University of Reading

Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice

Article

Published Version

Open Access (Online Open)

Grant, A. D., Cottrell, G. S. ORCID: https://orcid.org/0000-0001-9098-7627, Amadesi, S., Trevisani, M., Nicoletti, P., Materazzi, S., Altier, C., Cenac, N., Zamponi, G. W., Bautista-Cruz, F., Lopez, C. B., Joseph, E. K., Levine, J. D., Liedtke, W., Vanner, S., Vergnolle, N., Geppetti, P. and Bunnett, N. W. (2007) Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice. Journal of Physiology, 578 (3). pp. 715-733. ISSN 0022-3751 doi: 10.1113/jphysiol.2006.121111 Available at https://centaur.reading.ac.uk/30269/

It is advisable to refer to the publisher's version if you intend to cite from the work. See Guidance on citing.

To link to this article DOI: http://dx.doi.org/10.1113/jphysiol.2006.121111

Publisher: Wiley-Blackwell

University of Reading

All outputs in CentAUR are protected by Intellectual Property Rights law, including copyright law. Copyright and IPR is retained by the creators or other copyright holders. Terms and conditions for use of this material are defined in the End User Agreement.

www.reading.ac.uk/centaur

CentAUR

Central Archive at the University of Reading

Reading's research outputs online

Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice

Andrew D. Grant[1], Graeme S. Cottrell[1], Silvia Amadesi[1], Marcello Trevisani[2], Paola Nicoletti[2], Serena Materazzi[2], Christophe Altier[3], Nicolas Cenac[3], Gerald W. Zamponi[4], Francisco Bautista-Cruz[5,6], Carlos Barajas Lopez[5,6], Elizabeth K. Joseph[7], Jon D. Levine[7], Wolfgang Liedtke[8], Stephen Vanner[6], Nathalie Vergnolle[3], Pierangelo Geppetti[2] and Nigel W. Bunnett[1]

[1] *Departments of Surgery and Physiology, University of California, San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143-0660, USA*
[2] *Department of Critical Care Medicine and Surgery, University of Florence, Florence, Italy*
[3] *Department of Physiology and Biophysics and* [4] *Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada*
[5] *Instituto Potosino de Investigacion Cientifica y Tecnologica San Luis Potosi, SLP, Mexico*
[6] *Gastrointestinal Diseases Research Unit, Division of Gastroenterology, Queen's University, Kingston, Ontario, Canada*
[7] *Departments of Oral and Maxofacial Surgery and Medicine, University of California, San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143-0660, USA*
[8] *Department of Medicine and Neurobiology, Duke University Medical Center 2900, Durham, NC 27710, USA*

Exacerbated sensitivity to mechanical stimuli that are normally innocuous or mildly painful (mechanical allodynia and hyperalgesia) occurs during inflammation and underlies painful diseases. Proteases that are generated during inflammation and disease cleave protease-activated receptor 2 (PAR_2) on afferent nerves to cause mechanical hyperalgesia in the skin and intestine by unknown mechanisms. We hypothesized that PAR_2-mediated mechanical hyperalgesia requires sensitization of the ion channel transient receptor potential vanilloid 4 (TRPV4). Immunoreactive TRPV4 was coexpressed by rat dorsal root ganglia (DRG) neurons with PAR_2, substance P (SP) and calcitonin gene-related peptide (CGRP), mediators of pain transmission. In PAR_2-expressing cell lines that either naturally expressed TRPV4 (bronchial epithelial cells) or that were transfected to express TRPV4 (HEK cells), pretreatment with a PAR_2 agonist enhanced Ca^{2+} and current responses to the TRPV4 agonists phorbol ester 4α-phorbol 12,13-didecanoate (4αPDD) and hypotonic solutions. PAR_2-agonist similarly sensitized TRPV4 Ca^{2+} signals and currents in DRG neurons. Antagonists of phospholipase Cβ and protein kinases A, C and D inhibited PAR_2-induced sensitization of TRPV4 Ca^{2+} signals and currents. 4αPDD and hypotonic solutions stimulated SP and CGRP release from dorsal horn of rat spinal cord, and pretreatment with PAR_2 agonist sensitized TRPV4-dependent peptide release. Intraplantar injection of PAR_2 agonist caused mechanical hyperalgesia in mice and sensitized pain responses to the TRPV4 agonists 4αPDD and hypotonic solutions. Deletion of TRPV4 prevented PAR_2 agonist-induced mechanical hyperalgesia and sensitization. This novel mechanism, by which PAR_2 activates a second messenger to sensitize TRPV4-dependent release of nociceptive peptides and induce mechanical hyperalgesia, may underlie inflammatory hyperalgesia in diseases where proteases are activated and released.

(Received 12 September 2006; accepted after revision 21 November 2006; first published online 23 November 2006)
Corresponding author N. W. Bunnett: UCSF, 513 Parnassus Ave., Room S1268, San Francisco, CA 94143-0660, USA. Email: nigel.bunnett@ucsf.edu

The ability to detect mechanical stimuli allows organisms to respond to their environment. High-intensity mechanical stimuli can damage tissue and provoke pain, leading to avoidance behaviours. Inflammatory mediators enhance sensitivity to mechanical stimuli that are normally innocuous or mildly painful (mechanical allodynia or hyperalgesia, respectively), resulting in pain associated with disorders such as arthritis, inflammatory bowel disease and irritable bowel syndrome. However, the ion channels that transduce mechanical stimuli are

© 2007 The Authors. Journal compilation © 2007 The Physiological Society DOI: 10.1113/jphysiol.2006.121111

not unequivocally identified, and the mechanisms by which inflammation causes mechanical allodynia and hyperalgesia are incompletely understood. Consequently, the treatments for these painful conditions are inadequate.

Proteases are prominent mediators of inflammation and pain. Injury, inflammation and disease trigger the production of numerous serine proteases from the circulation (e.g. coagulation factors), inflammatory cells (e.g. mast cell tryptase, neutrophil cathepsin G) and epithelial tissues (e.g. trypsin IV, kallikreins) that regulate cells by cleaving protease-activated receptors (PARs), a family of four G protein-coupled receptors (Ossovskaya & Bunnett, 2004). Proteolysis unmasks a tethered ligand domain, which binds to and activates the receptor. This irreversible mechanism of activation controls haemostasis, inflammation, pain and repair after tissue injury. PAR_2, a receptor for trypsins (Nystedt *et al.* 1994; Bohm *et al.* 1996*b*; Cottrell *et al.* 2004), tryptase (Corvera *et al.* 1997; Molino *et al.* 1997), coagulation factors FVIIa and FXa (Camerer *et al.* 2000) and kallikreins (Oikonomopoulou *et al.* 2006), is an important proinflammatory and nociceptive mediator. PAR_2 is expressed by primary spinal afferent neurons of dorsal root ganglia (DRG) containing the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) (Steinhoff *et al.* 2000). These neurons contribute to neurogenic inflammation and nociception. Agonists of PAR_2 (e.g. tryptase, secreted by mast cells adjacent to nerve fibres) stimulate the release of SP and CGRP from afferent nerves (Steinhoff *et al.* 2000). When released from peripheral nerve endings in the skin and intestine, SP and CGRP cause plasma extravasation, granulocyte infiltration and hyperaemia (i.e. neurogenic inflammation) (Steinhoff *et al.* 2000; Cenac *et al.* 2003; Nguyen *et al.* 2003). PAR_2 agonists also stimulate peptide release from the central endings of afferent nerves in the dorsal horn of the spinal cord, to cause thermal and mechanical hyperalgesia (Vergnolle *et al.* 2001; Coelho *et al.* 2002). This thermal hyperalgesia depends on sensitization of the transient receptor potential vanilloid 1 (TRPV1) ion channel, which enhances the activity of nociceptive fibres and consequent peptide release (Amadesi *et al.* 2004, 2006; Dai *et al.* 2004). The mechanism of PAR_2-induced mechanical hyperalgesia is unknown.

TRPV4, the mammalian homologue of the *C. elegans* gene *Osm-9* (Liedtke *et al.* 2003), is a potential mediator of mechanical hyperalgesia. TRPV4 is gated by altered tonicity and by temperatures $>27^{\circ}$C (Liedtke *et al.* 2000; Guler *et al.* 2002). Hypo-osmotic stimuli cause cell swelling, phospholipase A_2 activation and generation of arachidonic acid (Pedersen *et al.* 2000). A cytochrome P450 product of arachidonic acid, 5′,6′-epoxyeicosatrienoic acid, activates TRPV4 and is a potential endogenous agonist (Watanabe *et al.* 2003). The phorbol ester 4α-phorbol 12,13-didecanoate (4αPDD) is a synthetic TRPV4 agonist (Watanabe *et al.* 2002). The expression of TRPV4 by neurosensory structures, including circumventricular organs, inner ear hair cells, Merkel cells and sensory neurons, and its activation by hypotonic stimuli, suggest that it functions to detect osmotic and mechanical stimuli. $TRPV4^{-/-}$ mice show abnormal osmotic regulation and decreased nociceptive responses to pressure (Liedtke & Friedman, 2003; Suzuki *et al.* 2003), and TRPV4 knockdown or deletion reduces nociceptive responses to hypotonic and mildly hypertonic stimuli (Alessandri-Haber *et al.* 2003, 2005). Moreover, inflammatory agents can sensitize TRPV4 by mechanisms that are not fully characterized (Alessandri-Haber *et al.* 2003, 2006), suggesting that this channel mediates inflammatory hyperalgesia.

