Behavioral/Systems/Cognitive

Changes in the Effect of Spinal Prostaglandin E_2 during Inflammation: Prostaglandin E (EP1–EP4) Receptors in Spinal Nociceptive Processing of Input from the Normal or Inflamed Knee Joint

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Inflammatory pain is caused by sensitization of peripheral and central nociceptive neurons. Prostaglandins substantially contribute to neuronal sensitization at both sites. Prostaglandin E_2 (PGE_2) applied to the spinal cord causes neuronal hyperexcitability similar to peripheral inflammation. Because PGE_2 can act through EP1–EP4 receptors, we addressed the role of these receptors in the spinal cord on the development of spinal hyperexcitability. Recordings were made from nociceptive dorsal horn neurons with main input from the knee joint, and responses of the neurons to noxious and innocuous stimulation of the knee, ankle, and paw were studied after spinal application of recently developed specific EP1–EP4 receptor agonists. Under normal conditions, spinal application of agonists at EP1, EP2, and EP4 receptors induced spinal hyperexcitability similar to PGE_2. Interestingly, the effect of spinal EP receptor activation changed during joint inflammation. When the knee joint had been inflamed 7–11 hr before the recordings, only activation of the EP1 receptor caused additional facilitation, whereas spinal application of EP2 and EP4 receptor agonists had no effect. Additionally, an EP3α receptor agonist reduced responses to mechanical stimulation. The latter also attenuated spinal hyperexcitability induced by spinal PGE_2. In isolated DRG neurons, the EP3α agonist reduced the facilitatory effect of PGE_2 on TTX-resistant sodium currents. Thus pronociceptive effects of spinal PGE_2 can be limited, particularly under inflammatory conditions, through activation of an inhibitory splice variant of the EP3 receptor. The latter might be an interesting target for controlling spinal hyperexcitability in inflammatory pain states.

Key words: **pain; nociception; central sensitization; prostaglandin; EP receptor; inflammation**

Introduction

Prostaglandin E_2 (PGE_2) is a local mediator in the CNS that affects synaptic processing, particularly under pathophysiological conditions. In the spinal cord, research has focused on the role of PGE_2 in the synaptic processing of noxious inputs (Vanegas and Schaible, 2001; Svensson and Yaksh, 2002). PGE_2 is not only an important mediator in the development of peripheral inflammation and peripheral sensitization (Vane, 1971; Julius and Basbaum, 2001), but it also causes allodynia and hyperalgesia when it is applied to the spinal cord (Taiwo and Levine, 1988; Uda et al., 1990; Minami et al., 1994, 1996; Malmberg and Yaksh, 1995a,b; Ferreira and Lorenzetti, 1996). Recent research has established an important role of endogenous spinal PGE_2 in spinal nociceptive processing during peripheral inflammation. Cyclooxygenases 1 and 2 are present both in dorsal root ganglia (DRGs) and in the spinal cord (Willingale et al., 1997; Inoue et al., 1999). Importantly, in particular, cyclooxygenase 2 is markedly upregulated during peripheral inflammation (Beiche et al., 1996, 1998b; Hay and de Belleroche, 1997; Hay et al., 1997; Goppelt-Struebe and Beiche, 1998; Samad et al., 2001), and the intraspinal release of PGE_2 is enhanced (Yang et al., 1996; Ebersberger et al., 1999). Topical application of PGE_2 to the exposed spinal cord induces a state of hyperexcitability in dorsal horn neurons similar to peripheral inflammation, and spinal application of a prostaglandin synthesis inhibitor attenuates the generation of inflammation-evoked central sensitization (Vasquez et al., 2001).

The effects of PGE_2 are mediated by G-protein-coupled EP (EP1–EP4) receptors. Interestingly, all four receptors are present in the spinal cord, and therefore, it is of considerable interest to explore their role in spinal hyperexcitability. EP1 (Oida et al., 1995), EP3 (Sugimoto et al., 1994; Oida et al., 1995; Beiche et al., 1998a), and EP4 receptors (Oida et al., 1995) are expressed in dorsal root ganglion neurons, indicating that prostaglandins influence the release of transmitters from primary afferent fibers. EP2 receptors are expressed in spinal cord neurons (Kawamura et

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al., 1997), indicating postsynaptic effects of PGE_2 (Baba et al., 2001; Ahmadi et al., 2002). Activation of the EP1 receptor leads to calcium influx, and stimulation of EP2, some isoforms of EP3, and of EP4 receptors enhances intracellular cAMP through adenylate cyclase, but activation of other isoforms of the EP3 receptor (e.g., the EP3α subtype) reduces the intracellular cAMP concentration (Negishi et al., 1995a,b; Narumiya et al., 1999; Funk, 2001).

In the present experiments, we made extracellular recordings from rat spinal cord neurons *in vivo*. We explored how the activation of different EP receptors by spinal application of recently developed selective EP receptor agonists affects the response and receptive field properties of nociceptive neurons. In addition, we investigated whether the role of different EP receptors is modified during peripheral inflammation. Indeed, effects of the agonists were significantly different under normal and inflammatory conditions, and most interestingly, application of an EP3α receptor agonist reduced inflammation- or PGE_2-evoked hyperexcitablity. In patch-clamp recordings from isolated dorsal root ganglion neurons, the inhibitory effect of the EP3α receptor agonist was substantiated further.

Materials and Methods

In vivo *experiments*

Preparation. Experiments were performed on 75 male Wistar rats (200–350 gm; University of Jena, Jena, Germany) that were anesthetized with sodium thiopentone (Trapanal; initial dose, 85–115 mg/kg, i.p.; Byk Gulden, Konstanz, Germany). The trachea was cannulated, and a gentle jet of oxygen was blown toward the opening of the tracheal cannula. The animals breathed spontaneously. A catheter was inserted into the common carotid artery to measure blood pressure. Another catheter was inserted into the external jugular vein to give compounds intravenously. Usually, this catheter was only used to give a lethal dose of sodium thiopentone at the end of the experiment. 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 90–110 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 μl capacity over the spinal segments in which the recordings were to be performed. This trough was 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 24 rats, inflammation was induced in the left knee joint 7–11 hr before recordings. A 27 gauge needle was introduced through the patellar ligament, and 70 μl 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 μl 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. Action potentials were also stored on hard disk using the Spike–Spidi software (Forster and Handwerker, 1990). Final spike discrimination was performed off-line according to shape and size.

Dorsal horn neurons with input from the ipsilateral knee joint were selected for study. To identify neurons with knee input, we used manual mediolateral compression of the joint as a searching stimulus during the tracking procedure. For searching, the pressure was moderate, but at each depth range, noxious compression was used so that we could identify both wide dynamic range and nociceptive-specific neurons. If a neuron responded to pressure applied to the ipsilateral knee, we brushed the skin and squeezed skin folds over the knee. If a neuron responded to stimulation of the skin over the knee, it was not studied further. Neurons with deep input from the knee were characterized further. To determine the size of the receptive field, we applied moderate and noxious compression of the knee, thigh, lower leg, and paw and used brushing, squeezing of skin folds with forceps, and squeezing of the deep tissue to classify the neuron as a wide dynamic range or a nociceptive-specific neuron. Wide dynamic-range neurons responded to innocuous stimuli and showed graded responses to innocuous and noxious stimuli, whereas nociceptive specific neurons responded only to noxious stimulus intensities.

When a neuron was chosen for additional study, we used the following protocol for testing. Mechanical test stimuli of two standard intensities were 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 mm^2 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 mm^2 (or with 5.9 N/40 mm^2 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 mm^2 for innocuous stimulation; 5.8 N/20 mm^2 for noxious stimulation).

