

# Mechanisms of protease-activated receptor2-evoked hyperexcitability of nociceptive neurons innervating the mouse colon

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Agonists of protease-activated receptor 2 (PAR<sub>2</sub>) evoke hyperexcitability of dorsal root ganglia (DRG) neurons by unknown mechanisms. We examined the cellular mechanisms underlying PAR<sub>2</sub>-evoked hyperexcitability of mouse colonic DRG neurons to determine their potential role in pain syndromes such as visceral hyperalgesia. Colonic DRG neurons were identified by injecting Fast Blue and DiI retrograde tracers into the mouse colon. Using immunofluorescence, we found that DiI-labelled neurons contained PAR<sub>2</sub> immunoreactivity, confirming the presence of receptors on colonic neurons. Whole-cell current-clamp recordings of acutely dissociated neurons demonstrated that PAR<sub>2</sub> activation with a brief application (3 min) of PAR<sub>2</sub> agonists, SLIGRL-NH<sub>2</sub> and trypsin, evoked sustained depolarizations (up to 60 min) which were associated with increased input resistance and a marked reduction in rheobase (50% at 30 min). In voltage clamp, SLIGRL-NH<sub>2</sub> markedly suppressed delayed rectifier  $I_K$  currents (55% at 10 min), but had no effect on the transient  $I_A$  current or TTX-resistant Na<sup>+</sup> currents. In whole-cell current-clamp recordings, the sustained excitability evoked by PAR<sub>2</sub> activation was blocked by the PKC inhibitor, calphostin, and the ERK<sub>1/2</sub> inhibitor PD98059. Studies of ERK<sub>1/2</sub> phosphorylation using confocal microscopy demonstrated that SLIGRL-NH<sub>2</sub> increased levels of immunoreactive pERK<sub>1/2</sub> in DRG neurons, particularly in proximity to the plasma membrane. Thus, activation of PAR<sub>2</sub> receptors on colonic nociceptive neurons causes sustained hyperexcitability that is related, at least in part, to suppression of delayed rectifier  $I_K$  currents. Both PKC and ERK<sub>1/2</sub> mediate the PAR<sub>2</sub>-induced hyperexcitability. These studies describe a novel mechanism of sensitization of colonic nociceptive neurons that may be implicated in conditions of visceral hyperalgesia such as irritable bowel syndrome.

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The irritable bowel syndrome (IBS) is a common disorder affecting daily living and quality of life (Lembo *et al.* 2005). Of those symptoms that characterize IBS (i.e. abdominal pain, altered bowel pattern such as constipation and diarrhoea, abdominal bloating and altered defecation), abdominal pain is reported to be most troublesome and best correlates with severity of illness (Sandler *et al.* 1984). The lack of available therapies to effectively manage this pain continues to stimulate studies of the mechanisms that underlie this pain and to define novel therapeutic targets.

Visceral hypersensitivity (i.e. an increased sensitivity to stimuli arising from the intestinal wall) is widely recognized to underlie abdominal pain in IBS, at least in a significant subset of patients (gado-Aros & Camilleri, 2005). Both central and peripheral mechanisms have

been implicated and their relative role may be dependent on the clinical setting. In the periphery, sensitization of nociceptive nerve endings in the wall of the colon appears to be important. This may result from signalling from low levels of persisting cytokines in conditions such as post-infectious IBS (Spiller & Campbell, 2006), although there is also growing evidence that release of mast cell mediators may contribute to peripheral sensitization in IBS (Barbara *et al.* 2004).

Of the many substances released from mast cells, proteases such as tryptase may play a particularly important role in signalling to neurons (Barbara *et al.* 2004; Spiller & Campbell, 2006). The number of tryptase-containing mast cells is increased in intestinal tissues of patients with IBS (Barbara *et al.* 2004; 2006).

Moreover, tryptase levels in tissues, and tryptase release from biopsies are increased in IBS patients (Barbara *et al.* 2004). Proteases such as tryptase can signal to enteric (Reed *et al.* 2003) and dorsal root ganglia (DRG) (Vergnolle *et al.* 2001) neurons by cleaving and activating protease-activated receptors (PARs) to induce sustained increases in their excitability. While hyperexcitability of enteric neurons could result in disturbances in intestinal secretion and motility, hyperexcitability of DRG neurons could be an important mechanism of peripheral sensitization in conditions such as IBS (Mawe *et al.* 2004; Beyak & Vanner, 2005; Lomax *et al.* 2006). Although whole-animal studies have demonstrated a role for mast cell proteases signalling to PARs in the genesis of visceral hypersensitivity (Vergnolle *et al.* 2001), the precise mechanism(s) by which this occurs remain unclear.

Certain serine proteases that are generated and released during inflammation can signal to cells by cleaving protease-activated receptors (PARs), a family of four G-protein-coupled receptors. Cleavage exposes a tethered ligand domain that binds to and activates the cleaved receptors. Synthetic peptides that correspond to the tethered ligand domain of PAR<sub>1</sub>, PAR<sub>2</sub>, and PAR<sub>4</sub> directly activate these receptors and are useful tools to probe receptor function. Of the four cloned PARs, tryptase selectively activates PAR<sub>2</sub>, and trypsin is also a potent activator of this receptor (Saito & Bunnett, 2005). PAR<sub>2</sub> is expressed by DRG and enteric neurons, and PAR<sub>2</sub>-selective activating peptides induce hyperexcitability of these neurons. Although the digestive tract is the richest source of proteases that can activate PAR<sub>2</sub>, such as tryptase and trypsin, the effect of PAR<sub>2</sub> agonists on hyperexcitability of colonic nociceptive DRG neurons has not been examined. Moreover, although PAR<sub>2</sub> agonists are known to cause hyperexcitability of DRG neurons (Amadesi *et al.* 2004), the mechanisms of this effect are unknown. In the present study we examined the effects of PAR<sub>2</sub> activation with a selective agonist on the neuronal excitability of mouse colonic DRG nociceptive neurons. Our aims were to (1) determine if colonic DRG neurons express immunoreactive PAR<sub>2</sub>, using retrograde tracing and immunofluorescence; (2) examine whether activation of PAR<sub>2</sub> results in hyperexcitability of these neurons, and to identify any effects upon voltage-gated potassium or sodium currents using electrophysiological approaches; (3) identify the kinases that mediate PAR<sub>2</sub>-induced hyperexcitability using pharmacological approaches; and (4) determine if activated kinases are appropriately localized to regulate the affected ion channels, using immunofluorescence and confocal microscopy. Small DRG neurons were studied because these neurons express properties associated with nociceptors (Gold *et al.* 1996a; Yoshimura & de Groat, 1999; Moore *et al.* 2002; Beyak & Vanner, 2005). We found that PAR<sub>2</sub> activation evoked a sustained hyperexcitability of these colonic neurons, and

sought to determine the mechanism(s) that underlies this action.

## Methods

### Drugs and reagents

Synthetic peptides corresponding to the tethered ligand of rat and mouse PAR<sub>2</sub> (PAR<sub>2</sub>-activating peptide, PAR<sub>2</sub>-AP, SLIGRL-NH<sub>2</sub>) and the reverse peptide sequence that does not activate PAR<sub>2</sub> (PAR<sub>2</sub>-reverse peptide, PAR<sub>2</sub>-RP, LRGILS-NH<sub>2</sub>, control) were from Sigma Genosys (The Woodlands, TX, USA). All other chemicals were obtained from Sigma, with the exception of TTX and PD98059 (Calbiochem). All agonists were applied to the bath using a fast-flow solution-switching system (VC6; Warner Instruments) and a three-barrel square glass (Harvard Apparatus). In electrophysiological studies involving PD98059, the cells were incubated with the drugs for a minimum of 2 h before the experiment began. Both PD98059 and calphostin C were included in the bath and pipette solutions.

### Animals

Electrophysiological studies were conducted on CD-1 mice (Charles River Laboratories, Montreal, QC, Canada) of either sex, weighing 30–40 g. Immunohistochemical studies were conducted on C57Bl6 mice (6–8 weeks) from Jackson Laboratories (Bar Harbour, ME, USA) and Sprague-Dawley rats (male, 200–250 g) from Charles River Laboratories (Wilmington, MA, USA). The Queen's University Animal Care Committee and the UCSF Institutional Animal Care and Use Committees approved and monitored all procedures with these animals. At the end of experiments, animals were killed using sodium pentobarbitone (200 mg kg<sup>-1</sup>, i.p.) and bilateral thoracotomy.

### Retrograde labelling of colonic neurons

Mice or rats were anaesthetized with isoflurane (5%) or a combination of ketamine hydrochloride (Pfizer; New York, NY, USA) 18.75 mg ml<sup>-1</sup> and xylazine (Bayer; Etobicoke, ON, USA) 1.25 mg ml<sup>-1</sup> injected i.p. (0.1 ml (10 g body weight)<sup>-1</sup>). The descending colon was exposed by a midline laparotomy. A 25 µl syringe (Hamilton, Reno, Nevada, USA) with a 30–32 gauge needle was used to inject Fast Blue (17 mg ml<sup>-1</sup>, volume = 1.0 µl per injection) or 1,1-dioctadecyl-3,3'-3'-tetramethylindocarbocyanine (DiI) (5–17 mg ml<sup>-1</sup> in DMSO) into 5–10 sites along the colon wall (volume = 10 µl per injection site), as previously described (Beyak *et al.* 2004). Excess dye was removed using a cotton swab to prevent the dye leaking to other tissues. The abdomen was irrigated with saline and sutured closed. After surgery, animals were allowed to recover on a warming blanket and were given free

access to water and food. After recovery from anaesthesia, animals were monitored for signs of pain, feeding, and weight loss. Animals that displayed behaviour consistent with ongoing pain or failure to thrive were killed.