We examined the hypothesis that PAR_2 agonists sensitize TRPV4 and thereby enhance release of SP and CGRP in the dorsal horn of the spinal cord to cause mechanical hyperalgesia. To do so we (a) determined if PAR_2 agonists sensitize TRPV4 Ca^{2+} signals and currents in cell lines and DRG neurons; (b) characterized signalling pathways that mediate sensitization; (c) examined whether TRPV4 is expressed in nociceptive neurons with PAR_2, SP and CGRP; (d) determined if PAR_2 agonists sensitize TRPV4-induced SP and CGRP release in the spinal cord; and (e) examined whether TRPV4 deletion prevents PAR_2-induced mechanical hyperalgesia.

Methods

Animals

Sprague-Dawley rats (male, 200–300 g) and C57BL6 mice (male, 20–25 g) were from Charles River Laboratories (Wilmington, MA). $TRPV4^{+/+}$ and $TRPV4^{-/-}$ mice (male, 20–30 g) have been described (Liedtke & Friedman, 2003). Institutional Animal Care and Use Committees approved all procedures. Animals were killed by sodium pentobarbital (200 mg kg^{-1}, I.P.) and bilateral thoracotomy.

Agonists and antagonists

Peptides corresponding to the tethered ligand domains of PARs (activating peptides, APs) can selectively activate these receptors, whereas the reverse sequences (reverse peptides, RPs) are inactive control reagents. Mouse/rat PAR_2-AP (SLIGRL-NH2) and inactive PAR_2-RP (LRGILS-NH2) were from SynPep Corp. (Dublin, CA, USA). The PAR_1-selective agonist *Xenopus* PAR_1-AP (TFLLRN-NH_2) and inactive PAR1-RP (NRLLFT-NH_2) were from Sigma Genosys (Woodland, TX, USA). Capsaicin, 4αPDD, H-89 (*N*-[2-((*p*-bromocinnamyl)-amino)ethyl]-5-isoquinolinesulphonamide) and calphostin C were from Sigma (St Louis, MO, USA).

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

GF109203X (bisindolylmaleimide I), Gö6976 and Gö6983 were from Calbiochem (La Jolla, CA, USA). U73122 was from Tocris (Ellisville, MO, USA).

TRPV4 and PAR$_2$ RT-PCR and TRPV4 cloning

Total RNA was extracted from 16HBE14o$^-$ (HBE) cells and rat and mouse DRG (T12-L6) using Trizol®. (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed with oligo(dT)$_{12-18}$ or random hexamers and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) or TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). Control reactions omitted reverse transcriptase. Products were amplified using primers specific to human, rat and mouse TRPV4 or human PAR$_2$ (human TRPV4: forward 5′-ACATGCGGGAGTTCATTAAC-3′, reverse 5′-CACAGCCAGCATCTCGTGGCG-3′; rat TRPV4: forward 5′-TGGGAGGTATCACCCTTCTG-3′, reverse 5′-AGCCAGCATCTCATGGCG-3′; mouse TRPV4: forward 5′-ATCAACTCGCCCTTCAGAGA-3′, reverse 5′-CCCAAACTTACGCCACTTGT-3′; human PAR$_2$: forward 5′-CCCTTTGTATGTCGTGAAGCAGAC-3′, reverse 5′-TTCCTGGAGTGTTTCTTTGAGGTG-3′). Products were separated by electrophoresis (2% agarose gel), detected using ethidium bromide and sequenced to confirm identity. Human TRPV4 was cloned by PCR from HBE cells using the primers forward 5′-ATTGGATCCCCACCATGGCGGATTCCAGCGAAGG-3′ and reverse 5′-AATCTCGAGCTAGAGCGGGGCGTCATCAGTCC-3′. An HA.11 tag was added to the C-terminus of human TRPV4 by PCR using the primer 5′-AATCTCGAGCTAGGCGTAGTCGGGCACGTCGTAGGGGTAGAGCGGGGCGTCATCAGTCC-3′. The PCR product was subcloned into the *Hin*dIII and *Xho*I sites of pcDNA5/FRT or the tetracycline-inducible vector pcDNA5/FRT/TO (Invitrogen, Carlsbad, CA, USA).

Cell lines

The human bronchial epithelial cell line 16HBE14o- (HBE) was maintained in Modified Eagle's Medium (MEM) supplemented with 10% fetal bovine serum, penicillin (100 u ml^{-1}) and streptomycin (100 μg ml^{-1}). Human embryonic kidney (HEK) 293 cells were maintained in MEM with 10% fetal bovine serum. Cells were grown in 95% air, 5% CO_2 at 37°C. HEK cells were transiently transfected with TRPV4 using Lipofectamine^{2000} (Invitrogen) and designated HEK-TRPV4 cells. A tetracycline-inducible system was used to generate stable cell lines expressing TRPV4, since continuous overexpression of this channel was toxic to cells. HEK-FLPTREX cells were stably transfected with pcDNA5/FRT/TO + hTRPV4 (designated HEK-FLPTREX-TRPV4 cells) and grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% tetracycline-free fetal bovine serum, hygromycin (50 μg ml^{-1}) and blasticidin (10 μg ml^{-1}). To induce TRPV4 expression, tetracycline (0.1 μg ml^{-1}) was added to the medium 16 h before use.

Dispersion and culture of rat DRG neurons

DRG from thoracic and lumbar spinal cord of rats were minced in cold Hanks' Balanced Salt Solution (HBSS) and incubated for 60–90 min at 37°C in DMEM containing 0.5 mg ml^{-1} of trypsin, 1 mg ml^{-1} of collagenase type IA and 0.1 mg ml^{-1} of DNAse type IV (Sigma) (Steinhoff *et al.* 2000). Soybean trypsin inhibitor (Sigma) was added to neutralize trypsin. Neurons were pelleted, suspended in DMEM containing 10% fetal bovine serum, 10% horse serum, 100 u ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, 2 mM glutamine and 2.5 μg ml^{-1} DNAse type IV, plated on glass coverslips coated with Matrigel (BD Biosciences, Bedford, MA, USA), and cultured for 2–3 days.

Dispersion and culture of mouse DRG neurons

DRG from thoracic and lumbar spinal cord of mice were minced in cold HBSS, incubated for 15 min at 37°C in HBSS containing 0.5% papain, washed with Leibovitz's L-15 medium (supplemented with 2 mM glutamine, 0.2% glucose and 2.5% fetal bovine serum), and incubated for 10 min at 37°C in HBSS containing 1 mg ml^{-1} collagenase type I and 4 mg ml^{-1} dispase II (Sigma). Neurons were pelleted, suspended in DMEM containing 2.5% fetal bovine serum, 1% penicillin/streptomycin, 1% dextrose, 2 mM glutamine and 10 μM of arabinocytidine hydrochloride, floxuridine and uridine. Neurons were plated in poly L-orthinine-laminine-treated glass-bottomed dishes and studied immediately.

Immunofluorescence

HEK cells (transiently transfected with wild-type or HA.11-tagged TRPV4 or vector without insert) and cultured rat DRG were fixed in 4% paraformaldehyde in 100 mM PBS pH 7.4, for 20 min at 4°C. Rats were transcardially perfused with 4% paraformaldehyde in 100 mM PBS pH 7.4. DRG and spinal cord (L3–L6) were removed and fixed in 4% paraformaldehyde for 6 h at room temperature. Tissues were washed, incubated in 25% sucrose in PBS overnight at 4°C, and embedded in OCT. Sections of DRG (16–18 μm) were cut and mounted on poly L-lysine-coated slides. Sections of spinal cord (16 μm) were processed as floating sections. Cultured cells were washed and incubated with 100 mM PBS, pH 7.4 containing 1% normal goat serum and 0.1%

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

saponin. Tissue sections were washed and incubated in 100 mM PBS, pH 7.4, containing 5% normal goat serum and 0.3% Triton X-100. Cells and tissues were incubated with the following primary antibodies: rat anti-HA.11 (Roche, IN; 1 : 1000); rabbit anti-TRPV4 (Alomone, Israel; 1 : 750); mouse anti-PAR_2 (SAM11, Santa Cruz, CA, USA; 1 : 250); mouse anti-CGRP (no. 4901, CURE-UCLA, CA; 1 : 500); guinea pig antisubstance P (Chemicon, Temecula, CA, USA; 1 : 1000) (all overnight, 4°C). In controls, the TRPV4 antiserum was preabsorbed by preincubation with the antigen (10 μM, 24 h, 4°C) before staining. Cells and tissues were washed and incubated with goat antirat, antirabbit, antimouse or antiguinea pig IgG conjugated to fluorescein isothiocyanate or rhodamine red X (Jackson Immuno-Research, West Grove, PA, USA; 1 : 200; 2 h, room temperature). Specimens were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) for cultured cells or Prolong Gold (Invitrogen) for tissue sections.

Confocal microscopy

Specimens were observed using a Zeiss Axiovert microscope with a Bio-Rad MRC1000 confocal microscope and Zeiss Fluar Plan Apo ×40 (NA 1.4) or ×100 (NA 1.3) objectives. Images were collected with an iris of <3, zoom of 1–2 and typically 5–10 optical sections were taken at intervals of 0.5–1.0 μm. Presented images are single optical sections. Images were processed to adjust contrast and brightness and were coloured to represent appropriate fluorophores, using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA). Images of stained and control slides were collected and processed identically.