Experimental protocol. Specific EP receptor agonists were used to assess the functional effects of EP1–EP4 receptor stimulation. 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 the agonists were taken as the predrug baseline period. After having established the baseline responses, the spinal trough was rinsed and filled with 30 μl of a solution containing a specific EP receptor agonist. First, the lowest concentration was administered, and during the 50 min after the drug application, the mechanical stimulation of the knee, ankle, and paw was continued. The solution was then removed from the trough, the next higher concentration of the agonist was administered, and the mechanical stimulation protocol went on for another 50 min. This was continued with additional applications of the agonist at higher concentrations. In each experiment, only one neuron was tested, and only one agonist was applied.

Each agonist was tested in normal rats and in rats with inflammation of the knee. The following agonists were used: the EP1 receptor agonist ONO-DI-004 [doses in the present experiments were 0.1 ng/μl (2.36 μM) to 100 ng/μl (2.36 mM); Ono Pharmaceutical, Osaka, Japan], the EP2 receptor agonist butaprost [doses in the present experiments were 0.1 ng/μl (2.44 μM) to 100 ng/μl (2.44 mM); Cayman Chemical, Ann Arbor, MI], the EP3α receptor agonist ONO-AE-248 [doses in the present experiments were 1 ng/μl (26.28 μM) to 100 ng/μl (2.628 mM); Ono Pharmaceutical], and the EP4 receptor agonist ONO-AE1-329 [doses in the present experiments were 1 ng/μl (21.9 μM) to 100 ng/μl (2.199 mM); Ono Pharmaceutical]. The compounds ONO-DI-004, ONO-AE-248, and ONO-AE1-329 have been generated and characterized recently and have been used in different studies (Yamamoto et al., 1999; Zacharowski et al., 1999; Suzawa et al., 2000; Maruyama et al., 2001; Shinomiya et al., 2001). In membranes of Chinese hamster ovary cells expressing the respective mouse EP receptors, the new agonists had the following K_i values in [^{3}H]PGE_2 binding assays: ONO-DI-004, 0.15 μM at the EP1 receptor; ONO-AE-248, 0.0075 μM at the EP3α receptor; ONO-AE1-329, 0.0097 μM at the EP4 receptor. For comparison, PGE_2 had K_i values of 0.018 μM at the EP1 receptor, 0.038 μM at the EP2 receptor, 0.005 μM at the EP3 receptor, and 0.0031 μM at the EP4 receptor (Yamamoto et al., 1999; Suzawa et al., 2000). EC_{50} values are as follows: ONO-DI-004, 0.42 μM at the EP1 receptor; ONO-AE-248, 0.0052 μM at the EP3α receptor; ONO-AE1-329, 0.0031 μM at the EP4 receptor (Yamamoto et al., 1999).

The following experimental groups were used. In four different groups of normal rats, we tested the effects of the EP1, EP2, EP3α, or EP4 agonist on the responses of spinal cord neurons to stimulation of normal knee, normal ankle, and normal paw. In another four groups of rats, we induced inflammation in the knee joint and tested the EP1, EP2, EP3α, or EP4 agonist on the responses of spinal cord neurons to stimulation of the

inflamed knee, the normal ankle, and the normal paw. In another six experiments on normal rats, the effect of the vehicle alone was tested (i.e., the mechanical stimulation protocol was conducted for 3 hr 25 min with only the vehicle on the spinal cord).

Finally, in a group of 14 rats, we tested whether the EP3α receptor agonist would alter the facilitatory effect of PGE_2 on responses to mechanical stimulation. After having established the baseline of the responses to mechanical stimulation, 30 μl of PGE_2 [100 ng/μl (3.525 mM)] was administered to the spinal cord, and the mechanical stimulation protocol was continued. After 50 min, the solution in the trough was changed to a solution containing PGE_2 [100 ng/μl (3.525 mM)] plus the EP3α receptor agonist ONO-AE-248 [10 ng/μl (0.263 mM) or 100 ng/μl (2.628 mM)], and additional responses to the mechanical stimuli were monitored. In 6 of these 14 experiments, only PGE_2 [100 ng/μl (3.525 mM)] was applied for 150 min to assess the effects of long-term application of PGE_2 alone.

Data analysis. To assess the response of a neuron to a mechanical stimulus, we counted the action potentials that were elicited by the stimulus during the 15 sec application. To evaluate statistically the effects of the agonists on the responses to mechanical stimulation, we averaged all of the responses to each type of stimulus in the 25 min preceding drug application (baseline) as well as the responses during the last 25 min of EP receptor agonist application. Values are given as mean ± SE (SEM). Baseline values and the values after drug application were compared by using the Wilcoxon matched pairs signed rank test. The difference between the responses after the highest dose of the agonist and the baseline responses was tested first. If this difference was significant, the effect of the next lower concentration was tested for significance and so on. Significance was accepted at $p < 0.05$.

In vitro *experiments*

Whole-cell patch-clamp experiments on cultured dorsal root ganglion neurons. To study the interaction of the EP3α receptor agonist and PGE_2 on voltage-gated Na^+ currents in isolated DRG neurons, DRG cells were prepared from spinal segments from adult male Wistar rats (60 d of age) as described previously (Segond von Banchet et al., 2002). Briefly, DRGs were incubated at 37°C with 215 U/mg collagen type II (Paesel & Lorei, Hanau, Germany) dissolved in Ham's F-12 medium (Sigma) for 100 min. After washing with Ca^{2+}- and Mg^{2+}-free PBS, pH 7.4 (Invitrogen, Eggenstein-Leopoldshafen, Germany), the DRGs were incubated for 11 min at 37°C in DMEM (Sigma) containing 10,000 U/ml trypsin (Sigma). The ganglia were then dissociated into single cells by gentle agitation and by triturating through a fire-polished Pasteur pipette. The dispersed cells were collected by centrifugation (500 × *g* for 5 min), washed in DMEM, and recentrifuged. The neuron pellets were suspended in Ham's F-12 medium containing 10^{-3} M L-glutamine (Sigma), 10% heat-inactivated horse serum (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 100 ng/ml nerve growth factor (NGF) (NGF 7S; Boehringer Mannheim, Mannheim, Germany). The cells were centrifuged again, resuspended in Ham's F-12 medium (containing L-glutamine, inactivated horse serum, penicillin, streptomycin, and NGF), and then plated on 13-mm-diameter glass coverslips (cells from ~1 to 1.5 ganglion/coverslip) that had been precoated with poly-L-lysine (50 μg/ml; Sigma). The neurons were kept for 1–2 d at 37°C in a humidified incubator gassed with 3.5% CO_2 in air. Cultures were fed every day with Ham's F-12 medium (see above).

Currents were recorded from DRG neurons (1–2 d in culture) at room temperature using the whole-cell patch-clamp technique. The recordings were made with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Electrodes (1.0–3.0 MΩ) were fabricated from 1.7 mm capillary glass (Kimax; Kimble, Vineland, NJ) using a Sutter P-97 puller. The recording pipette solution contained the following (in mM): 140 CsCl, 10 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 2 Na_2-ATP, 5 EGTA, and 10 HEPES, pH 7.2. Stimuli were controlled with pClamp 7.01 software (Axon Instruments). Voltage errors were minimized using 75–80% series resistance compensation with amplifier circuitry. Current signals were filtered at 1 kHz (four pole Bessel) and sampled at 5 kHz with the interface (Digidata 1200; Axon Instruments). For presentation, data were filtered with a Gaussian filter at 500 and 250 Hz.

