Intrathecal Administration of Proteinase-Activated Receptor-2 Agonists Produces Hyperalgesia by Exciting the Cell Bodies of Primary Sensory Neurons

Kwai A. Alier, Jaclyn A. Endicott, Patrick L. Stemkowski, Nicolas Cenac, Laurie Cellars, Kevin Chapman, Patricia Andrade-Gordon, Nathalie Vergnolle, and Peter A. Smith

Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada (K.A.A., J.A.E., P.L.S., P.A.S.); Department of Pharmacology and Therapeutics, University of Calgary, Calgary, Alberta, Canada (N.C., L.C., K.C., N.V.); and Johnson & Johnson Pharmaceutical Research and Development, Spring House, Pennsylvania (P.A.-G.)

Received July 24, 2007; accepted October 3, 2007

ABSTRACT

Proteinase-activated receptors (PARs) are a family of G-protein-coupled receptors that are activated by endogenous serine proteinases that cleave the N-terminal domain of the receptor unmasking a “tethered ligand” sequence. Trypsin and other agonists at PAR2 act on peripheral nerves to augment the transfer of nociceptive information. We tested whether PAR2 agonists also exert a spinal pronociceptive effect by i.t. administering the selective ligand, Ser-Leu-Ile-Gly-Arg-Leu-NH₂ (SLIGRL). This produced thermal and mechanical hyperalgesia in rats and mice and augmented mechanical and thermal hyperalgesia seen in the formalin inflammatory pain test. Effects of SLIGRL were abrogated in PAR2-deficient mice and were not seen with the inactive control peptide, Leu-Arg-Gly-Ile-Leu-Ser-NH₂. Surprisingly, electrophysiological studies, using whole-cell recording from rat substantia gelatinosa neurons, failed to demonstrate an increase in excitatory transmission or neuronal excitability following treatment with SLIGRL or trypsin. In fact, the actions of trypsin were consistent with a decrease in dorsal horn excitability. SLIGRL and trypsin did, however, depolarize and increase the excitability of large, medium and small primary afferent, dorsal root ganglion neurons. The effects were associated with an increase in conductance at hyperpolarized potentials and a decrease in conductance at depolarized potentials. PAR2-like immunoreactivity was found in DRG but not in spinal dorsal horn. These results suggest that activation of DRG neuron cell bodies may account for the pronociceptive actions of i.t. applied PAR2 agonists. They also imply that pathophysiological release of PAR2-activating proteases in the vicinity of DRG neurons may produce profound effects on nociceptive processing in vivo.

Proteinase-activated receptors (PARs) are a novel family of G-protein-coupled receptors (Hoogerwerf et al., 2001; Steinhoff et al., 2005) that are found in various tissues throughout the body, including the central and peripheral nervous systems (Noorbakhsh et al., 2003; Vergnolle et al., 2003; Steinhoff et al., 2005). They have been implicated in the activation and/or modulation of nociceptive pathways (Vergnolle et al., 2001; Fiorucci and Distrutti, 2002; Cenac and Vergnolle, 2005). PARs are activated by endogenous serine proteinases that cleave the N-terminal domain of the receptor unmasking a “tethered ligand” sequence (Hoogerwerf et al., 2001; Vergnolle et al., 2003; Steinhoff et al., 2005). This tethered ligand subsequently binds to an active site within the receptor leading to activation and a downstream response (Hoogerwerf et al., 2001; Steinhoff et al., 2005). Activating enzymes are derived from cellular sources (trypsin and mast cell tryptase) as well as humoral sources (thrombin and factor Xa) and are released or generated during trauma and/or inflammation (Hoogerwerf et al., 2001). Trypsin may also be released from microglia following traumatic damage to the central nervous system (Noorbakhsh et al., 2003). So far, four types of PAR receptors, PAR₁, PAR₂, PAR₃, and PAR₄, have been identified (Hollenberg, 1999; Steinhoff et al., 2005). These can be distinguished functionally on the basis of the agonist effectiveness of various serine proteases. For example, thrombin selectively activates PAR₁ but not PAR₂, whereas PAR₃ is less sensitive to trypsin than PAR₂ (for review, see Vergnolle et al., 2003). Moreover, synthetic pep-
tides that mimic the structure of the tethered ligand also specifically activate various PAR subtypes. For example, Ser-Leu-Ile-Gly-Arg-Leu-NH₂ (SLIGRL) selectively activates PAR₂ (Vergnolle et al., 2001).

Intraplantar injection of PAR₂ agonists in rats causes marked and sustained hyperalgesia. This and other work strongly implicates PAR₂ in peripheral inflammatory pain (Hoogerwerf et al., 2001; Vergnolle et al., 2001; Fiorucci and Distrutti, 2002; Dai et al., 2004). Although PAR₂ agonists have also been reported to release substance P (SP) and calcitonin gene-related peptide from peripheral and spinal cord tissues (Vergnolle et al., 2001) and to augment capsaicin-induced neuropeptide release in spinal cord (Amadesi et al., 2004), the possibility that PAR₂ agonists act directly within the spinal cord to produce pronociceptive effects remains to be established. We have therefore directly applied PAR₂ agonists to the spinal cord via the i.t. route in vivo and examined their effects in various pain models. We have used whole-cell recording from DRG and dorsal horn (substantia gelatinosa) neurons to elucidate the underlying mechanism of the effects that we observed.