Western blotting

HEK-TRPV4a cells were washed with ice-cold PBS, scraped into 300 μl lysis buffer (50 mM Tris/HCl, 1% SDS, pH 7.4), boiled and centrifuged. Protein concentration in the supernatant was measured by BCA assay. Samples (10 μg protein) were separated by SDS-PAGE (8% gel), and transferred to Immobilon-FL membrane (Millipore, Billerica, MA, USA). Membranes were incubated overnight with rat anti-HA.11 (Roche, Indianapolis, IN; 1 : 5000), washed, and incubated for 1 h with goat antirat AlexaFluor680 antibody (Invitrogen, 1 : 20 000). Immunoreactive protein was detected using a Licor Odyssey Scanner (Licor, Lincoln, NE, USA).

Measurement of $[Ca^{2+}]_i$ in cell lines

HBE and HEK-TRPV4 cells grown on poly D-lysine-coated coverslips were washed and incubated in HBSS (pH 7.4) containing Ca^{2+} and Mg^{2+}, 20 mM Hepes buffer, 0.1% BSA, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, with 2.5 μM Fura-2AM (Invitrogen) for 20 min at 37°C. Cells were washed, and fluorescence was measured at 340 nm and 380 nm excitation and 510 nm emission in an F-2500 spectrophotometer (Hitachi Instruments, San Jose, CA, USA). Test substances were injected directly into the chamber (20 μl into 2 ml). Cells were challenged once with 4αPDD (0.1–10 μM) or osmotic stimuli (310–260 mosmol l^{-1}), or were pretreated with PAR_2-AP, PAR_2-RP, PAR_1-AP or PAR_1-RP (100 μM) for 5 min followed by 4αPDD or osmotic stimuli. In some experiments, cells were pretreated for 30 min with inhibitors (U73122, 10 μM; H-89, 10 μM; GF109203X, 10 μM; Gö6976, 0.1 μM; Gö6983, 0.1 μM) or vehicle (control) before the challenge with the test compound. In experiments where inhibitors were used, cells were pretreated with PAR_2-AP or PAR_2-RP for 10 min before TRPV4 agonists, since some inhibitors (e.g. GF109203X) delayed the return of $[Ca^{2+}]$ to baseline after stimulation with PAR_2 agonist. Results are expressed as the 340/380 nm emission ratio, which is proportional to the $[Ca^{2+}]_i$.

Measurement of $[Ca^{2+}]_i$ in DRG neurons

Rat DRG neurons grown on Matrigel-coated coverslips for 2–3 days after isolation were incubated in HBSS (pH 7.4) containing Ca^{2+} and Mg^{2+}, 20 mM Hepes, 0.1% BSA with 5 μM of Fura-2AM for 45 min at 37°C. Coverslips were mounted in an open chamber at room temperature. Fluorescence of individual cells was measured at 340 nm and 380 nm excitation and 510 nm emission using a Zeiss Axiovert microscope, an ICCD video camera (Stanford Photonics, Stanford, CA, USA) and a video microscopy acquisition program (Axon Instruments, Inc, Union City, CA, USA). Test substances were directly added to the chamber (50 μl into 350 μl). Neurons were preincubated with PAR_2-AP or PAR_2-RP (10 μM) for 20 min followed by 4αPDD (10 μM). In some experiments, cells were pretreated for 30 min with inhibitors (GF109203X, 1 μM; H-89, 10 μM) or vehicle (control) before the challenge with the test compound. DRG preparations were challenged with KCl at the end of each experiment, to identify neurons. Results are expressed as the 340/380 nm emission ratio.

Electrophysiology in DRG neurons

Whole-cell membrane currents of freshly dispersed mouse DRG neurons were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). The pipette solution contained (mM): 110 CsCl, 3 $MgCl_2$, 10 EGTA, 10 Hepes, 3 Mg-ATP, 0.6 GTP, pH 7.2 with CsOH, 315 mosmol l^{-1}. The extracellular solution contained (mM): 120 NaCl, 5 KCl, 5 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 Hepes, pH 7.4 with NaOH, 310 mOsm. Pipette resistance

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

was 2–4 MΩ. Whole-cell currents were recorded and analysed using Clampfit 9.2 software (Molecular Devices, Sunnyvale, CA, USA). Neurons were held at 0 mV to inactivate voltage-gated calcium and sodium channels, and a 150 ms linear ramp protocol was applied (−100 mV to +100 mV every 15 s). Current amplitude at −80 and +80 mV was normalized to cell capacitance to obtain current densities. Cells that did not display any detectable whole-cell currents were not included in the analysis. Sampling frequency for acquisition was 10 kHz, and data were filtered at 2 kHz. Neurons were preincubated with PAR$_2$-AP (10 μM) or vehicle for 20 min, and then challenged with 4αPDD (10 μM).

Electrophysiology in HEK-FLPTREX-TRPV4a cells

Whole-cell membrane currents of HEK-FLPTREX-TRPV4a cells were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) as described (Amadesi *et al.* 2006). The pipette solution contained (mM): KCl 140, $MgCl_2$ 3, EGTA 5, Hepes 5, and $ATPNa_2$ 5. The external solution contained (mM): NaCl 150, $CaCl_2$ 2, KCl 6, $MgCl_2$ 1, glucose 10, and Hepes 10. Patch pipette resistance was 2–5 MΩ, and the input resistance of the HEK-FLPTREX-TRPV4a cells was 1–10 GΩ. Whole-cell currents were recorded at room temperature (~23°C) using Clampex 9.2 software, and analysed using Clampfit 9.2 software (Molecular Devices). The recording chamber was continuously superfused with the external solution at approximately 2 ml min^{-1}, and rapid application of agonists in the external solution was made using an eight-barrelled fast-flow device. Membrane currents elicited by a linear ramp protocol from −100 mV to +100 mV (holding voltage 0 mV) repeated every 15 s were monitored at baseline and for 10 min following application of 4αPDD (0.5 μM). PAR$_2$-AP (100 μM) was applied for 2 min. Current amplitudes were measured at −80 mV and +80 mV in the ramp protocol from the maximal response to 4αPDD, and corrected for cell capacitance. In some experiments, calphostin C (2 μM) or H-89 (3 μM) were added to the pipette solution.

Neuropeptide release from the dorsal horn of the spinal cord

The spinal cords of rats were removed and slices (0.4 mm) from the dorsal part of the cervical and lumbar enlargements (without DRG) were prepared at 4°C (Amadesi *et al.* 2006). Slices (~100 mg) were placed in 2 ml chambers and superfused with oxygenated (95% O_2, 5% CO_2) Krebs' solution (mM: NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, $CaCl_2$ 2.5, KCl 4.7 and D-glucose 11) maintained at 37°C and containing 0.1% BSA, 1 μM phosphoramidon and 1 μM captopril. After a 60 min stabilization period, 5 min fractions were collected into acetic acid (2 M final concentration). Tissues were stimulated with 4αPDD (10 or 100 μM) or hypotonic solution (228 mosmol l^{-1}). In some experiments, slices were perfused with Ca^{2+}-free medium containing 1 mM EGTA, or afferent nerves were depleted of neuropeptides by preincubation with 10 μM capsaicin for 20 min before stimulation. To determine if PAR$_2$ sensitizes TRPV4 responses, slices were pretreated with PAR$_2$-AP, PAR$_2$-RP (10 μM) or vehicle for 20 min, washed and then challenged with TRPV4 agonists 20 min later. Freeze-dried fractions were reconstituted with assay buffer and analysed by enzyme immunoassays for CGRP and SP (Amadesi *et al.* 2006).

Paw withdrawal to mechanical stimuli

Mice were acclimatized for 15–20 min in a transparent box with a metal mesh floor (Alessandri-Haber *et al.* 2006). A calibrated von Frey hair monofilament (0.173 mN) (Stoelting Company, Wood Dale, IL, USA) was applied through the mesh floor to the plantar skin of the hindpaw. Paw withdrawal was assessed as the number of times the hind paw was withdrawn in response to five applications of the von Frey hair, expressed as a percentage (e.g. three withdrawals out of five was recorded as 60%). Basal measurements were made for all mice. After 15 min, PAR$_2$-AP, 4αPDD or hypotonic solution was injected (10 μl intraplantar injection), and paw withdrawal measurements repeated immediately. After an additional 5 min, 4αPDD or hypotonic solution was injected into paws previously treated with PAR$_2$-AP, and paw withdrawal measurements repeated immediately.

Statistical analysis

Results are expressed as mean ± S.E.M. and were compared by Student's *t* test or ANOVA with Bonferroni's or Dunnett's *post hoc* test. Differences were considered significant when $P < 0.05$.