Coverslips with cells were mounted on the stage of an upright microscope (Nikon, Tokyo, Japan) (equipped with a 63× water immersion objective). The cells were continuously superfused with a standard HEPES buffer (in mM): 60 NaCl, 50 choline-Cl, 5 KCl, 20 TEA-Cl, 2 $CaCl_2$, 2 $MgCl_2$, 0.1 $CdCl_2$, 10 glucose, and 10 HEPES, pH 7.4. Cadmium and TEA were included to block calcium and potassium currents, respectively. All recordings were conducted with 150 nM TTX in the bathing solution to minimize TTX-sensitive Na^+ currents. Whole-cell configuration was established after formation of a tight seal (>2 GΩ) and compensation of pipette capacitance with amplifier circuitry. Initially, the resting membrane potential (E_{res}) was measured. Starting from a holding potential of −70 mV, the neurons were depolarized from −70 up to +65 mV in voltage increments of 5 mV (1.2 sec intervals), and TTX-resistant (TTX-R) Na^+ currents were measured. The control value in each cell was established by recordings for *I–V* curves at 3 and 5 min after the initial procedures. Thereafter, the solution was switched to the test solution, and additional recordings for *I–V* curves were made at 2, 4, 6, and 8 min after application of the test solution. To test the effect of PGE_2, a solution was administered that contained standard HEPES buffer (see above) plus 2.5 μM PGE_2 (dissolved first in 0.07% ethanol). To test the effect of PGE_2 plus the EP3α agonist, we used standard HEPES containing 2.5 μM PGE_2 plus either the vehicle of the EP3α agonist (0.5% DMSO in 0.9% NaCl) or the EP3α receptor agonist at concentrations of 0.5, 2.0, or 10 μM. In 10 neurons, only the EP3α agonist was applied. Experiments were performed at room temperature.

The data were analyzed using the pClamp 7.01 (Axon Instruments) and Origin 6.1 (Microcal Software, Northampton, MA) software programs. Current densities were calculated by dividing the peak current (I_{peak}) evoked at each membrane potential (V_m) by the cell capacitance (C_m). All data are expressed as mean ± SEM (n = number of tested cells). For comparison of peak inward current densities within an experimental group, the paired *t* test was used. Increases in peak inward current densities in different experimental groups were compared using the Mann–Whitney *U* test. The effect of the highest dose of the EP3α agonist was tested for significance first. If this effect was significant, the effect of the next lower concentration of the agonist was tested for significance and so on. Significance was accepted at $p < 0.05$.

Results

Neurons with input from the knee joint were recorded in the deep dorsal horn of the segments L1–L4 at depths of 463–1322 μm (mean, 942 μm). Typically, the receptive field also included adjacent muscles and other deep tissue of the thigh and lower leg. In 51 rats with normal joints, 44 of 51 neurons were wide dynamic-range neurons that responded to innocuous and noxious stimulation of the knee in a graded manner, and 7 of 51 neurons were nociceptive specific and responded only to noxious pressure applied to the knee and other tissues. In 39 of these 51 neurons, the receptive field extended to the ankle, and in 26 neurons, the receptive field extended further to the paw. Seven neurons showed some spontaneous activity at a mean frequency of 0.9 ± 0.7 Hz. In the sample of 24 neurons with input from the inflamed knee joint, all neurons were wide dynamic-range neurons. In 18 neurons, the receptive field extended to the ankle, and in 12 neurons, it extended to the paw. In rats with inflamed knee joints, 6 of the 24 neurons were spontaneously active (1.83 ± 2.67 Hz). In total, only five neurons had cutaneous receptive fields located on the thigh. In each rat, only one neuron was recorded.

Induction of spinal hypersensitivity by agonists at the EP1, EP2, and EP4 receptor

When PGE_2 is applied to the spinal cord, nociceptive spinal cord neurons develop enhanced responses to mechanical stimulation of the tissue and an expansion of their receptive field (Vasquez et al., 2001). Topical application of the EP1 receptor agonist ONO-DI-004 to the spinal cord produced a similar effect (Fig. 1). Ini-

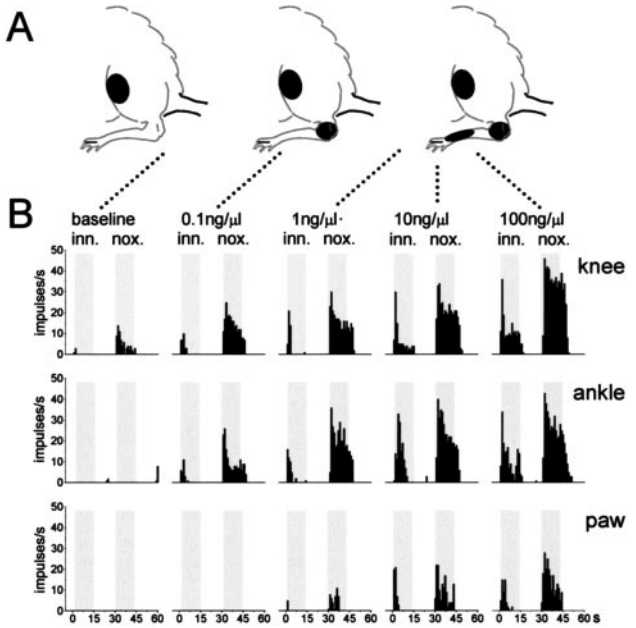

Figure 1. Facilitatory effect of the EP1 agonist ONO-DI-004 after spinal application. *A*, Expansion of the initial receptive field of the spinal cord neuron at the knee joint toward the ankle and paw during application of the EP1 agonist. Stimulation sites (circular areas) were filled black as soon as action potentials were elicited. *B*, Peristimulus time histograms showing the number of action potentials per second elicited during innocuous (inn.) and noxious (nox.) pressure applied to the knee, the ankle, and the paw (shaded boxes) before drug application (baseline) and after administration of increasing concentrations of the EP1 receptor agonist. The responses displayed were obtained 45–50 min after the application of each new concentration. Each stimulus lasted 15 sec.

tially, this neuron had its receptive field only at the knee (Fig. 1*A*, left, black area), and it responded to noxious pressure onto the knee, and only a few action potentials were evoked by innocuous pressure to the knee. Figure 1*B* shows the number of action potentials per second during application of innocuous and noxious pressure onto the knee (baseline). After the spinal application of the EP1 agonist, the response profile of the neuron changed. Figure 1*B* depicts the last responses after the application of the different concentrations of the agonist (recorded 45–50 min after administration). With increasing doses of the EP1 agonist ONO-DI-004, the responses to innocuous and noxious pressure applied to the knee showed a significant increase. Additionally, the neuron started to respond to ankle stimulation, and finally, after 1 ng/μl ONO-DI-004, even pressure to the paw evoked responses of the neuron (Fig. 1*A*,*B*). Thus, the EP1 receptor agonist induced a hypersensitivity pattern in this neuron that is usually seen when inflammation develops in the knee joint.