Materials and Methods

All experimental protocols were approved by the Health Sciences Animal Welfare Committee of the University of Alberta and the Animal Care Committee of the University of Calgary. They were performed in accordance with the guidelines established by the Canadian Council on Animal Care and the Committee for Research and Ethical Issues and are in concordance with the guidelines of the International Association for the Study of Pain.

Behavioral Experiments: Intrathecal and Intraplantar Injections. Male Wistar rats (175–200 g) were obtained from Charles River Laboratories (Montreal, Quebec, Canada). The rats had free access to food and water and were housed under constant temperature and light cycle. PAR₂-deficient mice and wild-type littermates used in the control experiments were originally obtained from Johnson and Johnson Pharmaceutical (Springhouse, PA). Both were bred using three von Frey filaments of different size and bending forces as described previously (Amadesi et al., 2004). The filaments were applied for 1 to 2 s, a minimum of three times randomly among the tested animals. A score was assigned based on the animal’s response: 0, no movement; 1, removal of the paw; and 2, removal of the paw and vocalization, licking or holding of the paw. Mechanical nociceptive score was expressed as a percentage of the maximal score that would be obtained for the number of tests at each time-point. Before all experiments, baseline measures of nociception were recorded (time 0) for all assays, and nociception was then measured again at different time-points after guideline rts.

Thermal and mechanical hyperalgesia were defined as a significant increase in withdrawal latency and nociceptive threshold or score, respectively. Mechanical allodynia was observed in mice when nociceptive score values > 0 were recorded using the smallest size von Frey filament (3.61), which under basal conditions did not provoke any nociceptive response.

Electrophysiological Experiments on Dorsal Root Ganglion Neurons. Sprague-Dawley rats, aged 18 to 26 days old, were anesthetized with 1.5 g/kg i.p. urethane. Laminectomy was performed under sterile conditions with carbogen-saturated dissection solution. The fourth, fifth, and sixth lumbar dorsal root ganglia (DRGs) were harvested with the aid of a dissection microscope. Dissection solution used throughout the DRG removal process contained 118 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO₄, 1.2 mM NaH₂PO₄, 5 mM MgCl₂, 25 mM H-glucose, 26 mM NaHCO₃, and 1.5 mM CaCl₂. DRGs were then enzymatically dissociated while shaking in a water bath at 35°C for 40 min. Digestion media was prepared in 6 ml of Dulbecco’s modified Eagle’s medium (DMEM) with GlutaMAX (Invitrogen, Carlsbad, CA) containing 0.5 mg/ml trypsin (Sigma), 1 mg/ml collagenase (Sigma), and 0.1 mg/ml deoxyribonuclease I (Sigma). Soybean trypsin inhibitor (Worthington Biochemicals, Lakewood, NJ), at a concentration high enough to neutralize 2 times the amount of trypsin present in the digestion medium, was dissolved in 1.5 ml of DMEM and added to the digestion medium after the 40 min of incubation. After trituration, the dissociated cells were plated in sterile 3.5-cm polystyrene culture dishes (Nalgene Nunc International, Rochester, NY). Cells were allowed to adhere to culture dishes for 2 min before 2 ml of culture medium was added to each dish. The culture medium used was made up in DMEM, including: 10% fetal bovine serum (Invitrogen), 10% horse serum (Invitrogen), 100 units/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 5 mg/ml mouse nerve growth factor 2.5× (Alomone Labs, Jerusalem, Israel). Culture medium was changed every 48 h. Experiments were done at room temperature (20°C) on neurons that had been maintained for 2 to 4 days in a tissue culture incubator at 37.0°C with 6% CO₂. Standard whole-cell recordings were made using an Axoclamp 2A amplifier in current-clamp mode or an Axopatch 1B amplifier in voltage-clamp mode. Borosilicate glass patch electrodes had a DC resistance of 4 to 6 MΩ for action potential (AP) recordings. Data were acquired and analyzed using pClamp 5.5 software, and final figures were produced using Origin 7.0 (OriginLab Corporation, Northampton, MA). The cells were continuously superfused with carbogen-saturated extracellular solution. For all recordings, the external solution contained the following: 127 mM NaCl, 2.5 mM KCl, 1.2 mM NaH₂PO₄, 1.3 mM MgSO₄, 26 mM NaHCO₃, 25 mM H-glucose, and 2.5 mM CaCl₂. Internal pipette solution contained: 130 mM t-glutamic acid, 10 mM EGTA, 4 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM HEPES, and 2 mM CaCl₂. The pH was adjusted to 7.2 with 1 M KOH. Single action potentials were generated using 20-ms depolarizing current pulses. In current-clamp recordings, changes in conductance were measured by means of 200-ms, 0.1-nA hyperpolarizing current pulses applied through the recording electrode at 2 Hz. Conductance changes in voltage clamp were measured from I-V relationships derived from slow ramp commands from −110 to +40 mV. Excitability was monitored by injecting depolarizing current ramps and observing the latency to discharge of the first action potential on the ramp. Drugs were applied by a rapid superfusion system constructed from 0.8-mm-diameter polyamide
Electrophysiological Experiments on Spinal Cord Slices. These were carried out according to our previously published methods (Moran et al., 2004; Balasubramanyan et al., 2006). In brief, male Sprague-Dawley rats (15–42 days old) were deeply anesthetized with urethane (1.5 g/kg i.p.), and a dorsal incision was made from the base of the tail to the skull. The incision site was immediately irrigated with −2 mL of ice-cold oxygenated (95% O2, 5% CO2) dissection solution containing 118 mM NaCl, 2.5 mM KCl, 26 mM NaHCO3, 1.3 mM MgSO4, 1.2 mM NaH2PO4, 1.5 mM CaCl2, 5 mM MgCl2, 25 mM d-glucose, and 1 mM kynurenic acid. A laminctomy was performed extending from the thoracic to the sacral portions of the spinal cord. The spinal cord was removed from the vertebral column, and the ventral roots and all dorsal roots except L4 to L5 were cut. Transverse slices (300–500 μm) were cut using a Vibratome (TPI, St. Louis, MO). Slices were incubated in a holding chamber (Sakmann and Stuart, 1995) at 36°C for 1 h and subsequently stored at room temperature (−22°C) in oxygenated dissection solution (see above, without 1 mM kynurenic acid).