Results

Epithelial cells express TRPV4 and PAR$_2$

PAR$_2$-induced sensitization of TRPV4 can be conveniently studied in cell lines expressing both proteins. Therefore, we determined whether epithelial cell lines coexpressed PAR$_2$ and TRPV4. Transcripts corresponding to PAR$_2$ (316 bp) were amplified by RT-PCR from the human bronchial epithelial cell line HBE (Fig. 1*A*, lane 1). Three isoforms of TRPV4 were amplified (Fig. 1*A*, lane 3), cloned and sequenced: full-length TRPV4a, TRPV4b (lacking amino acids 384–443, partially deleting the third of

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

three ankyrin repeat domains), and TRPV4c (lacking amino acids 237–283, corresponding to the first ankyrin repeat domain) (Fig. 1*B*). These isoforms correspond to reported sequences of TRPV4 (Arniges *et al.* 2006). Each isoform, tagged with a C-terminal HA.11 epitope for detection, was expressed in HEK cells, which naturally express PAR_2 (Amadesi *et al.* 2004). TRPV4a, TRPV4b and TRPV4c were detected at the plasma membrane and in intracellular locations by immunofluorescence using the HA.11 antibody (Fig. 1*C*, left panel). There was no detectable signal in HEK cells expressing vector without insert, confirming specificity. Western blotting confirmed expression of TRPV4a with the anticipated size (98 kDa) (Fig. 1*C*, right panel).

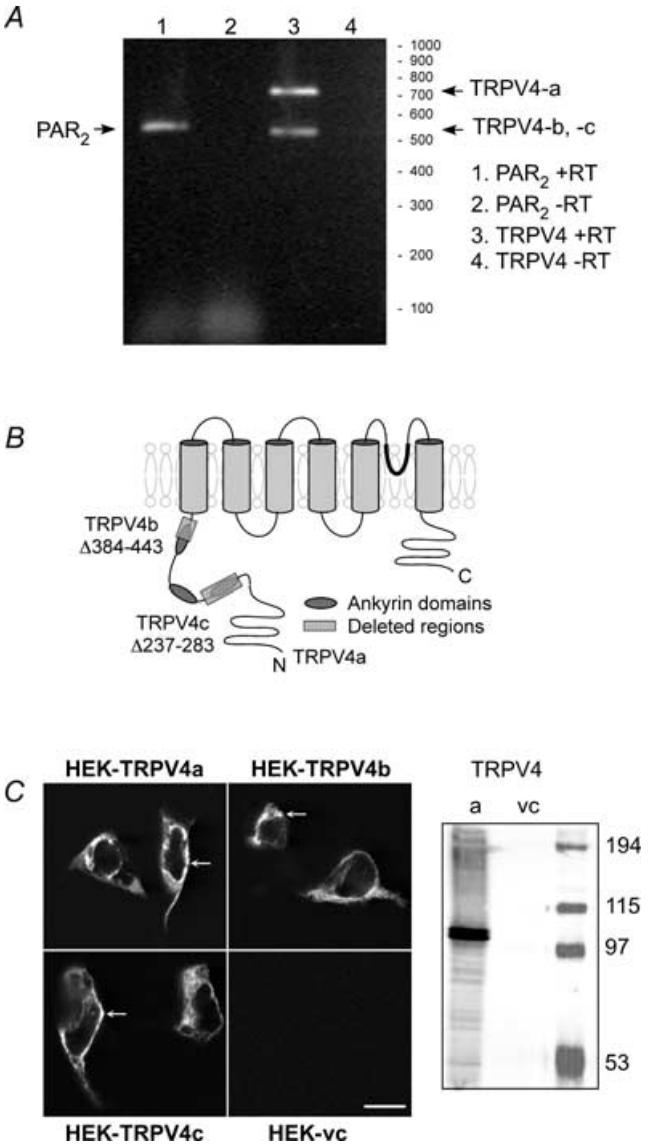

Figure 1. Expression of TRPV4 in HBE cells and HEK cells
A, RT-PCR of HBE cells showing amplification of transcripts for PAR_2 and TRPV4 isoforms a, b and c There were no signals when reverse transcriptase (RT) was omitted (control). *B*, structure of three TRPV4 isoforms cloned from HBE cells. *C*, transient expression of TRPV4 isoforms with HA.11 tag in HEK cells. Immunoreactive TRPV4 was detected by immunofluorescence (left) and Western blotting (right) using HA.11 antibody. vc, vector control without TRPV4 insert. Scale bar = 10 μm.

Since TRPV4 is a non-selective cation channel with preference for Ca^{2+} ions, measurement of $[Ca^{2+}]_i$ can be used to assess channel activity. To confirm expression of functional TRPV4 by HBE cells, we examined the effects of the TRPV4 agonists 4αPDD and hypotonic stimuli on $[Ca^{2+}]_i$ (Liedtke *et al.* 2000; Watanabe *et al.* 2002). 4αPDD (1–10 μM) stimulated a concentration-dependent increase in $[Ca^{2+}]_i$ over 250 s (Fig. 2*A*, upper panel). A decrease in osmolarity from 310 to 290 or 260 mosmol l^{-1} caused a tonicity-dependent increase in $[Ca^{2+}]_i$ over a similar period (Fig. 2*A*, lower panel). To confirm that these responses are mediated by TRPV4, we expressed the TRPV4 isoforms in HEK cells (Fig. 1*C*). 4αPDD (0.1–1 μM) and a hypotonic stimulus (260 mosmol l^{-1}) caused graded increases in $[Ca^{2+}]_i$ in HEK-TRPV4a cells (Fig. 2*B*). There were no detectable responses in HEK cells expressing TRPV4b, TRPV4c or empty vector (Fig. 2*B*). Thus, 4αPDD and hypotonic stimuli increase $[Ca^{2+}]_i$ in HBE and HEK-TRPV4a cells, and the response of HEK cells requires expression of the full-length TRPV4 channel. Although multiple mechanisms can increase $[Ca^{2+}]_i$, these data suggest that 4αPDD and hypotonic stimuli increase $[Ca^{2+}]_i$ by activating TRPV4.

PAR_2 sensitizes TRPV4 Ca^{2+} signalling in HBE and HEK-TRPV4a cells

Inflammatory mediators, including PAR_2 agonists, sensitize TRPV1 to cause thermal hyperalgesia (Lopshire & Nicol, 1997; Chuang *et al.* 2001; Tominaga *et al.* 2001; Vellani *et al.* 2001; Amadesi *et al.* 2004, 2006; Dai *et al.* 2004). Inflammatory agents also sensitize TRPV4, though the precise mechanism remains unclear (Alessandri-Haber *et al.* 2003, 2006). To examine whether PAR_2 agonists sensitize TRPV4, we measured their effects on Ca^{2+} responses to 4αPDD and a hypotonic stimulus in HBE cells. Pretreatment of HBE cells for 5 min with PAR_2-AP (100 μM) increased the magnitude of Ca^{2+} responses to 4αPDD (1 μM) or a hypotonic stimulus (260 mosmol l^{-1}) compared to pretreatment with inactive PAR_2-RP (100 μM, control) or vehicle, indicative of sensitization (Fig. 3*A*). PAR_2-AP similarly sensitized Ca^{2+} responses to 4αPDD (0.1 μM) or hypotonic stimulus (260 mosmol l^{-1}) in HEK-TRPV4a cells (Fig. 3*B*). Similar results were obtained whether cells were pretreated with PAR_2-AP for 5 min (Fig. 3*A* and *B*) or 10 min (Fig. 4*A*–*C*) before challenge with the TRPV4 agonists. After 10 min, Ca^{2+} responses to PAR_2-AP had returned to baseline. Thus, pretreatment of epithelial cells with a selective agonist of PAR_2 sensitizes Ca^{2+} signals to TRPV4 agonists.

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

We examined the selectivity of TRPV4 sensitization by studying responses in cells pretreated with an agonist of PAR_1. In contrast to PAR_2, agonists of PAR_1 do not sensitize TRPV1 (Amadesi *et al.* 2004), and induce analgesia rather than hyperalgesia to thermal or mechanical stimuli (Asfaha *et al.* 2002). We therefore determined if PAR_1-selective AP sensitized TRPV4 in HEK cells, which naturally express this receptor (Amadesi *et al.* 2004). Although PAR_1-AP strongly increased $[Ca^{2+}]_i$ in HEK-TRPV4a cells, it did not sensitize responses to 4αPDD (Fig. 3*C*) or a hypotonic stimulus (not shown) compared to PAR_1-RP or vehicle. HBE cells did not respond to PAR_1-AP (100 μM), and thus do not express functional PAR_1 (data not shown). Thus, activation of PAR_2 but not PAR_1 sensitizes TRPV4.