### Immunohistochemical localization of PAR<sub>2</sub>

Ten to fifteen days following DiI injection, animals were killed using sodium pentobarbital (200 mg kg<sup>-1</sup> i.p.). DRG (T9–L1, T5 for control) were immersion fixed in 4% paraformaldehyde in 100 mM PBS pH 7.4 for 4 h at 4°C, washed with PBS and placed in a 25% sucrose solution overnight at 4°C. DRG were embedded in OCT compound (Miles, Elkhart, IN, USA) and sectioned at 25 μm. Sections were washed in PBS containing 1–10% NGS, 1% BSA and 0.3–0.5% Triton X-100, and incubated with primary antibodies to PAR<sub>2</sub> (mouse DRG, A5, 1:750; rat DRG, SAM11, 1:250) overnight at 4°C. Sections were washed and incubated with goat anti-antirabbit or antimouse IgG conjugated to FITC (1:200, room temperature, 2 h). Sections were washed and mounted in Prolong (Molecular Probes, Eugene, OR, USA). Mouse monoclonal antibodies to PAR<sub>2</sub> (SAM11) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit antibody to PAR<sub>2</sub> (A5) was from Dr M. Hollenberg (Calgary University). Goat antimouse or antirabbit IgG conjugated to FITC was from Jackson ImmunoResearch (West Grove, PA, USA).

### Isolation and culture of DRG neurons for ERK phosphorylation studies

Mice and rats were killed using sodium pentobarbital (200 mg kg<sup>-1</sup> i.p.). DRG from thoracic and lumbar spinal cord were minced in cold Hank's balance salt solution (HBSS) (Steinhoff *et al.* 2000; Amadesi *et al.* 2004). Mouse DRG were digested by incubating in DMEM containing 1 mg ml<sup>-1</sup> collagenase type IA and 0.8 mg ml<sup>-1</sup> DNase type IV, for 60 min at 37°C. This solution was removed and neurons were incubated for 45 min at 37°C with DMEM containing 0.25% trypsin. Rat DRG were digested by incubating in Dulbecco's modified Eagle's medium (DMEM) containing 0.5 mg ml<sup>-1</sup> trypsin, 1 mg ml<sup>-1</sup> collagenase type IA and 0.1 mg ml<sup>-1</sup> DNase type IV (all from Sigma, St Louis, MO, USA) for 60–90 min at 37°C. After digestion, soybean trypsin inhibitor (Sigma, 0.5%) was added to neutralize trypsin. Neurons were pelleted, suspended in DMEM containing 10% fetal bovine serum, 10% horse serum, 100 u ml<sup>-1</sup> penicillin, 0.1 mg ml<sup>-1</sup> streptomycin, 2 mM glutamine and 2.5 μg ml<sup>-1</sup> DNase type IV, plated on glass coverslips coated with Matrigel (BD Biosciences, Bedford, MA, USA), and cultured for 2–3 days.

### ERK phosphorylation assays

Neurons in short-term culture (48–72 h) were incubated for 18 h at 37°C in DMEM containing 1% horse serum,

1% fetal bovine serum, 100 u ml<sup>-1</sup> penicillin, 0.1 mg ml<sup>-1</sup> streptomycin, 2 mM glutamine and 2.5 μg ml<sup>-1</sup> DNase IV. Neurons were washed and incubated for 30 min at 37°C in DMEM containing 0.1% BSA (protease-free). Neurons were challenged by addition of SLIGRL-NH<sub>2</sub> (PAR<sub>2</sub>-AP) or LRGILS-NH<sub>2</sub> (PAR<sub>2</sub>-RP) (both 100 μM) or PMA (1 μM) for 0–30 min. Neurons were immediately washed in ice-cold PBS (100 mM, pH 7.4), and were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Neurons were incubated for 30 min at room temperature in PBS containing 1% NGS and 0.1% saponin, and were incubated overnight at 4°C with pERK antibody (1:500). Neurons were washed and incubated with goat antimouse IgG conjugated to FITC (1:200, room-temperature, 1 h). Slides were washed, fixed for 20 min in paraformaldehyde, and mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Mouse monoclonal antibodies to phosphorylated ERK<sub>1/2</sub> (pERK<sub>1/2</sub>, E4) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### Confocal microscopy

Specimens were observed using a Zeiss Axiovert microscope, a Bio-Rad MRC1000 confocal microscope, with a Zeiss Plan Apo ×40 (NA 1.4) objective for tissue sections and a Zeiss Plan Apo ×100 (NA 1.3) objective for cultured neurons. Images were collected at laser intensities of 3–30%, an iris of 2.5–3 mm and a zoom of 1–2, and typically 5–10 optical sections were taken at intervals of 0.5–1.0 μm. Images were processed to adjust contrast and brightness using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA). In experiments to localize pERK<sub>1/2</sub> in cultured neurons, images were collected under identical conditions (laser intensity, iris and gain) for control and stimulated neurons.

### Electrophysiological studies

Neurons were acutely dissociated as previously described (Beyak *et al.* 2004). Briefly, tissue was incubated in collagenase (Worthington; Lakewood, NJ, USA) 1 mg ml<sup>-1</sup> and dispase (Roche; Indianapolis, IN, USA) (4 mg ml<sup>-1</sup>) for 10 min at 37°C, titrated with a fire-polished Pasteur pipette, and incubated for an additional 5 min at 37°C. Dissociated neurons were plated onto vitrogen-coated coverslips and stored in a humidified incubator at 37°C, under 95% air–5% CO<sub>2</sub> until retrieval (4–24 h) for electrophysiological studies. The incubating media was obtained from a stock solution containing 10 ml fetal bovine serum, 1 ml penicillin/streptomycin, 0.028 g glutamine, 0.6 g dextrose and 0.2 g NaHCO<sub>3</sub>, and made up to 100 ml with DMEM (pH 7.2–7.3).

Whole-cell or perforated-patch-clamp electrophysiological experiments were performed in current or

voltage clamp at room temperature. Neurons adhering to the glass coverslip were placed in an RC-26 recording chamber (Warner Instruments; Hamden, CT, USA), which was mounted on the stage of an inverted microscope (Olympus IX70; Tokyo, Japan) fitted for both bright-field and fluorescence microscopy. With the use of the U-MWIG2 filter (Olympus) for Fast Blue, labelled colonic neurons were identified by their bright blue fluorescence. All cells were labelled with Fast Blue except in some latter experiments where a few unlabelled neurons were studied because of relatively lower numbers of labelled neurons. Almost all of these latter experimental groups had ~50% or more labelled neurons, and no differences were observed between labelled and unlabelled neurons. Only small neurons ( $\leq 25 \mu\text{m}$  diameter and  $\leq 40 \text{ pF}$  capacitance) were studied, because these neurons express properties associated with nociceptors (i.e. capsaicin sensitivity, TTX-resistant action potentials) (Gold *et al.* 1996a; Yoshimura & de Groat, 1999; Moore *et al.* 2002; Beyak & Vanner, 2005) Recordings were obtained using G85165T-4 patch glass capillary tubing with flame-polished ends (Warner Instruments), pulled with a PP-830 micropipette puller from Narishige (East Meadow, NY, USA), and fire-polished by using an FP-830 fire polisher (Narishige). Final pipette resistance was between 2 and 5 M $\Omega$  for all experiments. Signals were amplified using an Axopatch 200B amplifier and digitized with a Digidata 1322A A/D converter from Axon Instruments (San Jose, CA, USA). Signals were low-pass filtered at 5 kHz, acquired at 20 kHz, and stored on disk using Clampex 8.2 (Axon Instruments).

Data were only analysed from recordings that exhibited the following properties. The starting seal resistance between the cell membrane and the electrode tip was  $\geq 1 \text{ G}\Omega$ . The starting series resistance was  $\leq 20 \text{ M}\Omega$ , and remained stable throughout the experiment. In current clamp, cells had a stable resting membrane potential more negative than  $-45 \text{ mV}$  for more than 5 min before recording, and displayed overshooting action potentials (peak  $\geq +50 \text{ mV}$ ) for the duration of the experiment. In voltage clamp, the leak current was  $\leq 0.5 \text{ nA}$  when tested at a holding potential of  $-100 \text{ mV}$  throughout the experiment. All perforated-patch recordings were obtained using amphotericin B from Sigma (St Louis, MO, USA) as previously described (Rae *et al.* 1991). Briefly, amphotericin B stock solution was made by diluting 6 mg of powder into 100  $\mu\text{l}$  dimethylsulfoxide (DMSO) (Sigma), followed by sonication and vortex-mixing. Immediately prior to experiments, 20  $\mu\text{l}$  of stock solution were added into 5 ml of internal pipette solution. The solution was vortexed until a uniform light-yellowish tint developed, then covered with aluminium foil to prevent exposure to light. The final amphotericin B concentration was 240  $\mu\text{g ml}^{-1}$ . Due to the compound's photosensitivity and rate of breakdown upon exposure to light, pipette

solutions with amphotericin B were replaced every 2 h. Pipette tips were dipped briefly into amphotericin-free internal pipette solution, then back-filled with unfiltered amphotericin-containing solution.

Current-clamp recordings were carried out in perforated-patch mode using the following solutions (mM): (pipette) 140 KCl, 10 Hepes, 5 EGTA, 4 Na<sub>2</sub>-ATP, 5 MgCl<sub>2</sub>, 2.5 CaCl<sub>2</sub> with pH adjusted to 7.2 using KOH; (bath), 140 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 Hepes, and 10 D-glucose, with pH adjusted to 7.4 using NaOH.

The membrane potential was continuously monitored using Clampex 8.2. After a stable membrane potential was recorded for 5 min, membrane potential, rheobase, twice rheobase, and input resistance were recorded. Cells were then superfused with PAR<sub>2</sub>-AP or PAR<sub>2</sub>-RP (both 100  $\mu\text{M}$ ) for 3 min and the parameters were again measured at 3, 10, 30 and 60 min after application of the peptide. Rheobase was recorded using 180 ms steps in 0.01 nA increments, which continued until an action potential was fired and the current needed to generate the action potential was recorded. Input resistance was recorded using a single 180 ms hyperpolarizing step command equal to the negative value of the cell's rheobase, and varied between  $-0.1$  and  $-0.3 \text{ nA}$ . The membrane response to the PAR<sub>2</sub> agonist trypsin (300 nM) was tested in a separate series of experiments.