Slices were placed in a circular glass-bottomed recording chamber and held in place with a U-shaped platinum wire frame with attached parallel nylon fibers. They were superfused at room temperature (−22°C) with 95% O2, 5% CO2 saturated artificial cerebrospinal fluid, which contained 127 mM NaCl, 2.5 mM KCl, 1.2 mM NaH2PO4, 26 mM NaHCO3, 1.3 mM MgSO4, 2.5 mM CaCl2, and 25 mM d-glucose, pH 7.4. Spinal cord slices were viewed with a Zeiss Axioskop FS upright microscope (Carl Zeiss GmbH, Jena, Germany) equipped with infrared differential interference contrast optics.

Whole-cell recordings from visually identified substantia gelatinosa neurons were made with an NPI SEC 05L amplifier (NPI Electronic, Tamm, Germany) in discontinuous single-electrode voltage-clamp or bridge-balance current-clamp mode. Switching frequencies were typically between 30 and 40 kHz. Signals were digitized between 5 and 20 kHz and filtered at 1 kHz. Patch pipettes were pulled from thin-walled borosilicate glass (TW-150F-4; WPI, Sarasota, FL). Pipettes for recording APs had resistances of 5 to 10 MΩ when filled with an internal solution containing 130 mM potassium gluconate, 1 mM MgCl2, 2 mM CaCl2, 10 mM HEPES, 10 mM EGTA, 4 mM Mg-ATP, and 0.3 mM Na-GTP, pH 7.2 (290–300 mOsm). For some experiments recording synaptic currents, a Cs+-based internal solution containing 140 mM CsCl, 5 mM HEPES, 10 mM EGTA, 2 mM CaCl2, 2 mM Mg-ATP, and 0.3 mM Na-GTP, pH 7.2 (290–300 mOsm) was used.

Data for I-V plots were obtained in voltage-clamp mode using incremental 10-mV, 800-ms commands. Current values were measured from the close-to-steady-state values attained at the end of these commands. As in DRG experiments, the excitability of substantia gelatinosa neurons was monitored by injecting depolarizing current ramps (60 pA/s) and observing the latency to discharge of the first action potential on the ramp (see also Balasubramanyan et al., 2006).

Spontaneous EPSCs (sEPSCs) were recorded at −70 mV, digitized at 5 kHz, and filtered at 1 kHz. Data were acquired for 3 min before, and 5 or 10 min after, superfusion of tryptsin or peptides. Mini Analysis Program software (Synaptosoft, Decatur, GA) was used to distinguish sEPSCs and spontaneous inhibitory postsynaptic currents from the baseline noise and to prepare cumulative probability plots or frequency histograms. Data were only included in the analysis if the series resistance was below 25 MΩ and did not change by >20% during the course of an experiment. Spontaneous post synaptic currents were detected automatically using an amplitude threshold of 10 pA and an area threshold of 15 femtocolombs. All detected events were then re-examined and visually accepted or rejected based on subjective visual examination. The Mini Analysis Program was used to further analyze the data and to generate figures. Approximately 50 events were used for analysis before and after agonist superfusion. Whenever possible, the first 50 events after 1 min of acquisition were used. In some cells, however, spontaneous activity was limited, so the whole 3-min acquisition period was used to provide data.