Phospholipase Cβ and protein kinases A, C and D mediate PAR_2-induced sensitization of TRPV4

Second messenger kinases including protein kinases A (PKA), C (PKC) and D (PKD) can phosphorylate TRPV1

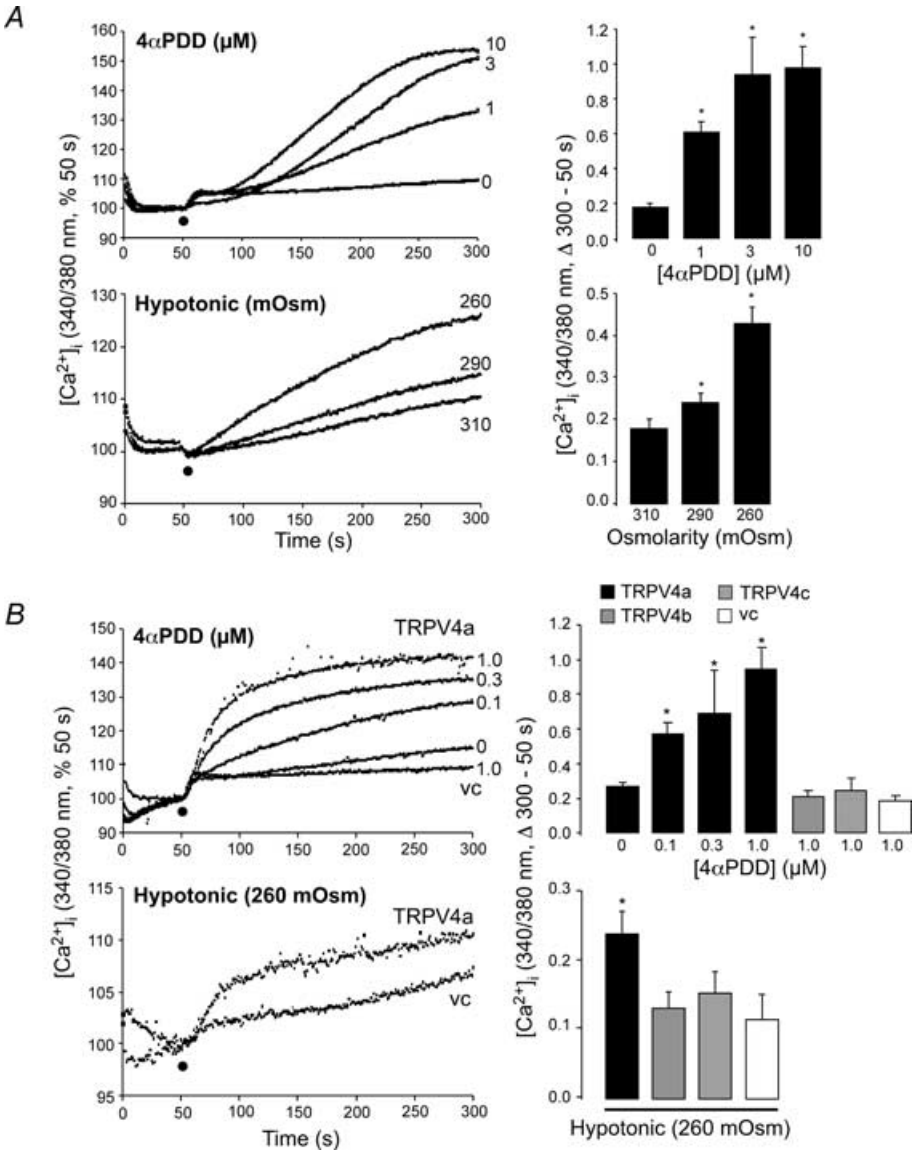

Figure 2. Activation of TRPV4 in HBE cells and HEK cells
Effects of 4αPDD (0.1–10 μM, upper traces) and hypotonic stimuli (260–310 mosmol l^{-1}, lower traces) on $[Ca^{2+}]_i$ in HBE cells (*A*) and HEK cells transiently expressing TRPV4 isoforms a, b or c or vector control (*B*). Left panels show records of $[Ca^{2+}]_i$ expressed as 340/380 nm emission ratio as a percentage of response measured at 50 s (prestimulus, 100%). Right panels show $[Ca^{2+}]_i$ responses as difference in 340/380 nm emission ratio between 50 s (prestimulus) and 300 s (maximal response). 4αPDD and hypotonic stimuli caused graded increases in $[Ca^{2+}]_i$ in HBE cells and HEK-TRPV4a cells, but had no effect in HEK cells expressing b or c TRPV4 isoforms or vector control. $^*P < 0.05$, ANOVA and Dunnett's test, $n = 9$ experiments.

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

to modify channel gating and thereby mediate hyperalgesia to inflammatory stimuli (Lopshire & Nicol, 1998; Premkumar & Ahern, 2000; Vellani *et al.* 2001; Bhave *et al.* 2002; Mohapatra & Nau, 2003; Wang *et al.* 2004). The mechanism of PAR_2-induced sensitization of TRPV4 is unknown. Since PAR_2 is known to couple to phospholipase Cβ (PLCβ) (Bohm *et al.* 1996*a*) and to activate PKC, PKA (Amadesi *et al.* 2006) and PKD (N.W. Burnett, unpublished observation), we examined the contributions of these enzymes to PAR_2-induced sensitization of TRPV4 Ca^{2+} signals in HBE and HEK cells by using inhibitors.

Inhibitors of PLCβ (U73122; 10 μM), PKA (H-89; 10 μM) and classic and novel PKCs (GF109203X; blocks PKCα, β, γ, δ, ϵ and ζ; 10 μM) all strongly inhibited PAR_2-AP-induced sensitization of Ca^{2+} responses to 4αPDD in HBE cells (Fig. 4*A*). Gö6976 (0.1 μM), which blocks PKCα and β and PKD (Martiny-Baron *et al.* 1993; Gschwendt *et al.* 1996), also inhibited this sensitization, whereas Gö6983 (0.1 μM), which blocks PKCα, β, γ, δ and ζ, but not PKD (Gschwendt *et al.* 1996), had no effect. U73122, H-89 and Gö6976 also inhibited PAR_2-AP-induced sensitization of Ca^{2+} responses to a hypotonic stimulus in HBE cells, whereas GF109203X and Gö6983 had no effect (Fig. 4*B*). U73122, H-89, GF109203X and Gö6976, but not Gö6983, inhibited PAR_2-induced sensitization of responses to 4αPDD in HEK-FLPTREX-TRPV4a cells (Fig. 4*C*). Thus, in HBE cells and HEK cells, activity of PLCβ, PKA, PKC and possibly PKD are required for PAR_2-induced sensitization of responses to 4αPDD. In HBE cells, activity of PLCβ, PKA and possibly PKD, but not PKC isozymes that are sensitive to GF109203X, are required for PAR_2-induced sensitization of responses to a hypotonic stimulus. Our

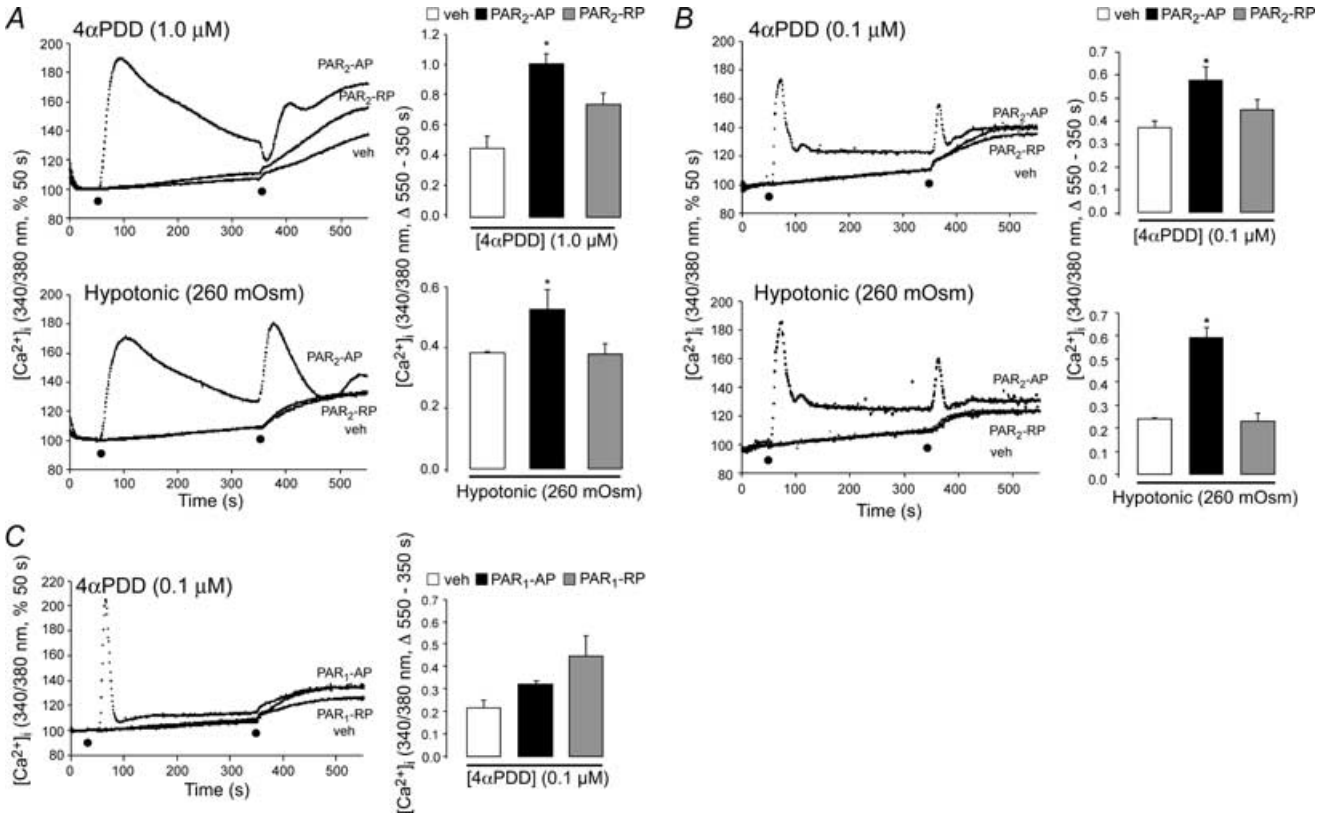

Figure 3. PAR-induced sensitization of TRPV4 Ca^{2+} signals
A, *B*, *C*, left panels show records of $[Ca^{2+}]_i$ after challenge with PAR_2-AP, PAR_2-RP (both 100 μM) or vehicle (veh) (*A* and *B*) or PAR_1-AP, PAR_1-RP (both 100 μM) or vehicle (*C*) at 50 s followed by 4αPDD (0.1 or 1 μM) or hypotonic stimulus (260 mosmol l^{-1}) at 350 s. Traces are from HBE cells (*A*) and HEK-TRPV4a cells (*B* and *C*). $[Ca^{2+}]_i$ is expressed as 340/380 nm emission ratio as a percentage of response measured at 50 s (prestimulus, 100%). Right panels show $[Ca^{2+}]_i$ responses as difference in 340/380 nm emission ratio between 350 s (prestimulus) and 550 s (maximal response). Pretreatment with PAR_2-AP but not PAR_1-AP increased Ca^{2+} responses to 4αPDD and hypotonic stimulus, indicative of TRPV4 sensitization. $^*P < 0.05$, ANOVA and Bonferroni's test, $n = 9$ experiments.