Agonists at the EP1 (ONO-DI-004), EP2 (butaprost), or EP4 (ONO-AE1-329) receptor dose-dependently increased the responses of the neurons to knee stimulation (Fig. 2). After having established the baseline with the vehicle on the cord, increasing doses of the particular agonist were administered to the spinal cord surface at intervals of 50 min. The columns in Figure 2*A* show the average increase above baseline (set to zero) of the responses of neurons tested to innocuous and noxious pressure applied to the knee in the last 25 min after the spinal application of increasing doses of the EP receptor agonists. Responses to innocuous pressure applied to the knee after the EP1 receptor agonist increased from an average baseline of 46 $\pm$ 21 impulses (imp)/15 sec (mean $\pm$ SEM) by 4 $\pm$ 3 imp/15 sec at 0.1 ng/μl, 38 $\pm$ 21 imp/15 sec at 1 ng/μl, 51 $\pm$ 19 imp/15 sec at 10 ng/μl, and 79 $\pm$ 25 imp/15 sec at 100 ng/μl, and the increase was significant at the dose of 1 ng/μl ($p < 0.05$; Wilcoxon matched pairs signed rank test). The responses to noxious pressure applied to the knee increased from a baseline of 153 $\pm$ 36 imp/15 sec by 38 $\pm$ 25 imp/15 sec at 0.1 ng/μl, 72 $\pm$ 28 imp/15 sec at 1 ng/μl, 147 $\pm$ 59 imp/15 sec at 10 ng/μl, and 192 $\pm$ 72 imp/15 sec at 100 ng/μl, and the increase was significant at the dose of 1 ng/μl ($p < 0.05$; Wilcoxon matched pairs signed rank test). The baseline in the experiments with the EP2 receptor agonist was 19 $\pm$ 11 imp/15 sec for innocuous pressure, and responses increased by 6 $\pm$ 5 imp/15 sec (0.1 ng/μl), 57 $\pm$ 17 imp/15 sec (1 ng/μl), and 83 $\pm$ 37 imp/sec (100 ng/μl). For noxious pressure applied to the knee, the baseline was 243 $\pm$ 98 imp/15 sec, and responses increased by 27 $\pm$ 26 imp/15 sec (0.1 ng/μl), 169 $\pm$ 44 imp/15 sec (1 ng/μl), and 163 $\pm$ 71 imp/15 sec (100 ng/μl). Increases were significant at 0.1 ng/μl ($p < 0.05$; Wilcoxon matched pairs signed rank test). When the EP4 receptor agonist was tested, the baseline response to innocuous pressure was 16 $\pm$ 11 imp/15 sec, and responses increased by 15 $\pm$ 12 imp/15 sec (1 ng/μl), 43 $\pm$ 33 imp/15 sec (10 ng/μl), and 110 $\pm$ 68 imp/15 sec (100 ng/μl), and the increase was significant at 100 ng/μl ($p < 0.05$). Responses to noxious pressure (baseline response, 183 $\pm$ 76 imp/15 sec) increased by 164 $\pm$ 114 imp/15 sec (1 ng/μl), 235 $\pm$ 174 imp/15 sec (10 ng/μl), and 327 $\pm$ 219 imp/15 sec (100 ng/μl), and the increase was significant at 1 ng/μl ($p < 0.05$; Wilcoxon matched pairs signed rank test).

The curves in Figure 2*B* show the time course of the effects of the agonists on the responses to knee stimulation and the concomitant increases over baseline of the responses to mechanical stimulation of the ankle (for baseline values, see the legend to Fig. 2). Because not all neurons with knee input had a receptive field in the ankle and paw (neither before nor after the administration of the agonists), the number of neurons that are displayed in Figure 2 for ankle stimulation is smaller than the number of neurons with knee stimulation in some cases. An expansion of the receptive field toward the ankle and/or paw (Fig. 1) was found in three neurons after the EP1 agonist and in three neurons after the EP2 agonist (these neurons are included in Fig. 2). The facilitation of the responses also included the lowering of thresholds. A reduction in threshold causing a neuronal response not only to noxious pressure but also to innocuous pressure applied to the ankle was seen in three neurons after the EP1 agonist, in three neurons after the EP2 agonist, and in one neuron after the EP4 agonist. The agonist at the EP3α receptor had no significant effect on the responses (but see below and Fig. 4). In the absence of agonists, the responses remained stable (see below).

After application of the EP1 agonist, spontaneous activity increased from 0.52 to 2.4 Hz in one neuron, and after the application of the EP4 agonist, spontaneous activity increased from 0.07 to 1.5 Hz in one neuron. In the other neurons, the agonists neither induced nor altered ongoing discharges.

Different pattern of effects of EP agonists during peripheral inflammation

When the knee joint is inflamed, the facilitation of the responses to mechanical stimulation by the topical application of PGE_2 to the spinal cord is less pronounced. On average, responses to knee stimulation were less facilitated by PGE_2, and responses to ankle and paw stimulation were not increased after PGE_2 (Vasquez et al., 2001). These data suggested differences in the spinal PGE_2 effects under inflammatory conditions. Therefore, we studied the spinal effects of the EP1–EP4 agonists in rats in which inflammation in the knee joint had been induced 7–11 hr before recording.

Figure 3 shows the responses to noxious and innocuous pressure onto the inflamed joint before and after topical application of agonists at the EP1, EP2, or EP4 receptor (same type of display as in Fig. 2). Under inflammatory conditions, only the spinal application of the EP1 agonist caused a progressive enhancement of the responses to mechanical stimulation, whereas the agonists at the EP2 and EP4 receptor did not significantly influence the responses of the neurons. In experiments with the EP1 agonist, the average baseline response to innocuous pressure applied to the inflamed knee was 247 ± 75 imp/15 sec, and the responses increased by 98 ± 41 imp/15 sec (1 ng/μl), 126 ± 57 imp/15 sec (10 ng/μl), and 124 ± 87 imp/15 sec (100 ng/μl). Responses to noxious pressure (baseline, 770 ± 162 imp/15 sec) increased by 144 ± 62 imp/15 sec (1 ng/μl), 162 ± 68 imp/15 sec (10 ng/μl), and 188 ± 73 imp/15 sec (100 ng/μl). For both stimuli, the increase was significant at 1 ng/μl ($p < 0.05$; Wilcoxon matched pairs signed rank test). In experiments with the EP2 agonist, average baseline responses were 79 ± 30 imp/15 sec (innocuous pressure) and 355 ± 98 imp/15 sec (noxious pressure), but neither changes in responses to innocuous pressure (increases of 16 ± 17 imp/15 sec at 0.1 ng/μl, 56 ± 45 imp/15 sec at 1 ng/μl, and 12 ± 10 imp/15 sec at 100 ng/μl) nor changes in responses to noxious pressure (decrease of 29 ± 21 imp/15 sec at 0.1 ng/μl and 0 ± 40 imp/15 sec at 1 ng/μl and a decrease of 15 ± 43 imp/15 sec at 100 ng/μl) were significant ($p > 0.05$; Wilcoxon matched pairs signed ranked test). Similar data were seen when the EP4 agonist was tested. Average baseline responses were 229 ± 77 imp/15 sec (innocuous pressure) and 602 ± 110 imp/15 sec (noxious pressure). Neither increases in the responses to innocuous pressure (by 38 ± 34 imp/15 sec at 1 ng/μl, 13 ± 41 imp/15 sec at 10 ng/μl, and 31 ± 55 imp/15 sec at 100 ng/μl) nor changes in the responses to noxious pressure (an increase of 61 ± 40 imp/15 sec at 1 ng/μl, a decrease of 6 ± 66 imp/15 sec at 10 ng/μl, and an increase of 16 ± 50 imp/15 sec at 100 ng/μl) reached significance ($p > 0.05$; Wilcoxon matched pairs signed rank test). Spontaneous activity increased in two neurons after application of the EP1 agonist (from 0.1 to 3.6 Hz and from 0.9 to 1.3 Hz), and after the application of the EP4 agonist, spontaneous activity decreased in one neuron (from 0.9 to 0.3 Hz) and increased in a second one (from 7 to 18 Hz).