Immunohistochemistry. To localize PAR2 in DRG and spinal cord sections, rats were anesthetized with sodium pentobarbital (200 mg/kg i.p.) and transcardially perfused with 4% paraformaldehyde in 100 mM PBS, pH 7.4. Spinal cord and DRG (T10-L5) were fixed overnight at 4°C, placed in 25% sucrose in PBS for 24 h at 4°C, embedded in orinthine carbamyl transferase compound (Miles, Elkhart, IN), and sectioned at 25 μm. A section of DRG neurons and spinal cord was adjacent fixed on each slide. Sections were washed in PBS containing 1% normal growth serum, 1% bovine serum albumin, and 0.3 to 0.5% Triton X-100 and incubated with primary antibodies to PAR2 (goat; Santa Cruz Biotechnology, Santa Cruz, CA, 1:200; or B5 antibody, 1:200, a generous gift from Dr. M.D. Hollenberg, University of Calgary) overnight at 4°C. Sections were washed and incubated with secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes, Invitrogen Canada Inc., Burlington, ON, Canada; 1:1000, room temperature, 2 h). Sections were washed and mounted in Prolong (Molecular Probes, Eugene, OR).

As a control for PAR2 specificity, the primary antisera was preincubated with the peptide used for immunization (10 μM) for 24 h at 4°C before staining. Confocal images were acquired using a Zeiss LSM-510 META confocal microscope using 10× and 20× objectives in the inverted configuration. For all confocal images, a regular phase transmission image was obtained. Images of stained and control cells were collected and processed identically.

Drugs and Chemicals. The PAR2-activating peptide, SLIGRL, and the inactive control peptide, LRGILS, were obtained from the Peptide Synthesis Facility of the University of Calgary (Calgary, Alberta, Canada). The composition and purity of peptides were confirmed by HPLC analysis. Formalin was purchased from VWR (Mississauga, ON, Canada). All other chemicals were from Sigma (Oakville, ON, Canada). SLIGRL and LRGILS were dissolved in external solution that contained 0.1% bovine albumin (Sigma). Tryptsin was also dissolved in external solution and molarities calculated assuming a molecular mass of 24 kDa.

Statistics. All data are presented as mean ± S.E.M. Statistical significance in electrophysiological experiments was assessed by the Student’s unpaired t test after passing a normality test. The Kolmogorov-Smirnov two-sample test was used to compare distributions of amplitudes and interevent intervals in neurons in acute slices. The withdrawal latency, nociceptive threshold, and nociceptive score data were analyzed using a one-way analysis of variance followed by a Dunnett’s test. With all statistical analyses, distributions were considered statistically significant if p < 0.05.

Results

Intrathecal Injection of PAR2 Agonists Causes Alldynia and Hyperalgesia. Intrathecal injection of the PAR2 agonist SLIGRL, but not the control peptide LRGILS, caused a decrease in withdrawal latency in response to a thermal stimulus, characteristic of thermal hyperalgesia both in rats (Fig. 1A) and in mice (Fig. 2A). Likewise, i.t. injection of SLIGRL, but not LRGILS, caused a significant decrease in nociceptive threshold in rats (Fig. 1B) and a significant increase in nociceptive score in mice (Fig. 2, B and C). This effect of the PAR2 agonist is characteristic of mechanical hyperalgesia. Although mice did not respond to the smallest size von Frey filament (3.61) in basal conditions, the PAR2 agonist SLIGRL, but not the control peptide LRGILS, injected i.t., caused a significant increase in nociceptive score characteristic of mechanical allodynia (Fig. 2B). The effects of SLIGRL on withdrawal latency and nociceptive score were...
completely abolished in PAR2-deficient mice, demonstrating the specificity of SLIGRL as a PAR2 agonist (Fig. 2, A–C).

**Intrathecal Injection of PAR2 Agonists Enhances Peripherally Induced Inflammatory Allodynia and Hyperalgesia.** Intraplantar injection of formalin caused thermal and mechanical hyperalgesia characterized by a decrease in withdrawal latency in response to a thermal stimulus compared with basal measurements (Fig. 3A) and an increased nociceptive score in response to von Frey filament application (Fig. 3C). In addition, formalin intraplantar injection caused mechanical allodynia, as measured by a nociceptive response to the smallest size von Frey filament (3.61) (Fig. 3B). Both thermal and mechanical hyperalgesia as well as mechanical allodynia induced by formalin were enhanced by i.t. injection of the PAR2 agonist SLIGRL compared with the effect of the control peptide LRGILS, as observed by a larger decrease in withdrawal latency and enhanced nociceptive scores to von Frey filament exposure (Fig. 3).

**Effects of Trypsin and PAR2 Agonists on Substantia Gelatinosa Neurons.** To understand the mechanism whereby i.t. applied PAR2 agonists facilitate nociception, we examined the effects of trypsin, SLIGRL, and LRGILS on the electrophysiological properties of second order sensory neurons in the substantia gelatinosa. There were no obvious effects of SLIGRL on the I-V relationship of substantia gelatinosa neurons. Because PAR2 resides on DRG cell bodies and on the peripheral sensory nerve endings (Steinhoff et al., 2000), it has been suggested that it also exists on primary afferent terminals (Fiorucci and Distrutti, 2002). We therefore examined the actions of PAR2 agonists on sEPSCs. The first series of experiments was done with Cs+-filled pipettes so as to maximize sEPSC amplitude (Moran et al., 2004). Trypsin (21 μM) affected 11 of 15 cells examined (Table 1), but its dominant effect was to decrease both sEPSC amplitude and frequency (Fig. 4, A–C). These effects, which were sustained for at least 10 min, could not be attributed to deterioration of recording conditions because sEPSC frequency and amplitude were sustained in the absence of trypsin (Fig. 4, D and E). SLIGRL (10 μM) also reduced sEPSC amplitude in two of five cells examined but had less effect on sEPSC frequency (Fig. 4, F and G). However, the effect of SLIGRL on sEPSC amplitude was also seen with the control compound, LRGILS, which also decreased SEPSC frequency (Fig. 4, H and I).