To record potassium currents, perforated patch was first established using the current-clamp bath solution described above. After perforation was complete, sodium-free bath solution was perfused onto the cell to isolate potassium currents. The composition (mM) was: 140 NMDG, 4 KCl, 1.8 Hepes, 1 D-glucose, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, pH adjusted to 7.4 using HCl. Pipette solution composition was (mM): 110 K-aspartate, 30 KCl, 10 EGTA, 10 Hepes, 2 Na<sub>2</sub>-ATP, 1 MgCl<sub>2</sub>, pH adjusted to 7.2 using KOH.

To study the effect of PAR<sub>2</sub> activation on  $I_A$  and  $I_K$  currents, neurons were superfused for 3 min with PAR<sub>2</sub>-AP or PAR<sub>2</sub>-RP (both 100  $\mu\text{M}$ ).  $I_A$  and  $I_K$  currents were separated using voltage protocols, as previously described (Stewart *et al.* 2003). Briefly,  $I_K$  was isolated using 400 ms depolarizing steps in 15 mV increments from  $-90 \text{ mV}$  to  $+45 \text{ mV}$  at a holding potential of  $-60 \text{ mV}$ , with the sustained  $I_K$  measured isochronally, 400 ms after the onset of the pulse, at which time  $I_A$  was largely inactivated, minimizing contamination by this current. The  $I_A$  was isolated by subtracting the sustained  $I_K$  from the total potassium current recorded using 400 ms depolarizing steps in 15 mV increments from  $-90 \text{ mV}$  to  $+45 \text{ mV}$  from a holding potential of  $-100 \text{ mV}$ . Peak  $I_A$  was measured as the peak of the transient component of this subtracted current. Recordings were made at the beginning of the experiment and at 3, 5, 10 and 15 min following the application of the peptide.

Voltage-clamp studies of Na<sup>+</sup> currents were carried out in whole-cell mode. All recordings were obtained with the

following solutions (mM): (pipette) 110 CsCl, 1 MgCl<sub>2</sub>, 11 EGTA, 10 Hepes, 10 NaCl, pH adjusted to 7.2 with CsOH; (bath), 55 NaCl, 80 cholineCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 Hepes, and 5 D-glucose, pH adjusted to 7.4 using NaOH. The low extracellular sodium concentration was used to reduce the size of the sodium currents, enabling them to be accurately clamped. Na<sup>+</sup> currents were recorded in voltage clamp using 30 ms depolarizing steps in 5 mV increments from -80 to +40 mV at a holding membrane potential of -100 mV. TTX-resistant Na<sup>+</sup> currents were isolated with TTX (1 μM). The PAR<sub>2</sub> agonist SLIGRL-NH<sub>2</sub> (100 μM) or LRGILS-NH<sub>2</sub> (100 μM) was then superfused for 3 min, and voltage protocols in the presence of TTX repeated at 3, 5, and 10 min following application of the peptide.

Conductance was determined using the relation:

$$G = I / (V_m - E_x)$$

where  $G$  is the conductance,  $I$  is the measured membrane current,  $V_m$  is the command voltage, and  $E_x$  is the equilibrium potential, which was calculated to be -84 mV for potassium and +89.82 mV for sodium in control solutions. Normalized average conductances were plotted against membrane potential and the resultant curve was fitted to a Boltzmann function of the form:

$$G / G_{\max} = 1 / (1 + \exp[V_{50} - V_m / k])$$

where  $G$  is the conductance,  $G_{\max}$  is the fitted maximal conductance,  $V_{50}$  is the membrane potential for half-activation,  $V_m$  is the command potential, and  $k$  is the slope factor.

Inactivation curves were measured using a two-pulse protocol, as previously described (Yoshimura & de Groat, 1999; Stewart *et al.* 2003).  $I_A$  inactivation curve studies used a 1 s prepulse varying between -120 and 0 mV, followed by a 400 ms test pulse of +50 mV.  $I_K$  inactivation curves were generated using a two-pulse protocol: an 8 s prepulse varying between -80 and 0 mV, followed by a 1 s test pulse of +50 mV. TTX-R sodium current inactivation curves were generated using a two-pulse protocol: a 1 s prepulse between -120 and 0 mV, followed by a 30 ms test pulse to 0 mV. Residual current amplitudes were normalized and plotted against conditioning pulse potential, and the continuous line in Fig. 4E is an average of fits to a negative Boltzmann function:

$$I / I_{\max} = 1 / (1 + \exp[V_{50} - V_m / k]),$$

where  $I$  is the current,  $I_{\max}$  is the maximal current,  $V_{50}$  is the membrane potential for half-activation,  $V_m$  is the command potential, and  $k$  is the slope factor.

### Statistical analysis

Electrophysiological recordings were analysed using Clampfit 8.2 software (Axon Instruments). Linear leak

subtraction was used for all experiments with the P/N Clampfit protocol. Values obtained at various time points were expressed as a difference (absolute or percentage) from the resting membrane potential at  $t = 0$  min. Data are expressed as means  $\pm$  s.e.m. A two-way analysis of variance (ANOVA) with a Bonferroni *post hoc* test for significance was carried out to compare the results at every time point, and data values were deemed statistically significant if  $P < 0.05$ . Data from Fast Blue and non-labelled cells were also compared at every time point and were always found to be statistically insignificant ( $P > 0.05$ ). Fitting of data was done with the least squares method using the fit function in Prism 4.0 (Graphpad Software). Voltages of half-activation ( $V_{50}$ ), time constants, and slope factors were obtained from means of the individual Boltzmann curve fits.

## Results

### PAR<sub>2</sub> is expressed in colonic neurons

We have previously reported that nociceptive neurons of rat DRG that express TRPV1, CGRP and SP also express PAR<sub>2</sub> (Steinhoff *et al.* 2000; Amadesi *et al.* 2004). To determine if those DRG neurons innervating the colon contain PAR<sub>2</sub>, we used retrograde tracing. When the tracer DiI was injected into the distal colon of mice, labelled DRG neurons could be detected in DRG from T9-L1 after 10–15 days (Fig. 1A). These neurons expressed immunoreactive PAR<sub>2</sub> (Fig. 1B and C). Since we have previously reported that PAR<sub>2</sub> is expressed by nociceptive DRG neurons in rats, and because intracolonic administration of PAR<sub>2</sub> activators causes hyperalgesia to distension of the rat colon, we also determined if DRG neurons innervating the colon express PAR<sub>2</sub> in this species. When DiI was injected into the distal colon of rats, labelled DRG neurons were detected in DRG from T9-L1 after 10–15 days (Fig. 1D). These neurons expressed immunoreactive PAR<sub>2</sub> (Fig. 1E and F). Together, these results show that DRG neurons that innervate the distal colon of mice and rats express PAR<sub>2</sub>.

### PAR<sub>2</sub> activation on mouse colonic DRG neurons evokes sustained hyperexcitability

Current-clamp recordings were obtained from 64 DRG neurons. Only small neurons were chosen for study (cell diameter  $\leq 25$  μm and a capacitance  $\leq 40$  pF) because these small cells have been shown to have properties exhibited by nociceptors (Beyak & Vanner, 2005). The mean resting membrane potential was  $-51.6 \pm 1.6$  mV. The mean input resistance was  $585.29 \pm 65.80$  MΩ. All action potentials obtained displayed a shoulder on the repolarizing phase of the action potential, characteristic of TTX-resistant action potentials on nociceptive DRG neurons (Beyak & Vanner,

2005). Fewer than 10% of cells displayed spontaneous action potentials.

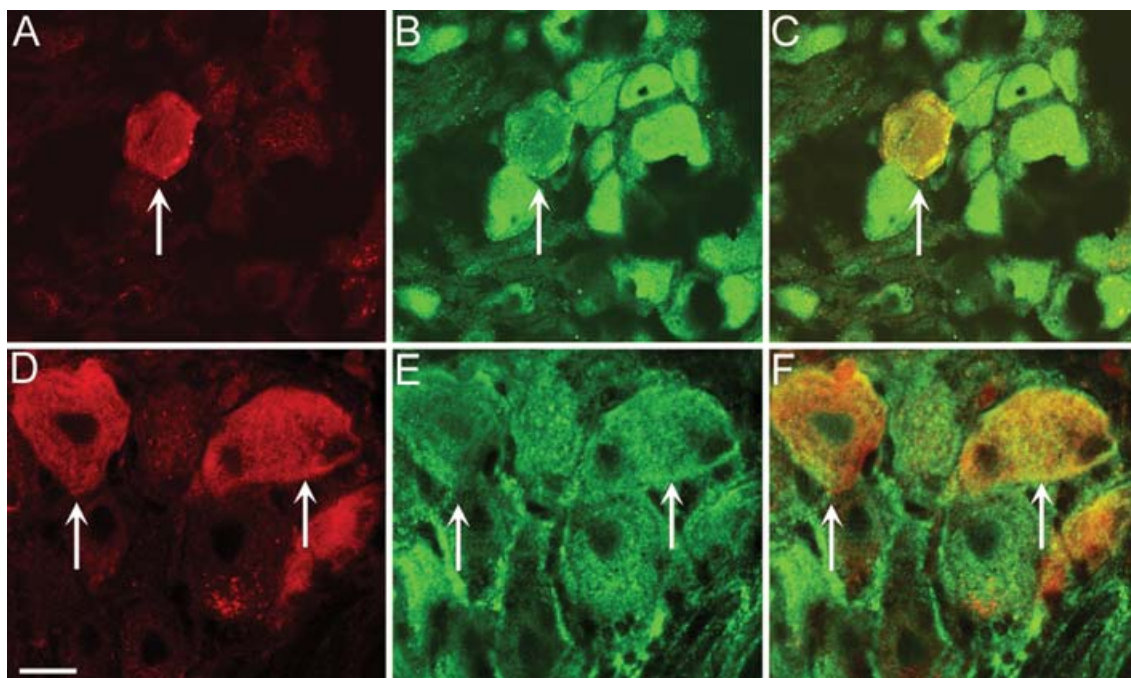
We have previously shown that PAR<sub>2</sub> activation on guinea-pig submucosal (Reed *et al.* 2003) and rat DRG neurons (Amadesi *et al.* 2004) caused a sustained decrease in rheobase and in rat DRGs a prolonged membrane depolarization. To evaluate the effect of PAR<sub>2</sub> activation on mouse colonic DRG neurons, Fast Blue-labelled neurons were superfused for 3 min with the PAR<sub>2</sub>-AP (100 μM) and changes in passive and active membrane properties were compared to baseline for 60 min. Superfusion of PAR<sub>2</sub>-AP (*n* = 5) caused a significant membrane potential depolarization at 3 min (mean = 6.8 mV; range: 2.1–12.4 mV) in all neurons tested, which peaked by 10 min (mean = 7.8 mV; range: 2–15.2 mV) (Fig. 2). This depolarization, although smaller, was still evident at 60 min. In contrast, superfusion with the reverse peptide PAR<sub>2</sub>-RP (100 μM; *n* = 6 Fast Blue-labelled) had no effect on membrane potential.