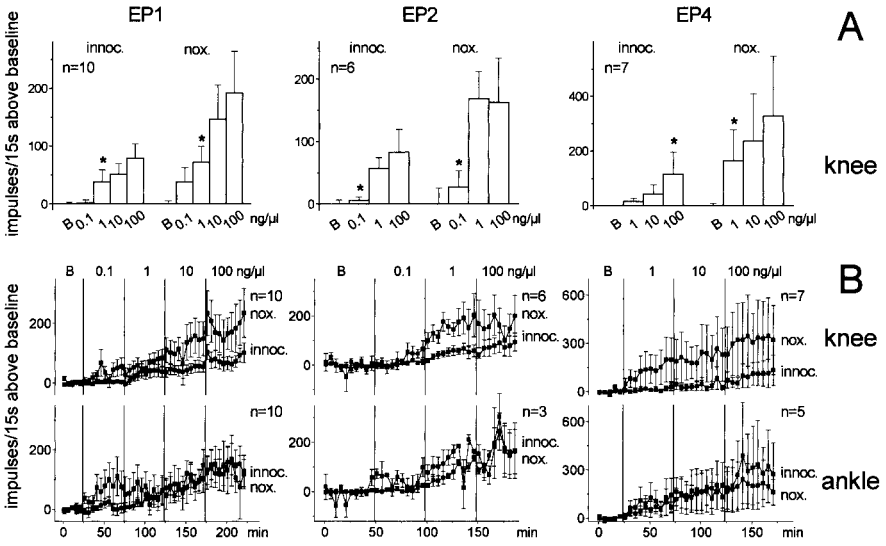

Figure 2. EP1, EP2, and EP4 receptor agonists (ONO-DI-004, butaprost, ONO-AE1 329) induce spinal hyperexcitability. *A*, Columns show the average increase (mean ± SEM) of the responses of neurons during innocuous (innoc.) and noxious (nox.) pressure onto the knee joint during the last 25 min under different concentrations of the agonists. The baseline (B) (absolute values, see below) was set to zero. Asterisks show the lowest concentration of the agonist that caused a significant increase in the response to mechanical stimulation compared with the baseline. *B*, Curves show the time course of the facilitation of the responses to noxious and innocuous pressure applied to the knee joint and to the ankle. The values after drug applications show dose-dependent increases (mean ± SEM) of the responses over B (set to zero) (n = number of neurons). Because not all neurons with responses to knee stimulation showed responses to ankle stimulation, n was lower for ankle stimulation in some cases. The predrug baseline values were as follows (measured as impulses per 15 sec; mean ± SEM): innocuous pressure knee joint, 46 ± 21 (EP1), 19 ± 11 (EP2), 16 ± 11 (EP4); noxious pressure knee joint, 153 ± 36 (EP1), 243 ± 98 (EP2), 183 ± 76 (EP4); innocuous pressure ankle, 32 ± 17 (EP1), 3 ± 2 (EP2), 19 ± 8 (EP4); noxious pressure ankle, 152 ± 60 (EP1), 94 ± 40 (EP2), 169 ± 109 (EP4).

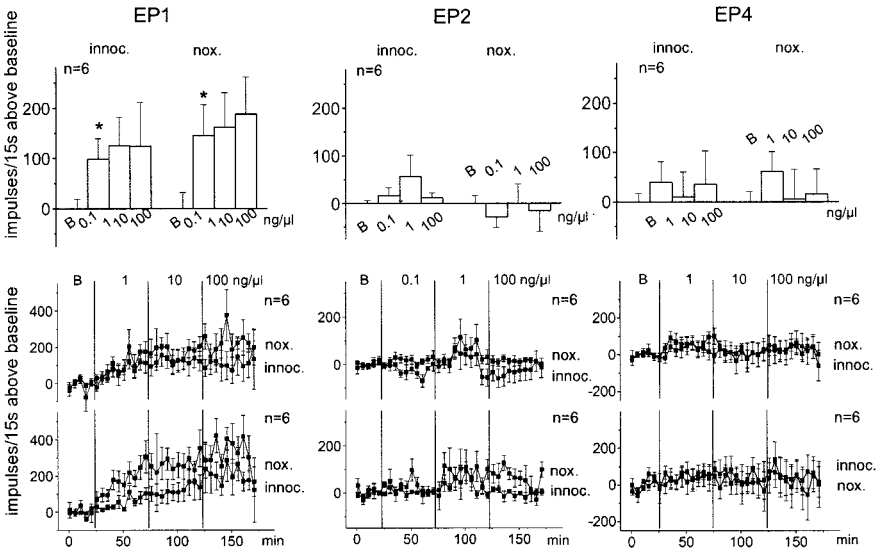

Figure 3. Effects of EP1, EP2, and EP4 receptor agonists under inflammatory conditions. Shown is the same type of display as in Figure 2. Asterisks show the lowest concentration of the agonist that caused a significant increase in the response to mechanical stimulation compared with the baseline. The inflammation in the knee joint was induced 7–11 hr before the recordings. The predrug baseline (B) values were as follows (measured as impulses per 15 sec; mean ± SEM): innocuous (innoc.) pressure knee joint, 247 ± 75 (EP1), 79 ± 30 (EP2), 299 ± 77 (EP4); noxious (nox.) pressure knee joint, 770 ± 162 (EP1), 355 ± 98 (EP2), 602 ± 110 (EP4); innocuous pressure ankle, 27 ± 14 (EP1), 62 ± 50 (EP2), 131 ± 65 (EP4); noxious pressure ankle, 448 ± 163 (EP1), 349 ± 174 (EP2), 423 ± 185 (EP4).

Of particular interest was the effect of the EP3α agonist under normal and inflammatory conditions. ONO-AE-248 is an agonist at the EP3α receptor that is coupled to G_i and decreases cAMP (Zacharowski et al., 1999). When the knee joint was normal, the EP3α agonist did not significantly change the responses of the neurons to mechanical stimulation of the knee joint and the ankle (Fig. 4*A*,*B*, left side, same type of display as in Figs. 2, 3). Responses to innocuous pressure (predrug baseline value, 61 ±

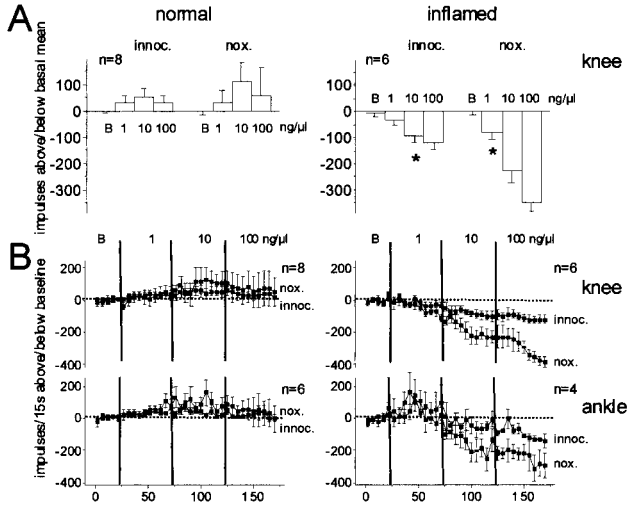

Figure 4. Different effects of the EP3α receptor agonist ONO-AE-248 under normal and inflammatory conditions. *A*, Dose-dependent effects of the EP3α receptor agonist ONO-AE-248 on the responses to innocuous (innoc.) and noxious (nox.) pressure onto the normal knee joint (left) and onto the inflamed knee joint (right). Shown is the same type of display as in Figures 2*A* and 3*A*. Asterisks show the lowest concentration of the agonist that caused a significant increase in the response to mechanical stimulation compared with the baseline. *B*, Time course of the effects of the EP3α receptor agonist on the responses to knee and ankle stimulation when the knee joint was normal (left) or inflamed (right). Shown is the same type of display as in Figures 2*B* and 3*B*. The predrug baseline (B) values were as follows (measured as impulses per 15 sec; mean ± SEM): innocuous pressure knee joint, 61 ± 30 (normal knee), 147 ± 29 (inflamed knee); noxious pressure knee joint, 345 ± 73 (normal knee), 506 ± 35 (inflamed knee); innocuous pressure ankle, 35 ± 18 (normal knee), 238 ± 54 (inflamed knee); noxious pressure ankle, 159 ± 58 (normal knee), 520 ± 114 (inflamed knee).