We also considered the possibility that trypsin and SLIGRL may exert different effects on different types of substantia gelatinosa neuron. Substantia gelatinosa neurons may be classified according to their discharge pattern in response to depolarizing current pulses as “tonic,” “phasic,” “delay,” “transient,” and “irregular” neurons (Balasubramanyan et al., 2006). The need to classify neurons on the basis of their AP firing pattern precluded the use of Cs+-filled electrodes for this series of experiments. Many tonic neurons (Fig. 4J) may be GABAergic and inhibitory, whereas delay neurons (Fig. 4K) may be excitatory (Lu and Perl, 2003, 2005). How-
ever, SLIGRL or trypsin had little effect on spontaneous synaptic activity in identified tonic neurons (Table 1) but reduced sEPSC frequency and amplitude in 50% of delay cells. Trypsin failed to alter excitability of delay or tonic cells because first spike latency on a 60 pA/s ramp in the presence of trypsin was unchanged in both cell types ($p > 0.4, n = 4$ for delay cells and $p > 0.2, n = 3$ for tonic cells). Typical experiments illustrating spike latency experiments are shown in Fig. 4, L and M.

Thus, the tendency for trypsin and SLIGRL to depress excitatory synaptic transmission in substantia gelatinosa as well as the tendency of trypsin to reduce membrane excitability seem at odds with the observation that i.t. applied PAR2 agonists have pronociceptive actions (Figs. 1–3). However, i.t. applied drugs likely have access to the DRG (Abram et al., 2006), so we next examined possible excitatory effects of trypsin, SLIGRL, and LRGILS on the cell bodies of primary afferent neurons.

**Classification of DRG Cells.** Because there is a reasonable correlation between AP shape and cell size of DRG neurons (Abdulla and Smith, 1997), “large” cells were defined as those with an AP duration $>3$ ms, “medium” cells had an AP duration of 3 to 5 ms, and “small” cells had an AP duration $<5$ ms (Fig. 5, A–C). Spike width was measured at 50% of the maximum amplitude. Both medium and small cells displayed a “hump” on the falling phase of their AP, whereas large cells did not.

**Effects of Trypsin and SLIGRL on the Membrane Potential of DRG Cells.** Twenty-eight of 43 (65%) of DRG neurons studied were depolarized in response to 30-s exposure to 42 $\mu$M trypsin. Responses to trypsin were not confined to any one cell type because it affected 16 of 23 large cells, 5 of 10 medium cells, and 7 of 10 small cells. Response amplitude was 6.8 ± 1.2 mV for large cells, 4.7 ± 1.3 mV for medium cells, and 5.2 ± 1.9 mV for small cells. It is likely that these responses were supramaximal because similar amplitude depolarizations were seen with much lower concentrations of trypsin (2.1 $\mu$M). A typical response to 42 $\mu$M trypsin is illustrated in Fig. 5D. In this experiment, hyperpolarizing current commands were passed through the recording electrode to monitor membrane conductance. Because the voltage responses observed in the presence of trypsin were reduced in amplitude, membrane conductance appeared to increase in this cell. The depolarizing response to trypsin following a second application to the same cell was rarely present and, if seen, was generally substantially lower in amplitude and shorter in duration. Application of trypsin occasionally produced a rapid and transient hyperpolarization before the onset of depolarization (Fig. 5D, arrow).

### Table 1

<table>
<thead>
<tr>
<th>Effectiveness of Drugs</th>
<th>Increase in sEPSC Amplitude</th>
<th>Decrease in sEPSC Amplitude</th>
<th>Increase in sEPSC Frequency</th>
<th>Decrease in sEPSC Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cs$^+$ internal</strong></td>
<td>2/15</td>
<td>9/15</td>
<td>2/10</td>
<td>5/15</td>
</tr>
<tr>
<td><strong>Trypsin, 5 min</strong></td>
<td>0/5</td>
<td>2/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td><strong>SLIGRL, 10 min</strong></td>
<td>0/5</td>
<td>2/5</td>
<td>0/5</td>
<td>1/5</td>
</tr>
<tr>
<td><strong>Delay cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trypsin, 10 min</strong></td>
<td>0/8</td>
<td>4/8</td>
<td>1/8</td>
<td>3/8</td>
</tr>
<tr>
<td><strong>SLIGRL, 5 min</strong></td>
<td>1/11</td>
<td>4/11</td>
<td>0/11</td>
<td>3/11</td>
</tr>
<tr>
<td><strong>SLIGRL, 10 min</strong></td>
<td>0/11</td>
<td>6/11</td>
<td>1/11</td>
<td>4/11</td>
</tr>
<tr>
<td><strong>Tonic cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trypsin, 10 min</strong></td>
<td>1/4</td>
<td>0/4</td>
<td>1/4</td>
<td>0/4</td>
</tr>
<tr>
<td><strong>Tonic cells</strong></td>
<td>1/11</td>
<td>1/11</td>
<td>1/11</td>
<td>2/11</td>
</tr>
<tr>
<td><strong>SLIGRL, 5 min</strong></td>
<td>1/11</td>
<td>1/11</td>
<td>2/11</td>
<td>1/11</td>
</tr>
<tr>
<td><strong>SLIGRL, 10 min</strong></td>
<td>3/11</td>
<td>1/11</td>
<td>2/11</td>
<td>1/11</td>
</tr>
</tbody>
</table>