PAR<sub>2</sub>-AP (100 μM) also caused a decrease in rheobase at 3 min (mean = 55.5%; range: 33.3–71.4%), which was maximal by 60 min (mean = 73.3%; range: 56.2–100%) (Fig. 3B). Changes in membrane potential were controlled for by manually applying DC current to return the membrane potential to the resting value at each time point before retesting the rheobase. PAR<sub>2</sub>-RP had no effect on rheobase. Changes in input resistance were monitored

with constant hyperpolarizing pulses (see trace in Fig. 2A). PAR<sub>2</sub> activation caused a significant increase in input resistance at 3 min (mean = 68.3%; range: 5.44–201.94%), which was maximal by 60 min (73.3%; range: 49.4–108%) (Fig. 3C). Conversely, the reverse peptide had no effect on input resistance. Most neurons fired one or two action potentials when depolarized at 2× rheobase. These numbers did not change following activation of PAR<sub>2</sub> receptors (data not shown).

In a separate series of experiments, the PAR<sub>2</sub>-AP (1–100 μM) displayed a dose-dependent effect on membrane potential (Fig. 2C). Two out of eight neurons did not respond to 30 μM and 100 μM PAR<sub>2</sub>-AP. Superfusion of the PAR<sub>2</sub> agonist trypsin (300 nM) also evoked slow depolarizations (mean peak amplitude = 3.8 ± 0.53 mV; *n* = 7).

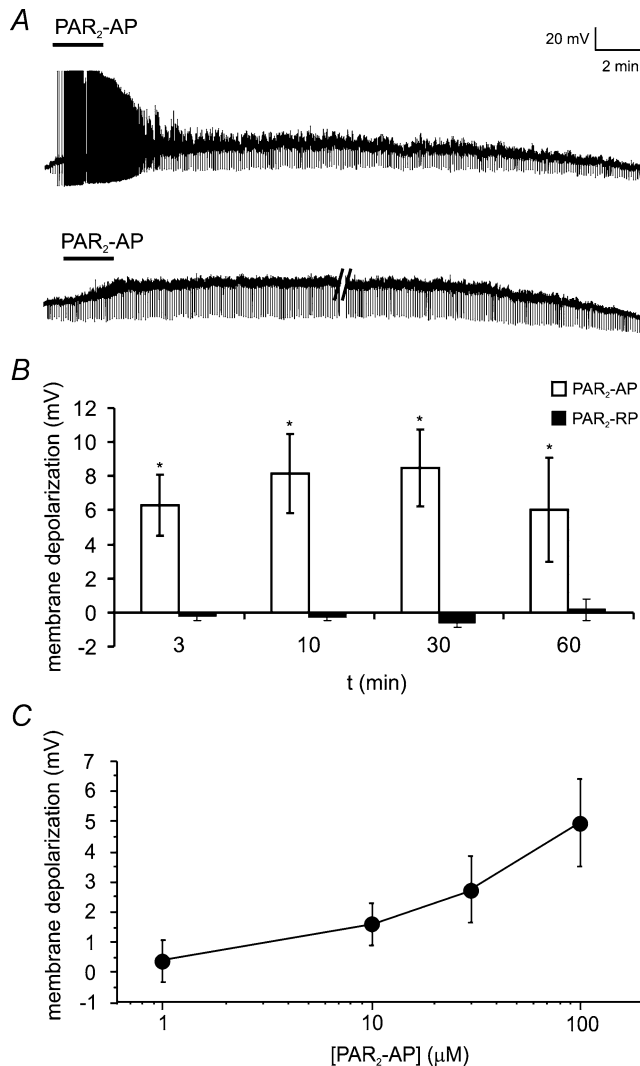
The effects of PAR<sub>2</sub>-AP on action potential duration and slope were also evaluated in a separate series of experiments, using short-duration depolarizing pulses (30 ms). Following PAR<sub>2</sub> activation, the mean action potential duration (*n* = 4) at one-half the peak amplitude (Fig. 3) was longer at all time points (3–15 min; *n* = 6) and statistically significant at 15 min (0 min mean = 3.18 ± 0.53 *versus* 15 min mean = 3.48 ± 0.50; *P* < 0.05, *n* = 6). The action potential duration was unchanged in control neurons (*n* = 4) over a similar time period (0 min mean = 2.49 ± 0.49 ms



**Figure 1.** Localization of immunoreactive PAR<sub>2</sub> in retrogradely labelled DRG from mouse (A–C) and rat (D–F) DRG

Dil was injected into the wall of the distal colon and PAR<sub>2</sub> was localized in DRG (T9–L1) 10–15 days later. A and D, Dil; B and E, immunoreactive PAR<sub>2</sub>; C and F, merge. Dil-labelled neurons innervating the colon expressed PAR<sub>2</sub> immunoreactivity. Representative images are shown from experiments on *n* = 3 animals. Scale bar = 20 μm. Arrows show labelled neuron.

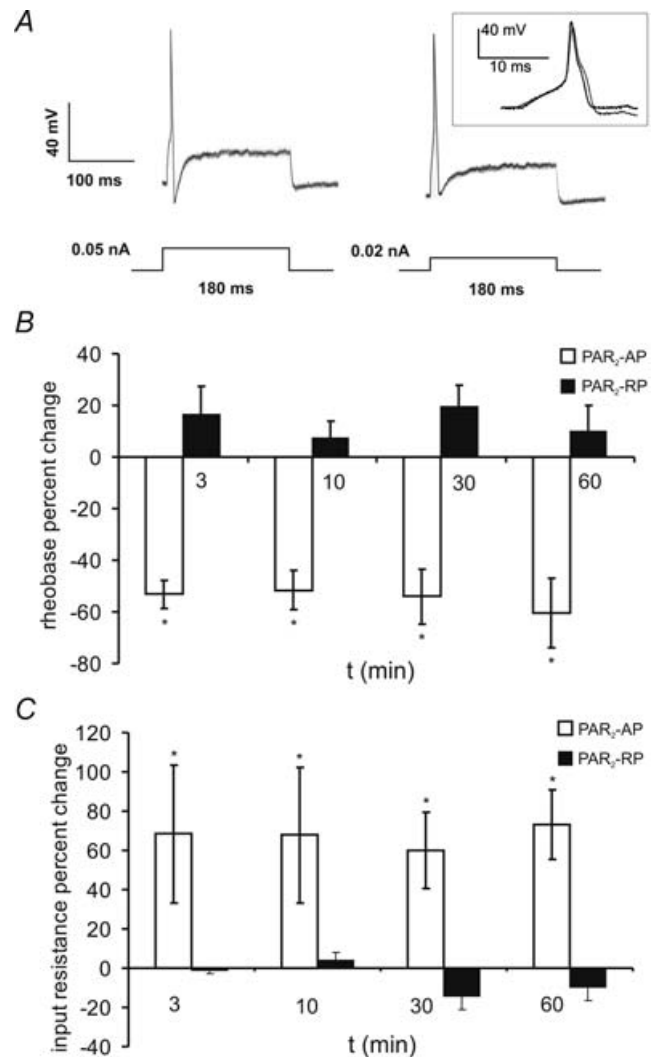
versus 10 min mean =  $2.49 \pm 0.46$  ms). The rising slope of the action potential was measured up to 10 min following PAR<sub>2</sub> activation ( $n = 5$ ), and was not altered (0 min mean =  $133.41 \pm 11.43$  mV ms<sup>-1</sup> versus 0 min mean =  $121.77 \pm 17.63$  mV ms<sup>-1</sup>).



