responses, elicited by “natural” innocuous and noxious stimuli. As mentioned, PGE₂ induced a pattern of effects on spinal cord neurons similar to that of central sensitization in general and of inflammation-induced hyperexcitability in particular. After PGE₂, like during peripheral inflammation (Woolf 1983; Hylden et al., 1989; Neugebauer and Schaible, 1990; Dougherty et al., 1992), and in agreement with behavioral experiments (see introductory remarks), the neurons showed an increased firing to noxious mechanical stimulation, which may lead to primary mechanical hyperalgesia, a decrease in the excitation threshold, and thus enhanced or novel responses to innocuous stimulation, which may lead to allodynia, and an expansion of the receptive field, which is typical of central sensitization and may lead to secondary mechanical hyperalgesia. Preliminary experiments show that butaprost, an EP2 agonist, produces similar effects as PGE₂ in a dose-dependent manner.

We also studied herein the consequences of reducing the effect of PGs during the induction of inflammation, because in inflammation, PG synthesis and release in the spinal cord are upregulated (see introductory remarks). Local transmitters and modulators may contribute to this upregulation, because NMDA (Sorkin, 1993), kainic acid (Yang et al., 1996b), substance P (Hua et al., 1999), depolarization by K⁺ (Dirig et al., 1997), and capsaicin (Dirig and Yaksh, 1999) have been shown to stimulate PG synthesis and release in the spinal cord (Vanegas and Schaible, 2001). During inflammation, interleukin-1 β -mediated induction of COX-2 may be particularly important (Samad et al., 2001). Because no specific antagonists are available for PG receptors, we applied indomethacin, a COX-1/COX-2 inhibitor, to the spinal cord before the inflammation began. This significantly attenuated the development of inflammation-induced neuronal hyperexcitability. Plausibly, the initial production of PGs in primary afferents and/or spinal cord intrinsic elements is essential for the full development of spinal cord plasticity. Indomethacin also reduces windup of dorsal horn neuronal discharges to electrical C-fiber stimulation (Willingale et al., 1997) and the discharges of dorsal horn neurons after subcutaneously applied formalin (Chapman and Dickenson, 1992). Although these and the present results are taken as attributable to COX inhibition, contribution by non-COX targets cannot be excluded (Vanegas and Schaible, 2001).

Effects of PGE₂ on the responses to AMPA and NMDA

The results discussed above could arise from a PG effect on presynaptic elements, e.g., primary afferent terminals, or on postsynaptic elements, e.g., intrinsic spinal neurons. PGE₂ acts on

G-protein-coupled receptors (EP1–4), and DRG neurons express EP1 (Oida et al., 1995), EP3 (Sugimoto et al., 1994; Oida et al., 1995; Beiche et al., 1998b), and EP4 receptors (Oida et al., 1995), whereas spinal cord neurons express EP2 receptors (Kawamura et al., 1997). Thus, the spinal effects of naturally released or experimentally applied PGE₂ on nociception may be exerted on both the primary afferents and dorsal horn neurons.

The evidence for presynaptic targets is considerable. For example, PGE₂ enhances the release of substance P and calcitonin gene-related peptide (CGRP) from cultured or native DRG neurons that is evoked by various chemical stimuli (Nicol et al., 1992; Andreeva and Rang, 1993; Vasko et al., 1993, 1994; Hingtgen and Vasko, 1994; Southall et al., 1998). Conversely, NSAIDs diminish the stimulus-induced release of substance P and CGRP from DRG neurons or spinal cord slices because this release is facilitated by PGs (Andreeva and Rang, 1993; Vasko et al., 1994). Furthermore, PGE₂ application increases the frequency but not the amplitude of miniature EPSCs mediated by AMPA receptors in spinal cord neurons (Minami et al., 1999). Recently, however, Baba et al. (2001) showed that PGE₂ excites spinal cord intrinsic neurons directly.

Here we show for the first time that PGE₂ increases firing of intrinsic neurons elicited by AMPA or NMDA. When the knees were normal, PGE₂ caused a significant facilitation of firing to application of NMDA close to the neurons. This is of particular interest because the activation of NMDA receptors of dorsal horn neurons is thought to be a key mechanism in the induction of central sensitization (see above) and in particular the increase in mechanosensitivity (Neugebauer et al., 1993). When the joint was inflamed, PGE₂ induced facilitated responses to AMPA and NMDA. This could result from presynaptic but also from postsynaptic effects of PGE₂. For example, PGE₂ might release compounds from primary afferents (e.g., substance P) that facilitate the responses to glutamate receptor agonists. Alternatively or additionally, the sensitivity of postsynaptic neurons to PGE₂ might increase during inflammation attributable to, for example, EP receptor sensitization. However, because the responses to AMPA and NMDA were not reduced by indomethacin after inflammation was established, a facilitation of glutamatergic synapses by endogenous PGs does not seem to be the mechanism whereby central sensitization is maintained (see also below).

Role of prostaglandins after development of inflammation

Unexpectedly, when the inflammation was established, the responses of spinal cord neurons to mechanical stimulation (and the responses to AMPA or NMDA) were not reduced by spinal administration of indomethacin. However, the responses to mechanical stimuli were reduced after systemic administration of indomethacin. Because inhibition of PG synthesis by indomethacin is rapid, we should have seen a reduction of the responses after spinal application if the continuous production of PGs in the spinal cord were important for the maintenance of hyperexcitability to mechanical stimuli during inflammation. Interestingly, Dirig et al. (1998) observed no reduction of thermal hyperalgesia with intrathecal administration of S(+)-ibuprofen or the specific COX-2 inhibitor SC58125 3 hr after induction of carrageenan inflammation. It thus seems that spinal PG synthesis is not required for the maintenance of central sensitization, at least in the time period studied, and that analgesic effects of NSAIDs in this period are exerted at sites other than spinal targets, at least with respect to mechanical stimulation. Such targets could be the

inflamed knee joint (Heppelmann et al., 1986) and supraspinal structures (Vanegas et al., 1997).

The lack of effect of spinal indomethacin after establishment of inflammation could have several reasons. First, there might be no additional spinal PG synthesis after the initial PG production, and hence indomethacin would not act. This seems unlikely, because upregulation of COX-2 in the spinal cord begins in the first hours of inflammation and lasts for many hours or days (Vanegas and Schaible, 2001). Second, PGs and/or other spinal mediators might, as inflammation progresses, cause persistent changes, including a decrease in the sensitivity of spinal neurons to PGs. Indeed, the effects of PGE₂ on the responses to mechanical stimulation were not fully reversible, a second application of PGE₂ in the same experiment did not further enhance these responses, and the effects of PGE₂ on these responses were much less pronounced in rats with a knee inflammation. Thus, PGE₂ could initiate cellular events that induce long-term changes in synaptic transmission that may contribute to the persistence of central sensitization. Because the activation of PG receptors sets in motion second messenger systems and enhances, for example cAMP (Vanegas and Schaible, 2001), it is likely that second messengers could induce these long-term changes. A desensitization of PG receptors or alterations in the transduction cascade may be part of these effects. It should be noted, however, that exogenous PGE₂ enhanced the responses to AMPA and NMDA in the spinal cord of rats with inflamed knees, so that a total insensitivity of neurons to PGE₂ is unlikely, and other explanations should be sought in the future.

In all, the present results highlight the involvement of PGs in the facilitation of somatosensory neuronal discharges, particularly during induction of central sensitization. A direct and/or indirect facilitation of glutamatergic synapses might underlie this effect, but other molecular players seem to be responsible for the sustained central hyperexcitability.

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