**Fig. 3.** Withdrawal latency (A) and nociceptive score (B and C) measured in mouse hindpaws, before (time 0) and after intraplantar injection of formalin in mice that have received (10 min before formalin) an i.t. injection of 1 $\mu$g of SLIGRL, LRGILS, or saline. Data are expressed as mean ± S.E.M. *, significantly different from LRGILS; #, significantly different from basal latency/score measured at time 0, for $p < 0.05$.
Fig. 4. Effects of trypsin and PAR2 agonists on rat substantia gelatinosa neurons. A, effect of trypsin on sEPSCs. Note decrease in frequency and amplitude. B to I, cumulative probability plots to illustrate the effect of trypsin (21 nM), time, SLIGRL (10 nM), and LRGILS (10 nM) on SEPS amplitude and interevent interval (reciprocal frequency). Note that trypsin, SLIGRL, and LRGILS decrease sEPSC amplitude, and trypsin and LRGILS decrease sEPSC frequency. Data in B to I obtained from approximately 50 events during 3-min recording in two to seven neurons that responded to agonists. J, characteristic firing pattern of a tonic neuron in response to depolarizing current command. K, characteristic firing pattern of a delay neuron in response to depolarizing current command. L and M, experiments to illustrate the lack of effect of 21 nM trypsin on spike latency in response to current ramps in tonic and delay cells, respectively. All data records in traces J to M are on the same time scale.
Like trypsin, the synthetic activating peptide SLIGRL (10 μM) was capable of depolarizing all types of DRG cells, and coincidentally, 65% of cells (13 of 20) were affected. Responses to SLIGRL were seen in 7 of 12 large cells, three of five medium cells, and three of three small cells. Response amplitudes were 6.6 ± 2.1 mV for large cells, 2.7 ± 0.4 mV for medium cells, and 1.9 ± 0.2 mV for small cells. A typical response is illustrated in Fig. 5E. In this cell, SLIGRL reduced the amplitude of voltage responses to hyperpolarizing current pulses, an observation consistent with an increase in membrane conductance. The response to SLIGRL following a second application to the same cell was generally similar in amplitude and duration to the first. Unlike trypsin, SLIGRL never produced transient hyperpolarization of DRG neurons. The synthetic peptide LRGILS, the reverse sequence of SLIGRL, was used as a
control. LRGILS (10 μM) failed to affect the membrane potential of seven of seven large cells and one of one medium cells (Fig. 5F).

Data from all cell types were pooled to compare the duration of depolarizing response between 42 μM trypsin (n = 9), 5.20 ± 0.93 min, and 10 μM SLIGRL (n = 7), 1.95 ± 0.56 min. The trypsin response was significantly longer than that produced by SLIGRL (p < 0.05; Fig. 5G).

After a 30-s exposure to 10 μM SLIGRL, some neurons also exhibited spontaneous action potential discharge at resting potential. To examine this phenomenon in more detail, neuronal excitability was examined in response to depolarizing current steps or ramps. Four of eight medium or large cells examined exhibited an increase in excitability in the presence of 42 μM trypsin. A typical experiment is illustrated in Fig. 5H. Before the application of trypsin, a series of depolarizing current ramps of increasing amplitude produces a modest discharge of action potentials. The most shallow current ramp fails to generate action potentials, and even with the steepest ramp, the latency to the first action potential is ~300 ms. In contrast, in the presence of trypsin, an action potential is seen on the shallowest ramp, and the latency to the first action potential on the steepest ramp is reduced to ~130 ms.

Conductance Changes in DRG Neurons in Response to PAR2 Agonists. When studied under current clamp, depolarizing responses to trypsin were associated with an increase in conductance in two of two small cells and four of six large cells. Conductance appeared unchanged in the remaining two large cells and in three of three medium cells. Increased conductances were produced by SLIGRL in five of five large cells, one medium cell, and two of two small cells. Application of the reverse sequence peptide, LRGILS, did not change conductance in four of four large cells.