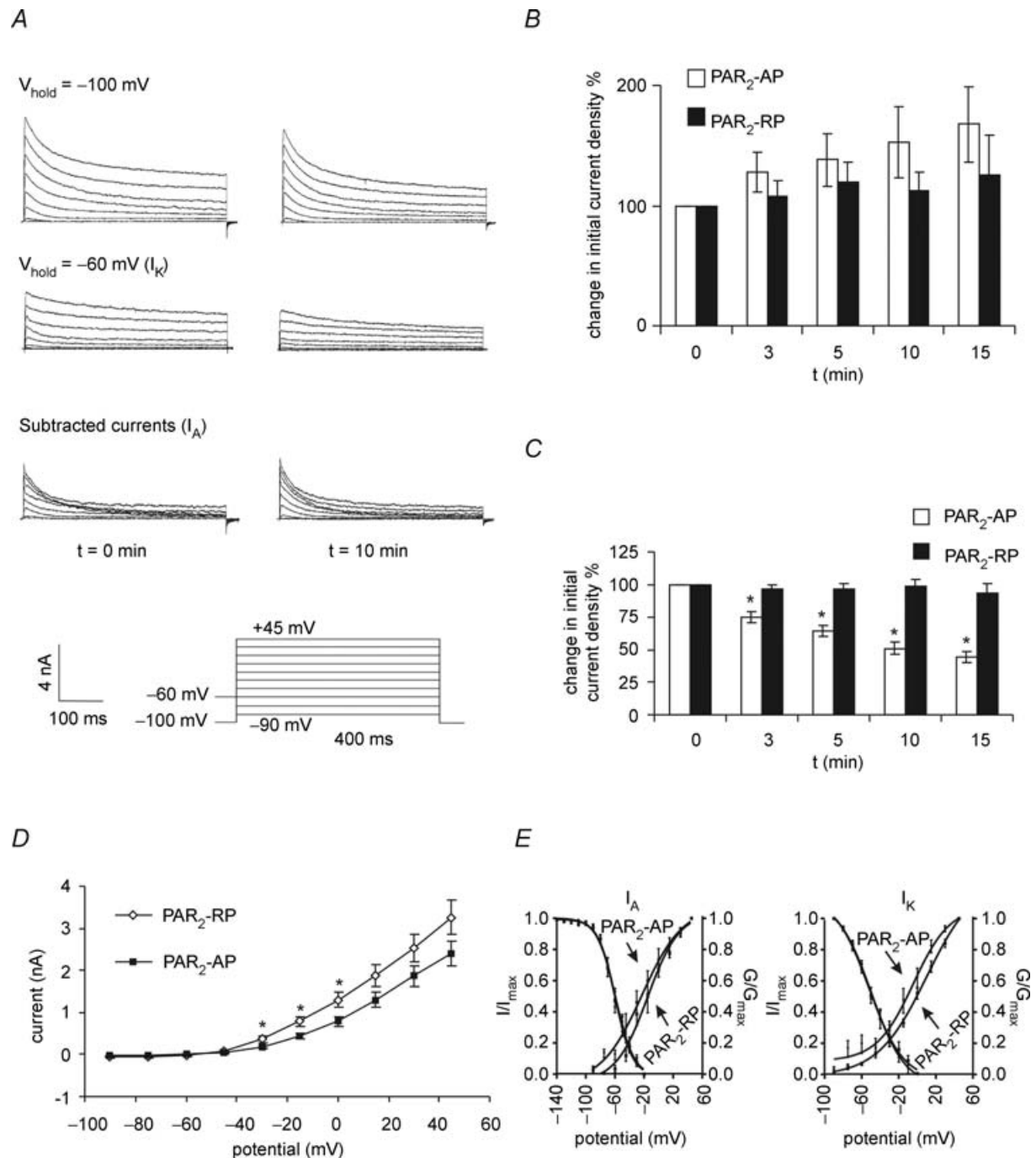
**Figure 2. PAR<sub>2</sub>-AP evokes sustained depolarization of mouse colonic DRG neurons**  
 A, representative traces showing PAR<sub>2</sub>-AP (SLIGRL; 100 μM) superfusion for 3 min caused a sustained depolarization of mouse DRG neurons for up to 60 min. This was associated with action potential discharge in a few neurons (upper trace) and an increase in input resistance (brief downward deflections evoked by constant hyperpolarizing pulse). Parallel lines (lower trace) represent 16 min break in recording. Each neuron was recorded for 5 min prior to application of the PAR<sub>2</sub>-AP (not shown) to ensure a stable resting membrane potential. B, summary of mean depolarization at each time point following superfusion of PAR<sub>2</sub>-AP (100 μM). The reverse peptide, which lacks biological activity at the PAR<sub>2</sub> (PAR<sub>2</sub>-RP, LRGILS; 100 μM), had no effect on membrane potential.  $n = 5$  or more for each time point. C, PAR<sub>2</sub>-AP (1–100 μM) evoked a dose-dependent depolarization.  $n = 5$  or more neurons at each point.

**Effect of PAR<sub>2</sub> activation on K<sup>+</sup> and Na<sup>+</sup> currents**

Numerous studies, including our own (Stewart *et al.* 2003; Beyak & Vanner, 2005), have shown that intestinal inflammation and specific inflammatory mediators causes a significant increase in TTX-resistant Na<sup>+</sup> currents



**Figure 3. PAR<sub>2</sub>-AP-evoked sustained hyperexcitability of mouse colonic DRG neurons**  
 A, representative recording showing rheobase was markedly reduced 10 min (right trace) following a 3 min superfusion of PAR<sub>2</sub>-AP (100 μM) compared to time 0 (left trace). The membrane potential was returned to the resting value by injecting depolarizing current at each time point before testing for changes in rheobase. Inset shows that the action potential duration was increased following PAR<sub>2</sub> activation. Rheobase was determined using a series of 0.01 current steps (not shown) and recording the current necessary to elicit an action potential. B, summary of percentage decrease in rheobase over time showing change was sustained up to 60 min. In contrast, the PAR<sub>2</sub>-RP has no effect.  $n = 5$  Fast Blue-labelled neurons at each time point. \* $P < 0.05$  C, summary of mean changes in input resistance at each time point showing sustained increase in input resistance. PAR<sub>2</sub>-RP had no effect.  $n = 5$  Fast Blue-labelled neurons at each time point. \* $P < 0.05$ .



**Figure 4. PAR<sub>2</sub> activation suppressed  $I_K$  currents**

**A**, representative recordings of transient ( $I_A$ ) and sustained ( $I_K$ ) K<sup>+</sup> currents before (left traces) and 10 min following the application of PAR<sub>2</sub>-AP (100  $\mu$ M) (right traces). Currents were measured in voltage clamp from a holding membrane potential of  $-100$  mV or  $-60$  mV using 400 ms steps of 15 mV from  $-90$  mV to  $+45$  mV.  $I_A$  was measured by subtracting recordings obtained at a holding membrane potential of  $-60$  mV from recordings obtained at a holding membrane potential of  $-100$  mV.  $I_A$  was measured as the peak current in the subtracted recordings.  $I_K$  was measured from the trace obtained at a holding membrane potential of  $-60$  mV at  $t = 400$  ms. **B** and **C**, summary of the mean current densities following PAR<sub>2</sub> activation for  $I_A$  and  $I_K$ , respectively.  $I_A$  peak current density was not altered, but the  $I_K$  peak current density was suppressed. Neurons were superfused for 3 min with PAR<sub>2</sub>-AP ( $n = 7$ ) or PAR<sub>2</sub>-RP ( $n = 8$ ) (100  $\mu$ M). \* $P < 0.05$ . **D**, current-voltage relationship for delayed rectifier  $I_K$  current showing onset of the PAR<sub>2</sub> effect at  $\sim -45$  mV \* $P < 0.05$ . **E**, PAR<sub>2</sub>-AP did not significantly alter  $I_A$  or  $I_K$  activation ( $G/G_{\max}$ ) or inactivation kinetics ( $I/I_{\max}$ ). Activation curves for  $I_A$  and  $I_K$  were measured using 5 mV steps from  $-80$  to  $+50$  mV, and for TTX-R sodium currents from  $-80$  to  $+10$  mV. Normalized conductance



**Table 1. The effect of PAR<sub>2</sub> activation on V<sub>50</sub> and k-values**

Experiment	PAR <sub>2</sub> -AP		PAR <sub>2</sub> -RP	
	V <sub>50</sub> (mV)	k	V <sub>50</sub> (mV)	k
<i>I<sub>A</sub></i>				
Activation	-22.7 ± 6.8	27.9 ± 9.2	-15.2 ± 4.4	21.8 ± 4.9
Inactivation	-59.2 ± 1.5	-12.7 ± 1.3	-59.6 ± 1.7	-11.9 ± 1.4
<i>I<sub>K</sub></i>				
Activation	-4.3 ± 5.2	19.8 ± 4.9	10.1 ± 5.4	26.6 ± 3.8
Inactivation	-49.7 ± 2.8	-18.2 ± 3.6	-52.1 ± 3.4	-18.5 ± 4.0
TTX-R Na <sub>v</sub>				
Activation	-43.4 ± 1.2	3.8 ± 1.0	-41.5 ± 1.0	3.9 ± 0.8
Inactivation	-29.9 ± 1.5	-5.7 ± 1.3	-31.3 ± 0.5	-5.3 ± 0.4

PAR<sub>2</sub> activation did not alter voltage of half-activation/inactivation (V<sub>50</sub>) or the slope (k) in *I<sub>A</sub>*, *I<sub>K</sub>*, or TTX-resistant Na<sup>+</sup> currents.

(Na<sub>v</sub>1.8) and/or a suppression of transient *I<sub>A</sub>*, and/or delayed rectifier *I<sub>K</sub>* potassium currents. We therefore examined whether changes in these currents may underlie the effects of PAR<sub>2</sub> activation on neuronal excitability.

***I<sub>A</sub>* and *I<sub>K</sub>* currents.** In the whole-cell voltage-clamp mode we observed a frequent run-down in K<sup>+</sup> membrane currents over a 5–10 min period. Preliminary studies using the perforated-patch-clamp mode demonstrated that, unlike in whole-cell mode, current run-down did not occur in perforated control neurons over the 15 min time period studied. The transient *I<sub>A</sub>* current was blocked by 4-aminopyridine (1 mM; *n* = 3), as previously described (Stewart *et al.* 2003).

Using the perforated-patch technique, superfusion of PAR<sub>2</sub>-AP (100 μM) (*n* = 7, 3 Fast Blue-labelled, 4 unlabelled neurons) caused a significant decrease in *I<sub>K</sub>* at 3 min (mean = 23.7%; *P* < 0.05) (Fig. 4). This persisted for the duration of the recording period and was maximal by 10 min (mean = 51.5%). No differences were observed between Fast Blue-labelled and non-labelled neurons. In contrast, PAR<sub>2</sub> activation had no effect on *I<sub>A</sub>* currents (Fig. 4). Similarly, superfusion of PAR<sub>2</sub>-RP (100 μM) had no effect on *I<sub>A</sub>* or *I<sub>K</sub>* (Fig. 4). Correlation of the current–voltage relationship (Fig. 4D) for *I<sub>K</sub>* demonstrated PAR<sub>2</sub> significantly decreased currents near -40 mV, suggesting this effect was active in the physiological range of membrane potentials.

Voltage-dependence of activation and inactivation (see Methods) were compared, to determine if changes in these parameters could contribute to the reduced current density observed following PAR<sub>2</sub> activation. There were

no significant differences in the activation curves for the *I<sub>A</sub>* or *I<sub>K</sub>* currents following superfusion of PAR<sub>2</sub>-AP (100 μM; *t* = 10 min) (*n* = 7 for activation curves, 3 Fast Blue-labelled cells) or reverse peptide (100 μM) (*n* = 8, 3 Fast Blue-labelled cells). We also found no significant difference in *I<sub>A</sub>*, or *I<sub>K</sub>* inactivation curves (Fig. 4) between cells superfused with PAR<sub>2</sub>-AP (100 μM) (*I<sub>A</sub>* and *I<sub>K</sub>* *n* = 5, unlabelled cells) and PAR<sub>2</sub>-RP. The voltage of half-activation and inactivation (V<sub>50</sub>) and corresponding slope factors (*k*) were determined from the mean of the individual curve fits and are summarized in Table 1. No significant difference was found between the PAR<sub>2</sub>-AP and corresponding PAR<sub>2</sub>-RP values for V<sub>50</sub> and *k*.