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

results are consistent with reports that 4αPDD and hypotonic stimuli activate TRPV4 by distinct mechanisms (Vriens *et al.* 2004).

TRPV4 is present in nociceptive neurons expressing PAR$_2$, CGRP and SP

PAR$_2$ could regulate TRPV4-dependent neuronal activity if these proteins are coexpressed in afferent neurons. PAR$_2$ is present in DRG neurons expressing SP, CGRP and TRPV1 (Steinhoff *et al.* 2000; Amadesi *et al.* 2004), but it is not known if TRPV4 is expressed by these nociceptive neurons. We used an antibody to the C-terminus of TRPV4 to localize this channel in rat DRG neurons. To characterize the antibody, we stained HEK cells expressing wild-type TRPV4a or TRPV4a with C-terminal HA.11 epitope. Both immunoreactive HA.11 and TRPV4 were detected at the plasma membrane and in intracellular locations (Fig. 5*A*). The C-terminal HA.11 epitope interfered with interaction of the TRPV4 antibody, which is directed to the C-terminus of this channel, which precluded simultaneous localization of HA.11 and TRPV4. Preabsorption of TRPV4 antibody abolished the signal (Fig. 5*A*), and neither antibody stained HEK cells expressing vector without TRPV4 insert (not shown), confirming specific detection.

Immunoreactive TRPV4 was detected at varying levels in the soma of rat DRG neurons, where it was present at the plasma membrane (Fig. 5*B*, arrowheads), in cytoplasmic vesicles and sometimes the nucleus (Fig. 5*B*, white arrows), and was also detected in fibres. We did not determine the number of neurons expressing immunoreactive TRPV4, since the large variability in expression levels precluded unequivocal discrimination between neurons expressing the channel at low levels and neurons that did not express detectable TRPV4. Some neurons expressing TRPV4 also expressed immunoreactive PAR$_2$, CGRP and SP (Fig. 5*B*, white arrows). However, TRPV4 was also found in neurons that did not contain these peptides (Fig. 5*B*, yellow asterisks), and some peptide-containing neurons did not express TRPV4 (Fig. 5*B*, yellow arrows). TRPV4 expression was retained by DRG in short-term culture, where immunoreactive TRPV4 was detected in the soma and fibres (Fig. 5*C*). Some cultured neurons expressing TRPV4 also expressed PAR$_2$, CGRP and SP (Fig. 5*C*). TRPV4 signals were abolished by preabsorption of the antibody. Transcripts corresponding to mouse (689 bp) and rat (723 bp) TRPV4 were amplified from whole DRG, and identified by sequencing (Fig. 5*D*). Only full-length TRPV4 was amplified. Thus, TRPV4 is present in DRG neurons, some of which express PAR$_2$, CGRP and SP. Since cultured neurons maintain coexpression of PAR$_2$ and TRPV4, they may be used to study the functional interactions of these proteins in nociceptive neurons.

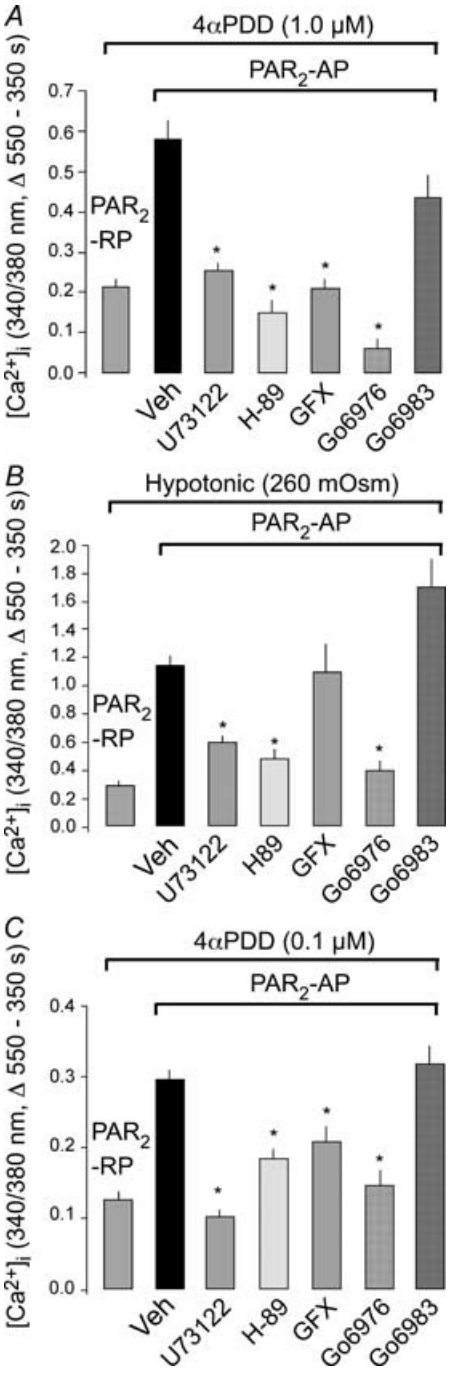

Figure 4. Mechanisms of PAR$_2$-induced sensitization of TRPV4 Ca^{2+} signals
Effects of antagonists of signalling pathways on PAR$_2$-induced sensitization of Ca^{2+} signals to 4αPDD (0.1 or 1 μM) (*A*) and hypotonic stimulus (260 mosmol l^{-1}) (*B*) in HBE cells and to 4αPDD in HEK-TRPV4a cells (*C*). Cells were pretreated with PAR$_2$-AP or PAR$_2$-RP (both 100 μM) for 10 min before challenge with TRPV4 agonists. U73122 (10 μM), H-89 (10 μM), GF109203X (GFX) 10 μM and Gö6976 (0.1 μM), but not Gö6963 (0.1 μM), inhibited PAR$_2$-induced sensitization of Ca^{2+} responses to 4αPDD in HBE cells and HEK-TRPV4a cells. U73122, H-89 and Gö6976, but not GF109203X or Gö6963, inhibited PAR$_2$-induced sensitization of Ca^{2+} responses to hypotonic stimulus in HBE cells. Veh, vehicle; $^*P < 0.05$; ANOVA followed by Dunnett's test; $n = 8$–12 experiments.

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

Agonists of PAR_2 sensitize TRPV4 Ca^{2+} signalling and currents in DRG neurons and HEK-TRPV4a cells

To determine if PAR_2 agonists sensitize TRPV4 in nociceptive neurons, we measured $[Ca^{2+}]_i$ in rat DRG neurons in short-term culture. In view of the divergent mechanisms of PAR_2-induced sensitization of responses to 4αPDD and hypotonic stimuli that we identified in epithelial cells, which require further experimentation to fully investigate, we focused on sensitization of responses to the TRPV4-selective agonist 4αPDD (Watanabe *et al.* 2002) in neurons. 4αPDD (10 μM) caused a gradual increase in $[Ca^{2+}]_i$ in DRG neurons pretreated for 20 min with PAR_2-RP (10 μM, control) (Fig. 6*a*). Pretreatment with PAR_2-AP (10 μM, 20 min) caused a >2-fold increase in the magnitude of Ca^{2+} response, indicative of sensitization (Fig. 6*b*). Inhibition of PKA with H-89 (10 μM) or PKC with GF109203X (1 μM) reduced this sensitization (Fig. 6*C*).

To confirm that PAR_2 activation sensitizes TRPV4, we measured TRPV4 currents in acutely dissociated mouse DRG neurons. In the whole-cell configuration, we recorded current upon application of voltage ramp from −100 to +100 mV. 4αPDD (10 μM) activated an outwardly rectifying current (Fig. 6*D*, left panel) with current densities of -14.04 ± 2.89 pA pF^{-1} at −80 mV and 27.40 ± 4.07 pA pF^{-1} at +80 mV (Fig. 6*E*), and shifted the reversal potential toward positive. Pretreatment with PAR_2-AP (10 μM) for 20 min enhanced the outward current (Fig. 6*D*, right panel), and increased current densities by >2-fold to -52.96 ± 15.89 pA pF^{-1} at −80 mV, and 62.27 ± 15.19 pA pF^{-1} at +80 mV (Fig. 6*E*). PAR_2-AP also increased the proportion of neurons that responded to 4αPDD by activation of an outwardly rectifying current from 66% in untreated neurons to 82% in neurons treated with PAR_2-AP (Fig. 6*F*). Thus, PAR_2-AP sensitizes TRPV4 Ca^{2+} signals and currents in DRG neurons, and activity of PKA and PKC is required for this sensitization.