To examine conductance changes in more detail, cells were studied under voltage clamp. Voltage-ramp commands from −110 to +40 mV were used to establish current-voltage relationships before and during application of trypsin or SLIGRL. A typical voltage-clamp experiment is illustrated in Fig. 5I. Multiple downward deflections at positive voltages reflect unclamped action potentials. When studied under voltage clamp, SLIGRL responses usually involved an increase in conductance at voltages more negative than −40 mV and a decrease in conductance at voltages positive to ~30 mV. Presumably, the latter effect, which was also seen with trypsin, reflects suppression of voltage-dependent K+ currents, which may in turn contribute to the increased excitability seen in the presence of trypsin (Fig. 5H). Careful examination of the voltage responses to current ramps in Fig. 5H is consistent with this possibility. Voltage responses to current ramps rise more rapidly in the presence of trypsin, indicating decreased conductance at depolarized voltages. The increase in conductance seen at hyperpolarized potentials under voltage clamp (Fig. 5I) would explain the increase in conductance often seen under current-clamp conditions (Fig. 5, D and E) because the hyperpolarizing current pulses passed throughout the response would assess conductance changes that are dominant at more negative voltages.

Localization of PAR2 Immunoreactivity. The above experiments suggest that although PAR2 receptors are absent from substantia gelatinosa, they are present on the cell bodies of DRG neurons. To confirm this, we investigated PAR2 expression by immunohistochemistry on rat spinal cord slides and DRGs, using a Santa Cruz Biotechnology anti-PAR2 antibody. Although PAR2 was detected in rat DRG neurons (Fig. 6B), no specific staining for PAR2 was detected from rat spinal cord slices (Fig. 6D). Additional immunohistochemistry stainings using another anti-PAR2 antibody (B5 antibody) were performed in spinal cord slices and confirmed the absence of PAR2 staining in those tissues (data not shown).

Fig. 6. PAR2 immunoreactivity in rat DRG (A and B) and spinal cord (C and D). DRG photographs without fluorescence (A) or with a fluorescent lamp exposure (B); typical distribution of PAR2 on DRG neurons can be seen on the cell membrane of 62% of DRG neurons (arrows). Spinal cord photographs without fluorescence (C) or with exposure to a fluorescent lamp (D); no specific fluorescent staining is observed on spinal cord section, demonstrating a lack of PAR2 expression at the spinal cord level.
Discussion

We have found that i.t. applied PAR₂ agonists produce mechanical and thermal hyperalgesia and augment the thermal and mechanical hyperalgesia produced in an inflammatory pain model (Figs. 1–3). The lack of effect of the control peptide, LRGILS, and the absence of SLIGRL effects in knockout animals strongly supports the notion that activation of PAR₂ augments the transfer of nociceptive information. Although neither SLIGRL nor trypsin exert excitatory actions within the substantia gelatinosa per se (Fig. 4), the changes produced in DRG neurons (Fig. 5) are consistent with an increased excitation, which may in turn contribute to the pronociceptive action. This implies that i.t. applied drugs must have access to the cell bodies of sensory neurons within the DRG. At least two studies suggest this is likely to be the case. Abram et al. (2006) showed that fluorescein, which does not cross the blood-brain barrier, can be detected in rat DRG after i.t. injection. Earlier work had shown that even fluorescein-labeled proteins accessed the DRG when administered by this route (Klatzo et al., 1964).

PAR₂-induced mechanical allodynia may reflect activation of Aβ fibers, whereas thermal and mechanical hyperalgesia may reflect activation of C-fibers. The ability of PAR₂ agonists to augment nociception in a variety of pain models may reflect their ability to increase the excitability of all types of DRG neurons (Fig. 5).

The presence of PAR₂ receptors in DRG neurons is confirmed and their absence from the spinal cord is suggested by our immunohistochemical studies (Fig. 6). To the best of our knowledge, there are no immunohistochemistry, immuno blot, or binding studies that demonstrate the presence of PAR₂ in the dorsal horn. This correlates with the inability of SLIGRL and trypsin to increase spontaneous synaptic events in our electrophysiological experiments (Fig. 4). These observations appear to contradict data from other functional studies that suggest that PAR₂ resides on the central terminals of primary afferent nerves (Fiorucci and Distrutti, 2002). The observation that SLIGRL releases SP from DRG neurons in culture (Steinhoff et al., 2000) may either reflect release from DRG cell bodies (Huang and Neher, 1996) or from neurites. In the latter case, it does not necessarily follow that PAR₂ receptors exist on the central terminals of primary afferent fibers because the release in vitro (Steinhoff et al., 2000) may come from neurites analogous to peripheral nerve endings. It is already established that PAR₂ agonists release SP from peripheral nerve endings in vivo (Steinhoff et al., 2000). It has been shown, however, that PAR₂ agonists augment TRPV1-induced increase of SP from spinal cord slices that contain neither DRG neuron bodies nor peripheral nerve endings (Amadesi et al., 2004). One possible explanation for this effect is that activation of TRPV1 receptors promotes a rapid appearance of PAR₂ within the dorsal horn. We have preliminary electrophysiological and histochemical data to suggest this may be the case. One might therefore speculate that the appearance of PAR₂ is dependent upon the prior or constitutive activation of TRPV1 receptors. If this is so, it may explain why PAR₂ is found on peripheral nerve terminals and DRG cell bodies but not usually on the central terminals of primary afferents.

Because our electrophysiological studies were confined to the substantia gelatinosa, it could be argued that spinal pronociceptive actions of PAR₂ agonists reflect actions within deeper sensory lamina, such as laminae III and IV, which also receive sensory information from pain fibers. This is unlikely to be the case because these areas, like lamina II, fail to display PAR₂-like immunoreactivity (Fig. 6D).