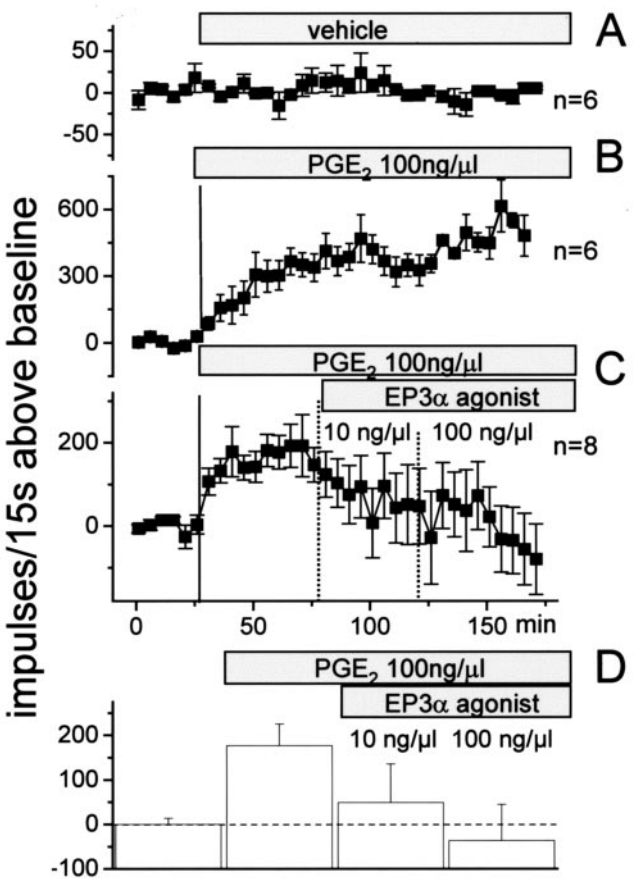

Figure 5. Effect of the EP3α agonist ONO-AE-248 on PGE_2-induced spinal hyperexcitability. *A*, No change in the responses to noxious pressure onto the normal knee when only vehicle solution was on the spinal cord. *B*, Long-term increase in the responses to noxious pressure onto the normal knee during topical application of PGE_2 to the cord surface. *C*, Facilitation of the responses to noxious pressure by PGE_2 and partial reversal of this effect by coadministration of the EP3α agonist at two doses. *D*, Display of the average increase in the responses to noxious pressure above baseline 26–50 min after PGE_2 (second column), 26–50 min after PGE_2 plus 10 ng/µl ONO-AE-248 (third column), and 26–50 min after PGE_2 plus 100 ng/µl ONO-AE-248 (fourth column).

30 imp/15 sec) increased by 32 ± 23 imp/15 sec (1 ng/µl), 54 ± 31 imp/15 sec (10 ng/µl), and 32 ± 24 imp/15 sec (100 ng/µl). Responses to noxious pressure (baseline response, 345 ± 73 imp/15 sec) increased by 33 ± 43 imp/15 sec (1 ng/µl), 112 ± 72 imp/15 sec (10 ng/µl), and 61 ± 103 imp/15 sec (100 ng/µl), but none of the changes in the responses were significant ($p > 0.05$; Wilcoxon matched pairs signed rank test). However, when the knee joint was inflamed, the EP3α agonist reduced the responses to knee stimulation significantly at a dose of 10 ng/µl (innocuous pressure) and 1 ng/µl (noxious pressure) (Fig. 4, right side). Responses to innocuous pressure to the inflamed knee (predrug baseline, 147 ± 29 imp/15 sec) decreased by 31 ± 19 imp/15 sec (1 ng/µl), 93 ± 27 imp/15 sec (10 ng/µl), and 119 ± 25 imp/15 sec (100 ng/µl), and this was significant at a dose of 10 ng/µl ($p < 0.05$; Wilcoxon matched pairs signed rank test). Responses to noxious pressure onto the inflamed knee (predrug baseline, 506 ± 35 imp/15 sec) were decreased by 76 ± 28 imp/15 sec (1 ng/µl), 225 ± 47 imp/15 sec (10 ng/µl), and 349 ± 32 imp/15 sec (100 ng/µl), and the reduction was significant at 1 ng/µl ($p < 0.05$; Wilcoxon matched pairs signed rank test). The time course of the effect and changes in responses to ankle stimulation are shown in Figure 4*B*. Thus, the activation of the EP3α receptor counteracted the hyperexcitability presumably caused, at least partly, by the inflammation-induced release of PGE_2 and its action on EP1, EP2, and EP4 receptors. Spontaneous activity was reduced in two neurons after application of the EP3α agonist (from 0.2 to 0.05 Hz and from 1.8 to 1 Hz).

Interaction between PGE_2 and the EP3α receptor agonist

As mentioned previously, activation of EP2 and EP4 receptors leads to an enhancement of cAMP, whereas EP3α receptor activation decreases cAMP. Therefore, we studied whether the overall facilitatory effect of PGE_2 can be reduced when the EP3α receptor is stimulated. Figure 5 shows the interaction of PGE_2 and the EP3α receptor agonist on the responses of neurons to noxious pressure. When only the vehicle was applied (Fig. 5*A*) ($n = 6$ neurons), the responses to noxious pressure onto the knee (baseline response, 115 ± 33 imp/15 sec, set to zero in Fig. 5*A*), ankle, and paw (data not shown) remained stable for the entire recording period. When only PGE_2 was applied to the spinal cord, the responses to noxious pressure showed a gradual and persistent increase (Fig. 5*B*) ($n = 6$ neurons from six experiments). The baseline response (set to zero in Fig. 5*B*) was 205 ± 55 imp/15 sec, and responses increased by 325 ± 44 imp/15 sec (26–50 min after PGE_2), 356 ± 17 imp/15 sec (76–100 min after PGE_2), and 475 ± 78 imp/15 sec (126–150 min after PGE_2). The increase in the responses was already significant in the period 1–25 min after PGE_2 application ($p < 0.05$; Wilcoxon matched pairs signed rank test). When PGE_2 was administered first, the subsequent coadministration of the EP3α agonist caused a significant and dose-dependent attenuation of the effect of PGE_2 ($n =$ 8 neurons from eight experiments). Figure 5*C* shows the time course of the responses, and Figure 5*D* displays the averaged

values. In these experiments, the predrug baseline response was 430 ± 379 imp/15 (set to zero in Fig. 5*C*,*D*), the response increased on average by 177 ± 48 imp/15 sec between 26–50 min after PGE_2 (Fig. 5*D*, second column). The increase was already significant in the period 1–25 min after PGE_2. The average response between 26 and 50 min after coadministration of PGE_2 and 10 ng/μl ONO-AE-248 was 49 ± 86 imp/15 sec above the initial baseline (Fig. 5*D*, third column), and the average response between 26 and 50 min after coadministration of PGE_2 and 100 ng/μl ONO-AE-248 was −36 ± 80 imp/15 sec compared with the initial baseline (Fig. 5*D*, fourth column). For statistical evaluation, we compared the values from the last 25 min after PGE_2 alone with the values from the last 25 min after PGE_2 (100 ng/μl) plus ONO-AE-248 (dose, 10 ng/μl) and after PGE_2 plus ONO-AE-248 (dose, 100 ng/μl). Already, after the low dose of the EP3α agonist, the responses fell significantly from the level of the responses after PGE_2 alone ($p < 0.05$; Wilcoxon matched pairs signed rank test).