We similarly evaluated sensitization of TRPV4 currents in HEK-FLPTREX-TRPV4a cells. 4αPDD (0.5 μM) activated an outwardly rectifying whole-cell current (Fig. 6*G*) with current densities of -28.51 ± 6.61 pA pF^{-1} at −80 mV and 58.41 ± 12.39 pA pF^{-1} at +80 mV (Fig. 6*H*). Since these currents desensitized in the continued presence of 4αPDD, cells were challenged only once with 4αPDD, to avoid desensitization. Pretreatment with PAR_2-AP (100 μM) for 2 min

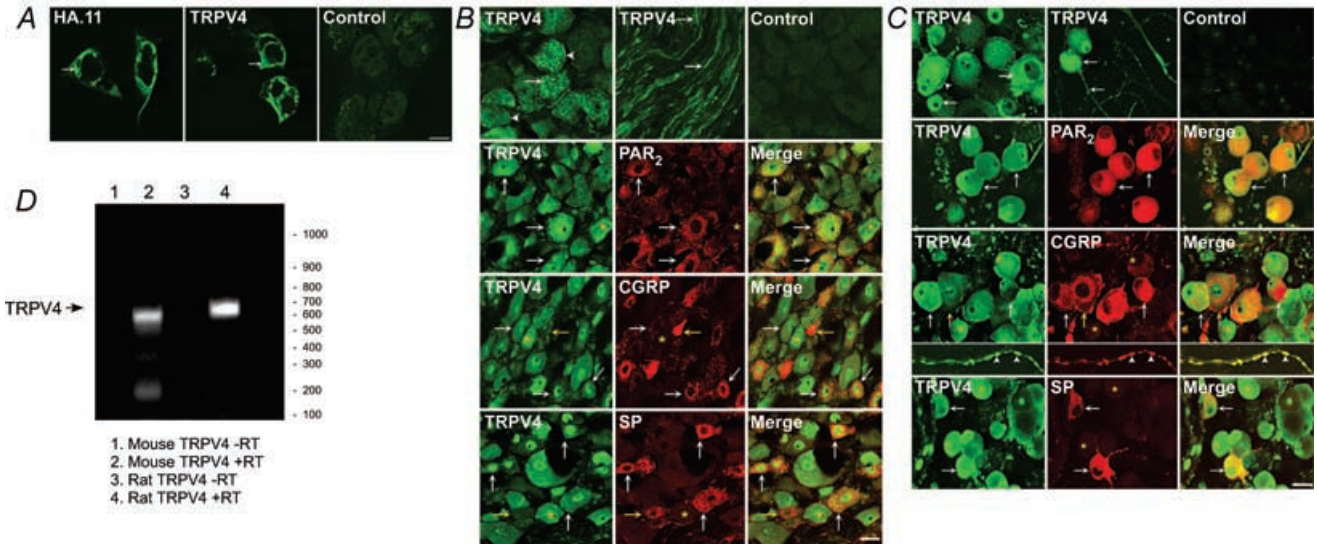

Figure 5. Localization and expression of TRPV4 in HEK cells and DRG neurons
A, localization of TRPV4a transiently expressed in HEK cells by immunofluorescence using antibodies to HA.11 epitope or TRPV4. Both antibodies detected immunoreactive TRPV4. Control shows preabsorption of TRPV4 antibody with antigen used for immunization. *B*, localization of immunoreactive TRPV4, PAR_2, CGRP or SP in sections of rat DRG. TRPV4 was detected in the soma at the plasma membrane (white arrowheads) and in intracellular locations (white arrows), and also in fibres (white arrows). White arrows show that some neurons coexpressed TRPV4 with PAR_2, CGRP or SP. Yellow arrows show that some neurons expressing CGRP or SP did not express TRPV4. Yellow asterisks show that some neurons expressing TRPV4 did not express PAR2, CGRP or SP. Control shows preabsorption of TRPV4 antibody. Scale bar = 10 μm c, Localization of immunoreactive TRPV4, PAR_2, CGRP or SP in rat DRG after 2 days in culture. White arrows denote colocalization of TRPV4 with PAR_2, CGRP or SP. Yellow arrows show that some neurons expressing CGRP did not express TRPV4. Yellow asterisks show that neurons expressing TRPV4 did not express CGRP or SP. Control shows preabsorption of TRPV4 antibody. Scale bar = 10 μm. *D*, RT-PCR of mouse and rat DRG showing amplification of transcripts for TRPV4. There were no signals when reverse transcriptase (RT) was omitted (control).

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

resulted in >2-fold increase in the maximal current induced by 4αPDD, and increased the currents by >2-fold to -61.90 ± 6.52 pA pF^{-1} at -80 mV, and 120.80 ± 12.70 pA pF at $+80$ mV, indicative of TRPV4 sensitization (Fig. 6*H*). Inhibition of PKA with H-89 (3 μM) or PKC with calphostin C (2 μM) reduced the current density at $+80$ mV following PAR$_2$-AP from 120.80 ± 12.70 pA pF^{-1} to 47.50 ± 30.28 pA pF^{-1} with H-89 and 26.00 ± 11.98 pA pF^{-1} with calphostin C (Fig. 6*I*). Thus, PAR$_2$ sensitizes TRPV4 Ca^{2+} signals and currents in DRG neurons and HEK-FLPTREX-TRPV4a cells by PKC- and PKA-dependent processes.

Agonists of PAR$_2$ sensitize TRPV4-stimulated release of neuropeptides

The release of SP and CGRP from the central projections of nociceptive DRG neurons in the dorsal horn correlates with pain transmission, and sensitization of this process may enhance pain transmission. The TRPV1 agonist capsaicin strongly stimulates SP and CGRP release within the dorsal horn (Mantyh *et al.* 1995; Marvizon *et al.* 1997, 2003; Amadesi *et al.* 2004). Agonists of PAR$_2$ also stimulate the release of CGRP and SP from nociceptive neurons (Steinhoff *et al.* 2000), and potentiate TRPV1-induced peptide release in the dorsal horn (Amadesi *et al.* 2004). However, it is not known if TRPV4 agonists stimulate neuropeptide release, and whether PAR2 sensitizes this effect. To examine these possibilities, we studied segments of rat spinal cord.

We first determined if TRPV4 was present in nerve fibres in superficial laminae of the dorsal horn that contain SP and CGRP. Immunoreactive TRPV4 was detected in cells bodies and in punctate structures that may represent nerve fibres in the superficial and deeper laminae of the dorsal horn of rat spinal cord (Fig. 7). Immunoreactive CGRP and SP were prominently detected in nerve fibres in superficial laminae I and II of the dorsal horn. Some fibres containing immunoreactive CGRP and SP also contained immunoreactive TRPV4 (Fig. 7). Thus, TRPV4 is present in neuropeptide-containing nerve fibres in the dorsal horn of the spinal cord.

To determine if TRPV4 agonists induce release of CGRP and SP, we incubated slices of dorsal horn of the rat spinal cord with 4αPDD or hypotonic solution, and measured release of immunoreactive CGRP and SP. 4αPDD (10 μM) stimulated a >9-fold increase in CGRP release and a >4-fold increase in SP release over basal values (basal: CGRP, 2.0 ± 1.0 fmol g (20 min)$^{-1}$, SP, 1.0 ± 1.0 fmol g (20 min)$^{-1}$; 4αPDD: CGRP, 18.7 ± 4.5 fmol g (20 min)$^{-1}$; SP, 4.2 ± 1.2 fmol g (20 min)$^{-1}$) (Fig. 8*A*). A higher concentration of 4αPDD (100 μM) produced a greater response (CGRP, 42.5 ± 10.2 fmol g (20 min)$^{-1}$; SP, 8.9 ± 1.8 fmol g (20 min)$^{-1}$). Hypotonic solution (228 mosmol l^{-1}) stimulated an 18-fold increase in CGRP release and a >10-fold increase in SP release (CGRP, 82.4 ± 5.6 fmol g (20 min)$^{-1}$; SP, 19.3 ± 2.6 fmol g (20 min)$^{-1}$) (Fig. 8*B*). Removal of extracellular Ca^{2+} ions, and capsaicin-desensitization of tissues inhibited the stimulatory effects of 4αPDD and hypotonic solution. Thus, TRPV4 agonists stimulate the Ca^{2+}-dependent release of proinflammatory and nociceptive peptides from capsaicin-sensitive afferent nerve endings.

A consequence of PAR$_2$-induced sensitization of TRPV4 may be to enhance the release of neuropeptides. To examine this possibility, we preincubated segments of the dorsal horn of the rat spinal cord with PAR$_2$-AP, PAR$_2$-RP (10 μM) or vehicle for 20 min, washed the segments, and then measured release of CGRP and SP in response to incubation of segments with 4αPDD (10 μM) or hypotonic solution (228 mosmol l^{-1}). PAR$_2$-AP enhanced the release of immunoreactive CGRP and SP to 4αPDD by >2-fold compared to PAR$_2$-RP or vehicle (Fig. 8*C*). Similarly, PAR$_2$-AP enhanced release of immunoreactive CGRP and SP to a hypotonic stimulus (Fig. 8*D*). Thus, agonists of PAR$_2$ sensitize TRPV4-induced release of nociceptive peptides in the dorsal horn.