**TTX-resistant Na<sup>+</sup> currents (Na<sub>v</sub>1.8).** TTX-resistant Na<sup>+</sup> currents were obtained using whole-cell recording techniques. In TTX (1 μM), superfusion of cells with PAR<sub>2</sub>-AP (100 μM) for 3 min had no effect on TTX-resistant Na<sup>+</sup> currents (Fig. 5; *n* = 5 Fast Blue-labelled cells). Similarly, currents were not altered by the reverse peptide (100 μM) (*n* = 4, 3 Fast Blue-labelled cells). The time course of these currents was similar to those described for slow-inactivating Na<sub>v</sub>1.8 currents as apposed to ultra-slow-inactivating Na<sub>v</sub>1.9 currents (Beyak *et al.* 2004). Previous studies have shown that ATP enhances TTX-resistant Na<sup>+</sup> currents in rat DRG neurons (Park *et al.* 2004). Therefore, we also examined whether ATP alters these currents in mouse DRG neurons. When ATP (1 μM) was superfused for 3 min, TTX-resistant Na<sup>+</sup> currents were significantly increased by 3 min following the drug application (285.5%; *n* = 3 Fast Blue-labelled cells; Fig. 5). Further

(*G/G<sub>max</sub>*) was plotted against test pulse voltage and fitted to a Boltzmann function. While the activation curves for PAR<sub>2</sub>-AP and PAR<sub>2</sub>-RP do not overlap, the differences at the various voltage steps are not statistically significant. *I<sub>A</sub>* inactivation curves were generated using a two-pulse protocol: a 1 s prepulse varying between -120 and 0 mV, followed by a 400 ms test pulse of +50 mV. *I<sub>K</sub>* inactivation curves were generated using a two-pulse protocol: an 8 s prepulse varying between -80 and 0 mV followed by a 1 s test pulse of +50 mV.

studies were also conducted using perforated-patch-clamp mode to provide an additional measure that effects of PAR<sub>2</sub> activation were not masked in the whole-cell mode. Using the perforated-patch technique, mean TTX-resistant Na<sup>+</sup> currents were not altered by PAR<sub>2</sub> activation (0 min mean = 2374.2 ± 148.9 pA versus 10 min 1797.1 ± 352.0 pA).

Activation curves for TTX-resistant Na<sup>+</sup> currents were not altered (Fig. 5) by prior application of PAR<sub>2</sub>-AP (100 μM; *n* = 5, Fast Blue-labelled) compared to cells superfused with PAR<sub>2</sub>-RP (100 μM) (*n* = 4, 3 Fast Blue-labelled cells). Similarly, inactivation curves were not altered (Fig. 5) (PAR<sub>2</sub>-AP; *n* = 3, all Fast Blue-labelled; PAR<sub>2</sub>-RP; *n* = 4, all Fast Blue-labelled). No significant differences were observed between *V*<sub>50</sub> and *k* values obtained with PAR<sub>2</sub>-AP compared to PAR<sub>2</sub>-RP (Table 1). The threshold for activation of these Na<sub>v</sub>1.8 currents is more hyperpolarized than that described for other species, as previously reported (Beyak *et al.* 2004).

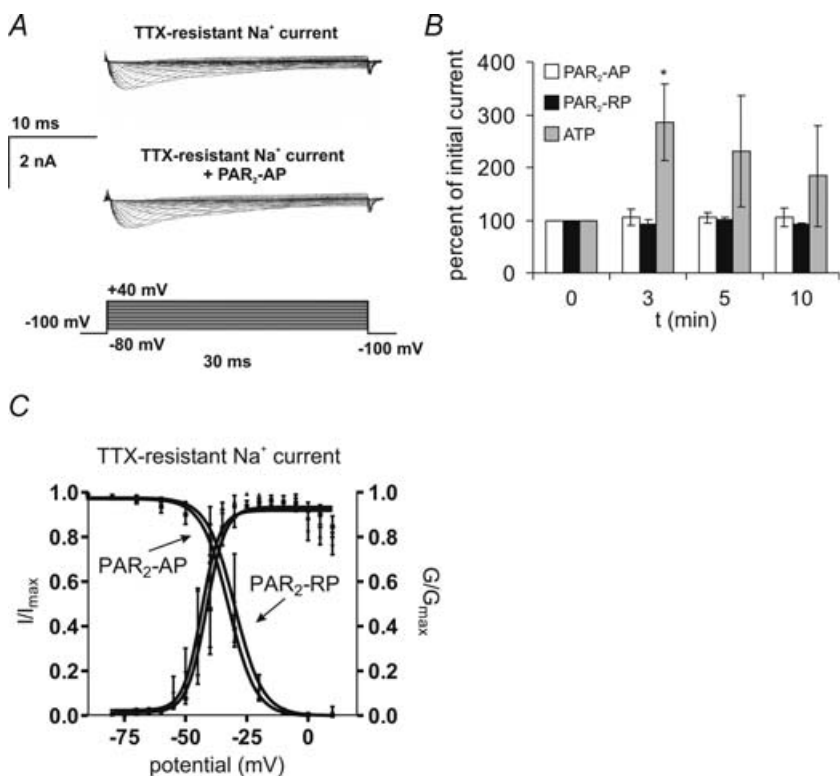
### PAR<sub>2</sub> activation of PKC and ERK<sub>1/2</sub>

**ERK<sub>1/2</sub> and PKC antagonists block PAR<sub>2</sub>-induced neuronal excitability.** To investigate whether the observed changes in DRG neuronal excitability were mediated by the activation of PKC and ERK<sub>1/2</sub>-dependent pathways, the effects of the PKC inhibitor calphostin C (1 μM) and the ERK<sub>1/2</sub> inhibitor PD98059 (100 μM) were studied. Calphostin C and PD98059 were added to the

intracellular pipette solution and superfused in the extracellular medium during electrophysiological recordings. Neurons were also incubated in PD98059 for 3 h prior to recordings.

Neurons were superfused for 3 min with the PAR<sub>2</sub>-AP (100 μM) or PAR<sub>2</sub>-RP (100 μM), and recordings were made at 3 and 10 min (Fig. 6). In the presence of either calphostin C (*n* = 5) or PD98059 (*n* = 5), superfusion of PAR<sub>2</sub>-AP had no effect on resting membrane potential, rheobase, or input resistance. These measurements of the membrane properties following PAR<sub>2</sub> activation (i.e. in the presence of inhibitors) did not differ from those obtained following the superfusion of the reverse peptide (100 μM) alone (*P* > 0.05), implying that the effects of PAR<sub>2</sub> proceed down PKC and ERK<sub>1/2</sub>-mediated pathways.

To examine the selectivity of PD98059 in DRG neurons, the PKA activator forskolin (1 μM) was studied to determine if PKA-mediated depolarizations were altered by the ERK<sub>1/2</sub> inhibitor PD98059 (Fig. 6). In these studies forskolin (1 μM) was superfused for 3 min, and changes in membrane potential were recorded at 3 and 10 min. Forskolin depolarized these neurons (*n* = 3, 2 Fast Blue-labelled) at *t* = 3 min (mean = 6.7 mV) and 10 min (mean = 6.7 mV). In separate cells, when forskolin was applied in the presence of PD98059 (100 μM; *n* = 3, 2 Fast Blue-labelled neurons), depolarizations were not substantively different (mean = 6.3 mV at *t* = 3 min, mean = 6.3 mV at *t* = 10 min) compared to those obtained with forskolin alone.

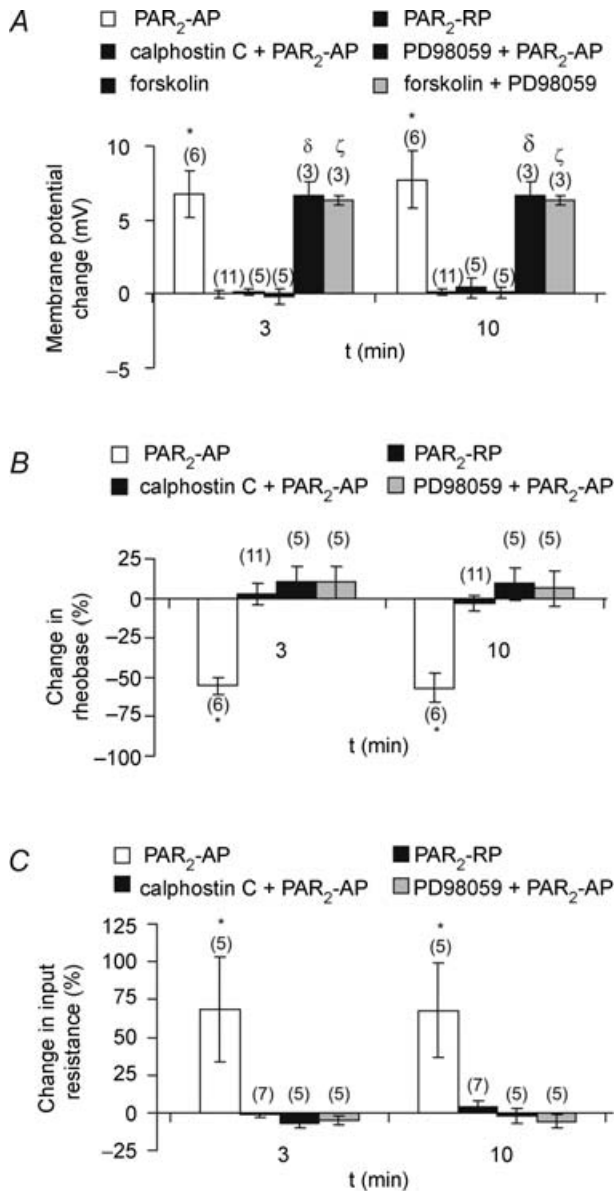


**Figure 5. The effect of PAR<sub>2</sub> activation on DRG TTX-R sodium currents**

PAR<sub>2</sub> activation did not alter TTX-resistant sodium currents (TTX-R) in DRG neurons. *A*, representative Na<sup>+</sup> currents elicited using 30 ms steps from -80 to +40 mV. TTX-resistant Na<sup>+</sup> currents were isolated with tetrodotoxin (TTX; 1 μM) applied for 3 min. PAR<sub>2</sub>-AP (100 μM) was superfused for 3 min and recordings obtained at *t* = 3, 5 and 10 min. PAR<sub>2</sub>-AP had no effect on TTX-R Na<sup>+</sup> currents. *B*, summary of change in peak Na<sup>+</sup> current evoked by application of PAR<sub>2</sub>-AP (*n* = 5) or PAR<sub>2</sub>-RP (*n* = 4). Using the protocol in *A*, cells were also superfused with ATP (1 μM; *n* = 3). ATP caused a significant increase in TTX-R Na<sup>+</sup> currents. \**P* < 0.05, two-way repeated ANOVA followed by Bonferroni post tests. *C*, PAR<sub>2</sub>-AP had no effect on TTX-R Na<sup>+</sup> activation and inactivation kinetics. TTX-R Na<sup>+</sup> current inactivation curves were generated using a two-pulse protocol, with a 1 s prepulse between -120 and 0 mV, followed by a 30 ms test pulse to 0 mV. Normalized current (*I*/*I*<sub>max</sub>) was plotted against prepulse voltage and fitted to a negative Boltzmann function.