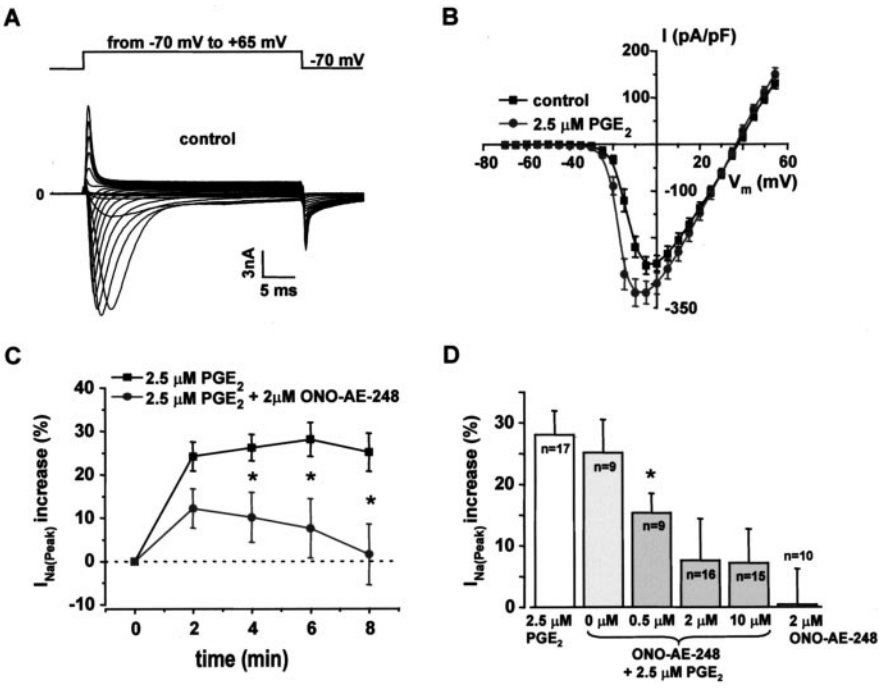

Figure 6. Effects of PGE_2 or PGE_2 plus the EP3α agonist on TTX-resistant Na^+ currents in dorsal root ganglion cells. *A*, Inward and outward currents in a DRG neuron evoked by stepwise depolarization from −70 to +65 mV in increments of 5 mV at intervals of 1.2 sec. *B*, Averaged current evoked by stepwise depolarization from −70 to −65 mV before PGE_2 application (squares) and 6 min after PGE_2 application (circles) ($n = 17$ neurons). Filled symbols show mean ± SEM. *C*, Average increase in the peak inward Na^+ current after PGE_2 alone or after PGE_2 plus the EP3α agonist at 2, 4, 6, and 8 min after application of the compound(s). The asterisk indicates a significant difference between effects of PGE_2 alone and PGE_2 plus ONO-AE-248. *D*, Average increase in peak inward Na^+ currents at 6 min after application of PGE_2 alone, PGE_2 plus vehicle of the EP3α agonist (0 μM ONO-AE-248 plus 2.5 μM PGE_2), PGE_2 plus different concentrations of the EP3α agonist, or the EP3α agonist alone (last column). Peak currents before drug application were similar in all experimental groups. The asterisk indicates the lowest concentration of the EP3α agonist that caused a significant reduction in the effect of PGE_2.

To further substantiate potential opposite effects of PGE_2 and the EP3α agonist, we assessed the interaction between PGE_2 and the EP3α receptor agonist in isolated DRG neurons. It is well established that PGE_2 enhances TTX-resistant Na^+ currents in small- to medium-sized DRG neurons, many of which are nociceptive (Gold et al., 1998, 2002; McCleskey and Gold, 1999). TTX-resistant Na^+ channels are involved in the action potential of DRG neurons (Blair and Bean, 2002; Fang et al., 2002), and they were demonstrated in DRG neurons, in peripheral axons and spinal processes of DRG neurons (Jeftinija, 1994; Quasthoff et al., 1995; Amaya et al., 2000), and in the dorsal horn, in which C-fiber afferent terminals and other afferents end to form synapses (Amaya et al., 2000). Here we tested whether PGE_2-evoked increases in TTX-resistant Na^+ currents are influenced by coadministration of the EP3α receptor agonist. Voltage-dependent Na^+ currents were tested in small- to medium-sized cultured DRG neurons [average diameter, 31.6 ± 4.5 μm (mean ± SD); $n = 76$] using whole-cell patch-clamp recordings. Figure 6*A* shows typical voltage-activated Na^+ currents in a DRG neuron. Figure 6*B* displays the *I–V* curves of 17 neurons before PGE_2 application and 6 min after bath application of PGE_2. During PGE_2 application, peak inward current densities increased significantly (paired *t* test), and a voltage shift of the peak current in the hyperpolarizing direction was observed, similar to previous studies (Gold et al., 1998, 2002). Figure 6*C* shows the average increase in the peak inward current densities in these 17 neurons 2, 4, 6, and 8 min after PGE_2 application and also the average increase in peak inward current densities in another 16 neurons in experiments in which PGE_2 was coadministered with the EP3α agonist ONO-AE-248. Both the administration of PGE_2 alone and the administration of PGE_2 plus the EP3α agonist produced a significant increase in the peak inward current densities at 2 min after application (paired *t* test). However, the effect of PGE_2 alone was significantly larger and more persistent than that of PGE_2 plus the EP3α agonist. The values were significantly different at 4, 6, and 8 min (Mann–Whitney *U* test). The bars in Figure 6*D* show the average changes in the peak inward Na^+ currents at 6 min after application of compounds from all experiments. PGE_2 plus the vehicle of the EP3α agonist (0 μM ONO-AE-248) had the same effect as PGE_2 alone. However, coadministration of PGE_2 plus the EP3α agonist caused smaller increases in the Na^+ currents than PGE_2 alone. Coadministration of 0.5 μM ONO-AE-248 was sufficient to reduce the effect of PGE_2 significantly (Mann–Whitney *U* test). Application of the EP3α receptor agonist alone did not influence Na^+ currents (Fig. 6*D*, last column).

Discussion

This study shows that the spinal application of agonists at the EP1, EP2, and EP4 receptor facilitated the responses of dorsal horn neurons to mechanical stimulation of the normal knee, ankle, and paw just as PGE_2 does (Vasquez et al., 2001). The EP3α receptor agonist had no effect when the knee joint was normal. When the knee joint was inflamed, only the agonist at the EP1 receptor caused an increase in the responses to mechanical stimulation, whereas the agonists at EP2 and EP4 receptors did not alter the responses. However, during inflammation, the spinal administration of the EP3α agonist caused a decrease in the responses to mechanical stimulation of the knee and ankle. Furthermore, the application of the EP3α agonist counteracted the overall facilitatory effect of PGE_2, the agonist at all EP receptors. Such an inhibitory action was also observed when the effect of

PGE_2 on TTX-R Na^+ currents was studied in DRG neurons. These data show differences in the functions of EP receptors in nociceptive spinal processing. Furthermore, the condition of the animal (i.e., normal vs inflamed knee) determines the impact of different EP receptors in nociceptive processing.