Unlike PAR₂ agonists, PAR₁ and PAR₄ agonists attenuate nociception (Steinhoff et al., 2000; Vergnolle et al., 2003; Asfaha et al., 2007), and these effects may be mediated both centrally and peripherally (Asfaha et al., 2002). The occasional transient hyperpolarization of DRG neurons seen with trypsin, but not with the PAR₂ agonist SLIGRL, may reflect activation of PAR₁ or PAR₄. The depressant actions of trypsin on eEPSC amplitude and frequency in putative excitatory delay cells in substantia gelatinosa may be mediated by PAR₁ or PAR₄ (Vergnolle et al., 2003), whereas depressant effects of SLIGRL may reflect nonspecific actions because these were also seen with the control peptide inactive on PAR₂, LRGILS (Fig. 4).

Effects on DRG Cells. Even at supramaximal concentrations, the membrane depolarizations produced by trypsin and SLIGRL were of quite small amplitude and unlikely to depolarize DRG neurons to the threshold for action potential generation, although spontaneous action potentials did appear in a few of the cells studied. It is probably more relevant that the agonists affected voltage-gated channels that control action potential discharge when neurons are subject to other depolarizing stimuli. Thus, in a pathological situation, where DRG neurons may be depolarized by a host of pronociceptive substances such as neuropeptides, cytokines, or even low pH, the concomitant activation of PAR₂ receptors would facilitate action potential generation in response to depolarization. This, in turn, would lead to increased firing of fibers that synapse in the dorsal horn. A variety of voltage-gated K⁺ currents are activated by depolarization of DRG neurons (Gold et al., 1996). During the ramp commands used in our voltage-clamp experiments (Fig. 5I), most of the outward current at positive voltages probably flows through delayed rectifier and voltage-dependent Ca²⁺-sensitive K⁺ channels (BK channels) (Abdulla and Smith, 2001). Suppression of conductance at these voltages is more likely to reflect an action on delayed rectifier currents than on BK channels because PAR₂ agonists are known to elevate intracellular Ca²⁺ in DRG cells (Steinhoff et al., 2000). Moreover, PAR₂-induced depolarization was not blocked by Ca²⁺ channel blockers such as Mg²⁺ or Ni²⁺ (J. A. Endicott and P. A. Smith, unpublished data). Decreased K⁺ conductance is likely to contribute to the PAR₂-mediated increase in DRG excitability (Fig. 5H). Because PAR₂ receptors are known to modulate TRPV1 channel activity (Amadesi et al., 2004; Dai et al., 2004), it is possible that the inward current seen at negative voltages reflects activation of these channels. This would seem unlikely because TRPV1 channels are confined to a subset of small DRG neurons, yet PAR₂ agonists produced increased conductance in “large” and “medium” DRG neurons that do not express TRPV1 channels. Moreover, TRPV1 channels exhibit outward rather than inward rectification so that would be more available at positive rather than negative voltages (Raisinghani et al., 2005). Another possibility is that PAR₂ agonists affect H-currents. These nonselective cation currents that are activated by hyperpolarization are found in some DRG cells (Abdulla and Smith, 2001; Chaplan et al., 2003). However, PAR₂ agonists failed to affect H-currents activated by standard hyperpolarizing voltage...
protocols (P. Stemkowski, unpublished data). Thus, the channels underlying PAR2-induced changes in conductance remain to be identified.

The observed overall 65% response rate of DRG neurons to PAR2 agonists fits well with the report that approximately 60% of them display PAR2 (Steinhoff et al., 2000). In trigeminal ganglion neurons, PAR2 receptors are found on neurons that do not express tachykinins (Dinh et al., 2005). This and the present observation that PAR2 depolarized large cells suggest that it is capable of affecting neurons that do not express tachykinins. (Dinh et al., 2005).

Significance of Findings. Our findings illustrate how selective stimulation of DRG neurons can produce hyperalgesia and underline the importance of DRG cell bodies as a site of action of i.t. applied drugs (Abram et al., 2006). Moreover, pathological or pharmacological excitation of DRG cell bodies is able to produce pain-like behavior in animal models. Although trypsin is regarded as the principal PAR2-activating proteinase, other activators include mast cell tryptase, trypsinogen IV, and a trypsin-like proteinase known as P22 that was identified in brain slices (Sawada et al., 2000).

Because mast cells are found in perivascular areas of the nervous system, including the sensory ganglia (Undem et al., 1993), mast cell tryptase is perhaps the most likely endogenous activator of PAR2 in DRG (Vergnolle et al., 2001). Although the actions of endogenous PAR2 agonists may be confined to DRG neurons, our findings suggest that such effects play a major role in nociceptive processing.

References


Moran TD, Colmers WF, and Smith PA (2004) Opioid-like actions of neuropeptide Y 2H7, Canada. E-mail: peter.a.smith@ualberta.ca


Address correspondence to: Dr. Peter Smith, Department of Pharmacology, University of Alberta, 97.95 Medical Sciences Building, Edmonton, AB T6G 2H7, Canada. E-mail: peter.a.smith@ualberta.ca