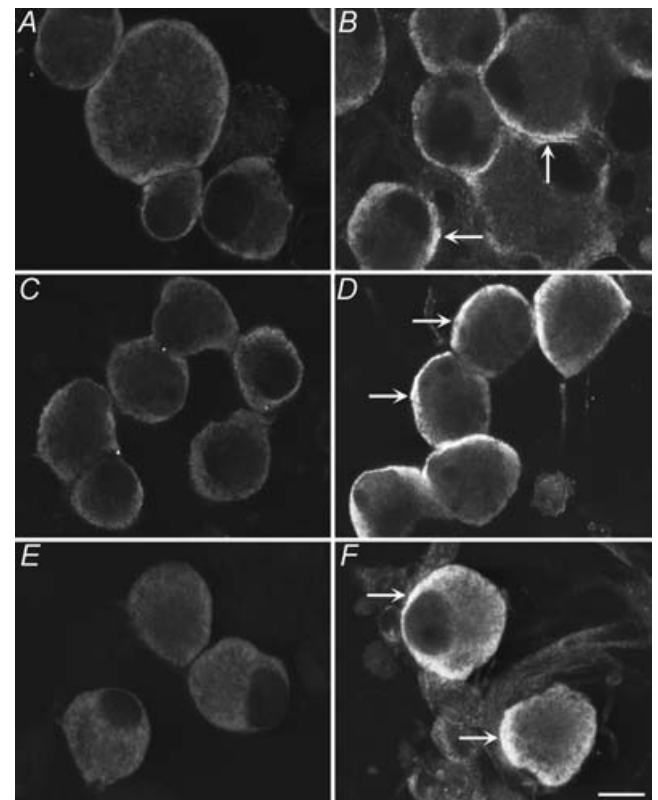
**PAR<sub>2</sub> agonists activate ERK<sub>1/2</sub> and induce phosphorylation of ERK<sub>1/2</sub> at the plasma membrane.** We have previously reported that PAR<sub>2</sub> agonists activate ERK<sub>1/2</sub> in transfected cell lines and in enterocytes that naturally express

this receptor (Defea *et al.* 2000). Since ERK<sub>1/2</sub> can phosphorylate and inhibit K<sub>v</sub>4.2 in the CNS and thereby enhance neuronal excitability (Adams *et al.* 2000), we determined if PAR<sub>2</sub> agonists phosphorylate and thus activate ERK<sub>1/2</sub> in cultured DRG neurons. In mouse DRG neurons, there were low levels of immunoreactive pERK<sub>1/2</sub> in the unstimulated state (Fig. 7). When neurons were incubated with PMA (1 μM, 5 min), as a positive control for ERK<sub>1/2</sub> activation, pERK<sub>1/2</sub> was observed in the cytosol of neurons, with the most intense signals in close proximity to the plasma membrane (Fig. 7B). Incubation of neurons with PAR<sub>2</sub>-RP (100 μM, 1–15 min), a control agonist that does not activate PAR<sub>2</sub>, had no effect on levels of detectable pERK<sub>1/2</sub> (Fig. 7C). However, when neurons were incubated with PAR<sub>2</sub>-AP (100 μM, 1–15 min), pERK<sub>1/2</sub> was detected in the cytosol of many neurons. PAR<sub>2</sub>-AP induced the appearance of pERK<sub>1/2</sub> within 1–5 min (not shown), and by 15 min there were strong signals in the cytosol in close proximity to the



**Figure 6. Calphostin C and PD98059 blocked PAR<sub>2</sub>-evoked DRG neuronal hyperexcitability**

A, summary of mean changes in membrane potential evoked by PAR<sub>2</sub>-AP at 3 and 10 min either alone or in presence of PKC inhibitor calphostin C (1 μM; applied for 3 min by superfusion and in pipette) or the ERK<sub>1/2</sub> inhibitor PD98059 (100 μM, applied for 3 h to cultures prior to application of PAR<sub>2</sub>-AP and in pipette). Depolarizations evoked by the PKA agonist forskolin (1 μM) were not inhibited by PK98059, supporting a selective action of this antagonist. B, summary of effects of inhibitors on rheobase. C, summary of effects on input resistance. For experimental protocol see Figs 2 and 3. The numbers in parentheses indicate the number of cells for every experiment. \*, δ and ζ indicate *P* < 0.05 versus the PAR<sub>2</sub>-RP; two-way repeated ANOVA followed by Bonferroni post tests.



**Figure 7. Localization of pERK<sub>1/2</sub> in cultured DRG neurons from mice (A–D) and rats (E and F)**

A, unstimulated neurons; B, PMA, 1 μM, 5 min; C, PAR<sub>2</sub>-RP, 100 μM, 15 min; D, PAR<sub>2</sub>-AP, 100 μM, 15 min; E, unstimulated neurons; F, PAR<sub>2</sub>-AP (100 μM, 15 min). When neurons were incubated with PMA or PAR<sub>2</sub>-AP, pERK<sub>1/2</sub> were detected in the cytosol in close proximity to the plasma membrane (arrows), whereas PAR<sub>2</sub>-RP (control) had no effect on the levels of pERK<sub>1/2</sub>. Representative images are shown from experiments with neurons prepared from *n* > 3 animals. Scale bar = 20 μm.

plasma membrane (Fig. 7D). Since we have previously reported that PAR<sub>2</sub> activators cause hyperexcitability of DRG neurons in rats, we also determined if they activate ERK<sub>1/2</sub> in DRG neurons from this species. There were low levels of detectable immunoreactive pERK<sub>1/2</sub> in unstimulated rat DRG neurons (Fig. 7E). When neurons were incubated with PMA (1 μM) or PAR<sub>2</sub>-AP (100 μM) for 1–15 min, pERK<sub>1/2</sub> was detected in the cytosol of many neurons in close proximity to the plasma membrane (PMA, not shown, PAR<sub>2</sub>-AP 15 min, Fig. 7F). As a control, PAR<sub>2</sub>-RP had no effect on levels of pERK<sub>1/2</sub> in rat DRG neurons (not shown). Together, these results suggest that PAR<sub>2</sub> agonists stimulate activation of ERK<sub>1/2</sub> in the region of the plasma membrane, where ERK<sub>1/2</sub> may phosphorylate and thereby control the activity of ion channels that regulate neuronal excitability.

## Discussion

This study demonstrates that PAR<sub>2</sub> agonists applied to neurons from mouse DRG caused a remarkably sustained hyperexcitability of these neurons. Several steps were taken to further clarify the relevance of this finding. Firstly, small neurons were targeted in these studies because they express properties associated with nociceptors (i.e. TTX-resistant action potentials, TRPV1 receptors, substance P and CGCP immunoreactivity) (Gold *et al.* 1996a; Yoshimura & de Groat, 1999; Moore *et al.* 2002; Stewart *et al.* 2003; Beyak *et al.* 2004; Beyak & Vanner, 2005). Secondly, electrophysiological recordings were obtained in neurons labelled with retrograde tracers injected into the wall of the colon, confirming that these findings were characteristic of nociceptors innervating the colon. Finally, we also demonstrated that labelled neurons from the colon expressed PAR<sub>2</sub> immunoreactivity. Together, these findings demonstrate that activation of PAR<sub>2</sub> on the plasma membrane of nociceptive DRG neurons innervating the mouse colon leads to sustained hyperexcitability.

### Sustained neuronal hyperexcitability

The long-lasting changes in membrane potential and rheobase observed in this study lasted up to 1 h after a brief application of the PAR<sub>2</sub> agonist. We have observed similar sustained effects on enteric neurons (Reed *et al.* 2003) and other studies of unidentified rat DRG neurons (Amadesi *et al.* 2004). The specificity of the PAR<sub>2</sub> agonists used in the current study has been carefully demonstrated in our previous studies (Reed *et al.* 2003; Amadesi *et al.* 2004) and others (Nystedt *et al.* 1994; Dai *et al.* 2004). Consistent with these observations, in the present study we found that PAR<sub>2</sub>-AP had a dose-dependent effect, which was mimicked by trypsin, whereas the reverse peptide, which lacks biological activity at PAR<sub>2</sub>, had no effect. This specificity has also been observed in studies

demonstrating that PAR<sub>2</sub>-AP directly activates intestinal afferents in anaesthetized animals (Kirkup *et al.* 2003).