Facilitatory effects of agonists at the EP1, EP2, and EP4 receptor

The spinal application of PGE_2 facilitates responses of nociceptive spinal cord neurons to mechanical stimulation of joints (Vasquez et al., 2001) and skin (Turnbach et al., 2002). Importantly, the spinal application of indomethacin significantly attenuates the development of inflammation-evoked spinal hyperexcitability, indicating that spinal effects of endogenous PGE_2 are relevant in the generation of hyperexcitability (Vasquez et al., 2001). Herein the spinal application of EP1, EP2, and EP4 receptor agonists mimicked the effects of spinal PGE_2 application. In the presence of these agonists, the neurons showed: (1) an increased firing to noxious mechanical stimulation, which may lead to primary mechanical hyperalgesia; (2) a decrease in the excitation threshold and thus enhanced or novel responses to innocuous stimulation, which may lead to allodynia; and (3) an expansion of the receptive field, which is typical of central sensitization and may lead to secondary mechanical hyperalgesia (Woolf, 1983; Hylden et al., 1989; Neugebauer and Schaible, 1990; Dougherty et al., 1992). The data suggest, therefore, that these receptors mediate the effect of PGE_2 on the generation of central sensitization. Unfortunately, it is currently not possible to assess the relative contribution of these EP receptors to the process of inflammation-evoked central sensitization, because no specific antagonists are available for the different EP receptors. Animals in which single EP receptors are knocked out are not suitable for this study, because the receptor deficiency could also interfere with the inflammatory process in the joint.

The EP3 receptor has different splice variants. These are coupled either to G_i-proteins that cause a decrease in cAMP or to G_s-proteins that cause an increase in cAMP (Negishi et al., 1995a,b). ONO-AE-248 is an EP3α receptor agonist that is coupled to G_i-proteins and decreases cAMP (Zacharowski et al., 1999). This agonist did not significantly influence the responses to mechanical stimulation of normal joints and paws. It is possible that an agonist at the EP3C receptor could have increased the responses to mechanical stimulation, because EP3C receptors (coupled to G_s-proteins) contribute to PGE_2-mediated sensitization of primary afferents (Southall and Vasko, 2001).

Spinal sensitization was similar regardless of whether the process was set in motion by agonists at the EP1, EP2, or EP4 receptor. Because EP1 and EP4 receptors are located in primary afferent neurons (Oida et al., 1995), and EP2 receptors are located on dorsal horn neurons (Kawamura et al., 1997), the present data suggest that spinal PGE_2 can initiate the process of facilitation by acting at presynaptic and postsynaptic sites. In cultured or native primary afferent neurons, PGE_2 enhances stimulation-evoked substance P and calcitonin gene-related peptide release (Nicol et al., 1992; Andreeva and Rang, 1993; Vasko et al., 1993, 1994; Hingtgen and Vasko, 1994; Southall et al., 1998), and cyclooxygenase inhibitors reduce the release of prostaglandins (Andreeva and Rang, 1993; Vasko et al., 1994). However, PGE_2 excites spinal cord intrinsic neurons directly (Baba et al., 2001) and blocks inhibitory glycinergic neurotransmission in dorsal horn neurons (Ahmadi et al., 2002). Although all of these actions may contribute to the facilitatory effect of PGE_2, the relative importance of these mechanisms in the generation of inflammation-evoked hyperexcitability has not been determined.

Different effects of agonists at EP receptors during peripheral inflammation

After inflammation, only the EP1 agonist facilitated the responses to mechanical stimulation, as in noninflamed animals, but not the EP2 and EP4 agonists. Conspicuously, the EP3α agonist actually reduced the responses to stimulation of the inflamed joint, although it had no effect on responses when the joint was normal. Thus, the net facilitatory effect of spinal PGE_2 should be reduced during inflammation. Indeed, we have shown previously that spinal application of PGE_2 still facilitates responses to mechanical stimulation when the joint is inflamed, but the effect is significantly smaller than in the uninflamed condition (Vasquez et al., 2001).

The EP3α agonist also reduced the enhanced responses of spinal cord neurons to mechanical stimulation that were evoked by spinal application of PGE_2, thus counteracting a PGE_2-mediated effect (Fig. 5). Such an inhibitory action was also observed in patch-clamp recordings from DRG neurons where the EP3α agonist reduced dose-dependently the facilitatory effect of PGE_2 on Na^+ currents but had no effect on Na^+ currents when administered alone. Thus both experimental approaches show the ability of the EP3α agonist to inhibit neuronal responses that have been facilitated either by PGE_2 or by inflammation. Although recordings from DRG neurons were primarily aimed to confirm the inhibitory action of the EP3α agonist in a second approach, the observed effects could nevertheless contribute to the *in vivo* effect of the EP3α agonist, because TTX-resistant Na^+ channels are also expressed in the spinal roots and in spinal terminations of afferent fibers (Jeftinija 1994; Amaya at al., 2000).

Interestingly, changes in the effect on neuronal activity during inflammation were only observed for the agonists at those receptors that are coupled to cAMP signaling, namely the EP2, EP3, and EP4 receptors (Negishi et al., 1995a,b; Narumiya et al., 1999). Generally, elevation of cAMP and actions of protein kinase A are thought to play an important role in spinal nociceptive processes and hyperexcitability (Cerne et al., 1992; 1993; Malmberg et al., 1997; Sluka and Willis, 1997), and thus, the regulation of cAMP seems to be an important mechanism involved in nociception. The following scenario could happen: During development of inflammation in the joint, mediators are released in the spinal cord that cause enhancement of cAMP levels. PGE_2 itself could contribute, because it is released in the spinal cord during inflammation (Yang et al., 1996; Ebersberger et al., 1999), and major effects of PGE_2 are mediated through the activation of adenylate cyclase (Vanegas and Schaible, 2001; Svensson and Yaksh, 2002). When inflammation and central sensitization have developed, PGE_2 activation of EP3 receptors that are coupled to G_i-proteins can now reduce cAMP.

Some suggestions can be made as to why the receptor activation is different under normal and inflammatory conditions. In rats with inflammation, the spinal application of exogenous EP2 and EP4 receptor agonists may be ineffective, because previously released endogenous PGE_2 has already triggered cellular effects that facilitate responses to mechanical stimulation. In fact, we had observed previously that only the first spinal application of PGE_2 in an experiment facilitated responses to mechanical stimulation but not a second application of PGE_2 (Vasquez et al., 2001). The molecular basis for this phenomenon is not known at present (ceiling effect? receptor desensitization? receptor downregulation?). In contrast, an EP3 receptor that is coupled to G_i-

proteins and reduces cAMP after stimulation should show its effect when the cAMP level is elevated. Indeed, the facilitatory role of PGE_2 on the responses of spinal cord neurons to mechanical stimulation was rapidly reduced after coadministration of the EP3α receptor agonist (Fig. 5). Antagonistic effects of the EP3α agonist on currents in DRG neurons were also seen within a few minutes. Because the effect of PGE_2 on Na^+ currents involves cAMP (England et al., 1996; Gold et al., 1998, 2002), these findings also support the idea that cAMP plays a major role.

In summary, this study provides insights into effects of the different PGE_2 receptors in the spinal cord. Most intriguing is that an inhibitory EP3 receptor comes into play when the joint is inflamed. Although spinal PGE_2 initially facilitates nociceptive processing during development of the inflammatory process, EP3 receptors with inhibitory actions could limit the increase in sensitivity and the facilitatory effect of PGE_2 once a certain state of hyperexcitability is established. Indeed, central sensitization usually reaches a maximum several hours after induction of inflammation (Neugebauer at al., 1993). Through activation of different EP receptors, spinal PGE_2 has pronociceptive effects as well as effects that may reduce nociceptive processing, but this depends on the condition of the animal. Additional studies should be conducted to clarify whether splice variants of EP3 receptors with inhibitory effects could become a target for analgesic treatment.

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