### Ionic mechanism(s) underlying sustained hyperexcitability

There is considerable evidence that voltage-gated Na<sup>+</sup> and K<sup>+</sup> channels underlying action potential electrogenesis are modulated by visceral inflammation or specific inflammatory mediators (Gold *et al.* 1996a; Yoshimura & de Groat, 1999; Gebhart *et al.* 2002; Beyak *et al.* 2004; Dang *et al.* 2004; Beyak & Vanner, 2005). The most common findings are that TTX-resistant Na<sup>+</sup> currents (Na<sub>v</sub>1.8) are increased and/or voltage-gated K<sup>+</sup> currents, namely I<sub>A</sub> and I<sub>K</sub>, are suppressed. However, the specific channel(s) affected may be dependent on the nature of the inflammation (Beyak & Vanner, 2005c) and/or the inflammatory mediator.

In the current study, mouse colonic DRG neurons exhibited transient I<sub>A</sub> and sustained delayed-rectifier I<sub>K</sub> voltage-gated K<sup>+</sup> currents (see Fig. 4) characteristic of other DRG neurons (McFarlane & Cooper, 1991; Stewart *et al.* 2003; Dang *et al.* 2004; Beyak & Vanner, 2005). The voltage and 4-AP sensitivity (Stewart *et al.* 2003) of the transient K<sup>+</sup> currents were typical of I<sub>A</sub> currents, and the kinetics of the I<sub>K</sub> currents match those described in other species (Stewart *et al.* 2003; Dang *et al.* 2004). We found that the hyperexcitability following PAR<sub>2</sub> activation was associated with a profound suppression of the I<sub>K</sub> currents (>50%) but not of I<sub>A</sub>. The effect was active in the physiological range of membrane potentials (Fig. 4) (i.e. around the threshold for action potential electrogenesis), and therefore may contribute to the decrease in rheobase observed with PAR<sub>2</sub> activation. However, models of visceral inflammation in the ileum, stomach and bladder (Yoshimura & de Groat, 1999; Stewart *et al.* 2003; Dang *et al.* 2004) have also shown that I<sub>A</sub> is suppressed in models of chronic inflammation, supporting the notion that individual mediators may signal preferentially to selective K<sup>+</sup> channels. Previous studies of the biophysical and pharmacological properties of I<sub>K</sub> currents suggest these currents may comprise three distinct currents (Gold *et al.* 1996b). The molecular determinants of the channels underlying these I<sub>K</sub> currents are currently unknown although studies suggest candidates of the α-subunits include K<sub>v</sub> 2.1/2.2 and 3.1/3.2 (Song, 2002; Kim *et al.* 2002).

In addition to the observed changes in I<sub>K</sub> currents, our findings also suggest that PAR<sub>2</sub> activation affects other K<sup>+</sup> currents. The increases in membrane depolarization and input resistance following PAR<sub>2</sub> activation cannot be accounted for by I<sub>K</sub> currents, because they were not open at the resting membrane potential. It is probable that these changes result from closure of background or 'leak' K<sup>+</sup>-selective channels. These channels may be members

of the two, and possibly four, transmembrane segment K<sup>+</sup> channels (Patel & Honore, 2001), which are important targets of neurotransmitters and paracrine agents.

An array of inflammatory mediators including adenosine, 5-HT, ATP, PGE<sub>2</sub> and NGF have been found to enhance TTX-resistant Na<sup>+</sup> currents (Na<sub>v</sub>1.8) in nociceptive DRG neurons (Gold *et al.* 1996a; Cardenas *et al.* 2001; Zhang *et al.* 2002; Park *et al.* 2004; Beyak & Vanner, 2005). In models of established inflammation in the stomach, ileum, colon, and bladder (Yoshimura & de Groat, 1999; Gebhart *et al.* 2002; Stewart *et al.* 2003; Beyak *et al.* 2004) these currents were also found to be increased in neurons innervating the inflamed segments. Somewhat surprisingly, in our study PAR<sub>2</sub> activation had no effect on this current. To demonstrate we could induce current increases in our neurons, we also tested ATP and found that this mediator enhanced currents, as predicted by other studies (Park *et al.* 2004). Recent studies have also suggested TTX-sensitive Na<sup>+</sup> currents (Na<sub>v</sub>1.7) may also be modulated by inflammation (Black *et al.* 2004). We cannot conclude that PAR<sub>2</sub> activation does not modulate these currents because they were not examined in our study. However, this seems unlikely given that previous studies examining the effects of inflammation either failed to observe an effect on TTX-sensitive Na<sup>+</sup> current (Yoshimura & de Groat, 1999; Gebhart *et al.* 2002; Beyak *et al.* 2004) or, in the recent study, suggested an effect on TTX-sensitive Na<sup>+</sup> currents (Black *et al.* 2004), which paralleled the effects on TTX-resistant Na<sup>+</sup> currents. We also did not examine the possibility that TTX-resistant Na<sub>v</sub>1.9 currents were modulated by PAR<sub>2</sub> activation. However our previous studies (Beyak *et al.* 2004) suggest these currents are not frequently found in nociceptive DRG neurons innervating the mouse colon, and furthermore other studies suggest that measurement of pharmacological effects on these currents can be extremely difficult (Maruyama *et al.* 2004). Finally, although our findings strongly suggest that PAR<sub>2</sub> activation does not directly modulate Na<sub>v</sub>1.8 currents, this may occur through indirect mechanisms in the whole animal because PAR<sub>2</sub> stimulates PGE<sub>2</sub> and CGRP release from nerve terminals (Steinhoff *et al.* 2000; Vergnolle *et al.* 2003) and these agents in turn can enhance the Na<sup>+</sup> currents (Baker, 2005; Natura *et al.* 2005).

### **PAR<sub>2</sub> signal transduction mechanisms of hyperexcitability in colonic DRG neurons**

The intracellular mechanism(s) that underlie the sustained hyperexcitability evoked by PAR<sub>2</sub> activation on DRG neurons described in this study and others is unknown. Our findings suggest ERK<sub>1/2</sub> signalling may play a major role because PAR<sub>2</sub> activation stimulates phosphorylation of ERK<sub>1/2</sub> at the plasma membrane (Fig. 7) where it could modulate ion channels, and the highly selective ERK<sub>1/2</sub>

inhibitor (PD98059) (Thomas & Huganir, 2004) blocked the PAR<sub>2</sub>-mediated effects on neuronal excitability (Fig. 6). We were specifically interested in examining the potential role of ERK<sub>1/2</sub> signalling because recent reports in the CNS (Thomas & Huganir, 2004) suggest that ERK<sub>1/2</sub> signals not only to nuclear pathways regulating gene expression but also to cytoplasmic proteins including voltage-gated K<sup>+</sup> channels. Moreover, phosphorylation of these channels in an ERK-dependent manner in the hippocampus underlies some forms of sustained excitability. Several recent studies have also demonstrated activation of ERK<sub>1/2</sub> in peripheral DRG neurons (Seino *et al.* 2006; Takahashi *et al.* 2006).

The signal transduction pathway by which PAR<sub>2</sub> agonists activate ERK<sub>1/2</sub> in neurons also remains to be defined. However, possible mechanisms include the trans-activation of growth factor receptors, activation of protein kinase C, and  $\beta$ -arrestin-dependent activation (Defea *et al.* 2000; Darmoul *et al.* 2004). We did observe that calphostin, a PKC blocker, also inhibited the PAR<sub>2</sub>-induced neuronal hyperexcitability (see Fig. 6), but further studies are needed to examine the interaction of PKC and ERK<sub>1/2</sub>.  $\beta$ -arrestin-dependent mechanisms may be of particular importance in targeting activated ERK<sub>1/2</sub> to the plasma membrane, where they may phosphorylate and regulate ion channels (Defea *et al.* 2000; Luttrell & Lefkowitz, 2002). Agonists of many G-protein-coupled receptors, including PAR<sub>2</sub>, stimulate the translocation of  $\beta$ -arrestins from the cytosol to phosphorylate receptors at the plasma membrane (Dery *et al.* 1999; Luttrell & Lefkowitz, 2002).  $\beta$ -Arrestins act to uncouple receptors from heterotrimeric G-proteins and thereby desensitize G-protein signalling, couple G-protein-coupled receptors to clathrin and AP<sub>2</sub>, and thereby mediate receptor endocytosis.  $\beta$ -Arrestins are also molecular scaffolds that recruit and organize components of the MAP kinase pathway to activated G-protein-coupled receptors, thereby determining the subcellular location and function of activated ERK<sub>1/2</sub>. We have shown that agonists of PAR<sub>2</sub> promote the assembly of a stable signalling complex in cells that includes PAR<sub>2</sub>,  $\beta$ -arrestins, raf-1, MEKK and pERK<sub>1/2</sub> (Defea *et al.* 2000). This complex acts to prevent the trafficking of activated ERK<sub>1/2</sub> to the nucleus and instead retain ERK<sub>1/2</sub> within the cytosol, where it could phosphorylate and regulate other targets, including ion channels. Whether this mechanism occurs in neurons and permits ERK<sub>1/2</sub> to regulate ion channels that mediate PAR<sub>2</sub>-induced hyperexcitability, such as those underlying I<sub>K</sub> currents, remains to be determined.

### **Implications of PAR<sub>2</sub>-evoked sustained hyperexcitability of nociceptive DRG neurons in the colon**

There is growing evidence that the release of proteases from mast cells plays an important role in the genesis of

visceral hyperalgesia in functional bowel disorders such as IBS (Barbara *et al.* 2004; Spiller & Campbell, 2006; Barbara *et al.* 2006). Studies have demonstrated that intestinal mast cell numbers are increased, at least in subsets of patients, and that there is evidence of increased degranulation and tissue content of mast cell tryptase. Moreover, preliminary studies have shown that tryptase in the supernatant of colonic biopsies from IBS patients can stimulate calcium signals in DRG neurons (Cenac *et al.* 2005), suggesting activation of these neurons. The proximity of mast cells to nerve terminals in the intestine has also been correlated with the severity of abdominal pain (Barbara *et al.* 2004). Numerous *in vitro* and *in vivo* animal studies have also shown that proteases, such as tryptase, released from degranulating mast cells have similar actions to those induced by the PAR<sub>2</sub> agonists used in this study. When taken together, the findings of the current study combined with these previous studies suggest it is possible that the sustained excitability of nociceptive DRG neurons following PAR<sub>2</sub> activation could be important in the genesis of abdominal pain in patients with IBS, and that repeated mast cell degranulation may lead to ongoing 're-priming' of these neurons.

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