TRPV4 mediates PAR$_2$-induced hypersensitivity to a mechanical stimulus

PAR$_2$ agonists cause mechanical allodynia and hyperalgesia by unknown mechanisms (Vergnolle *et al.* 2001; Coelho *et al.* 2002). Since deletion and downregulation of TRPV4 results in diminished pain to mechanical and hypoosmotic stimuli (Alessandri-Haber *et al.* 2003, 2006; Liedtke & Friedman, 2003; Suzuki *et al.* 2003), we examined the effects of PAR$_2$-AP on mechanical allodynia and hyperalgesia in TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice. We have previously reported that the hyperalgesic effect of PAR$_2$-AP requires expression of PAR$_2$, and that PAR$_2$-RP has no effect, confirming specificity (Vergnolle *et al.* 2001).

Intraplantar injection of 4αPDD (10 μl of 50 μM solution) or PAR$_2$-AP (1 μg in 10 μl saline) in TRPV4$^{+/+}$ mice produced an increase in paw withdrawal frequency to stimulation with a 0.173 mN von Frey hair, compared to basal measurements (Fig. 9*A* and *B*). These increases were not observed in TRPV4$^{-/-}$ mice. In contrast, intraplantar injection of hypotonic solution (10 μl of 17 mosmol l^{-1}) did not increase the number of paw withdrawals in TRPV4$^{+/+}$ or TRPV4$^{-/-}$ mice (Fig. 9*B*). Thus, TRPV4 is required for PAR$_2$- and 4αPDD-induced increases in mechanical sensitivity, which is indicative of mechanical allodynia and hyperalgesia.

To determine if activation of PAR$_2$ sensitizes pain responses to agonists of TRPV4, we administered PAR$_2$-AP

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

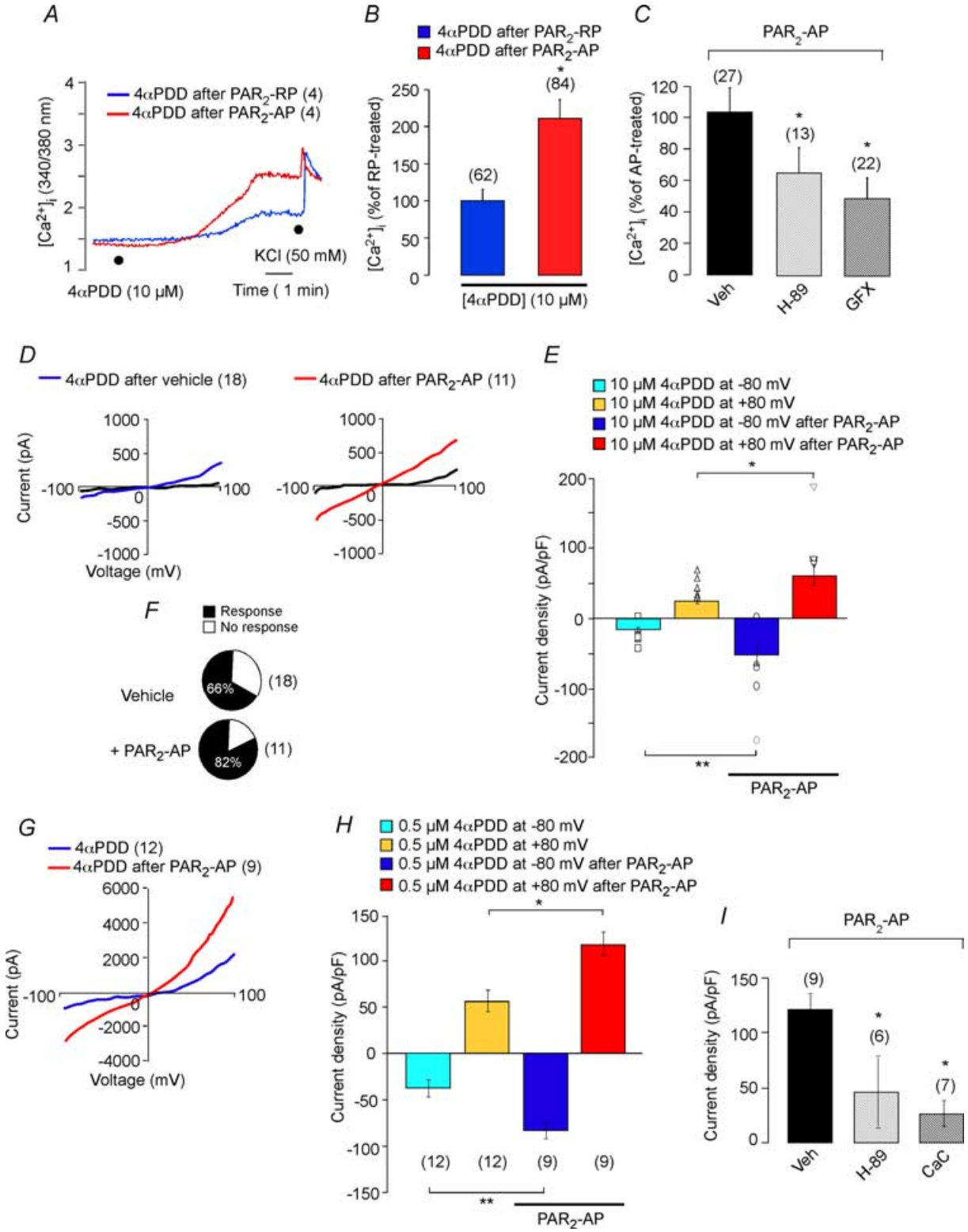

Figure 6. PAR_2-induced sensitization of TRPV4 Ca^{2+} signals and currents in DRG neurons (*A–F*) and HEK-FLPTREX-TRPV4a cells (*G–I*)

Neurons were pretreated for 20 min and HEK-FLPTREX-TRPV4a cells for 2 min with PAR_2-AP, PAR_2-RP or vehicle (Veh) before challenge with 4αPDD. *A–C*, $[Ca^{2+}]_i$ records in rat DRG neurons. *A*, records of $[Ca^{2+}]_i$ in DRG neurons challenged with 4αPDD (10 μM). *B*, maximal $[Ca^{2+}]_i$ responses to 4αPDD (10 μM) expressed as percentage response in neurons pretreated with PAR_2-RP (100%). Pretreatment with PAR_2-AP (10 μM) increased 4αPDD-stimulated Ca^{2+} response, indicative of TRPV4 sensitization (*$P < 0.05$, Student's *t* test). *C*, effects of inhibitors of second messenger

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

(1 μg in 10 μl saline) 5 min before 4αPDD (10 μl of 50 μM solution) or a hypotonic solution (10 μl of 17 mosmol l^{-1}). In TRPV4$^{+/+}$ mice, preinjection of PAR$_2$-AP caused a >2-fold increase in the frequency of paw withdrawal to 4αPDD alone (Fig. 9*A*), and a >3-fold increase in the frequency of paw withdrawal to hypotonic solution alone (Fig. 9*B*). This sensitization was not present in TRPV4$^{-/-}$ mice. Thus, PAR$_2$-AP sensitizes TRPV4 to increase sensitivity of the paw to a mechanical stimulus, indicative of mechanical allodynia and hyperalgesia.

Discussion

Our results show, for the first time, that TRPV4 mediates PAR$_2$-induced mechanical allodynia and hyperalgesia. PAR$_2$ sensitizes TRPV4 Ca^{2+} signals and currents in cell lines and afferent neurons by mechanisms requiring activity of PLCβ, PKA, PKC and perhaps PKD. TRPV4 agonists promote SP and CGRP release from afferent nerves in the spinal cord, where they can mediate nociceptive transmission. PAR$_2$ sensitizes both TRPV4-mediated neuropeptide release and TRPV4-induced mechanical hyperalgesia, and TRPV4 deletion prevents PAR$_2$-induced mechanical hyperalgesia. Thus, we have identified a novel mechanism by which proteases that are generated during injury and inflammation cleave PAR$_2$ on afferent nerve endings to activate second messenger kinases that sensitize TRPV4 Ca^{2+} signals; this sensitization enhances the release of nociceptive neuropeptides at the spinal level and causes mechanical allodynia and hyperalgesia (Fig. 10).

PAR-mediated sensitization of TRPV4

The observation that afferent nerves coexpress immunoreactive TRPV4 and PAR$_2$ suggests that PAR$_2$ can sensitize TRPV4 within an individual neuron, without involvement of other cell types. HBE cells also coexpressed TRPV4 and PAR$_2$, providing a convenient cell line in which to study interactions between these proteins. We identified three TRPV4 isoforms in these cells, corresponding to sequences also identified in human tracheal epithelial cells (Arniges *et al.* 2006). In this study, only full-length TRPV4 was detected at the plasma membrane, whereas truncated isoforms, lacking ankyrin domains, were retained in the endoplasmic reticulum (Arniges *et al.* 2006). In contrast, we found that all TRPV4 isoforms were present at the plasma membrane and in intracellular locations when transiently expressed in HEK cells. However, in both studies, only full-length TRPV4 protein with three complete ankyrin domains responded to 4αPDD or a hypotonic stimulus. The function of the other isoforms is unknown.

Expression of TRPV4a in HEK cells conferred responsiveness to 4αPDD and hypotonic stimuli, suggesting that these agents specifically activate TRPV4. Pretreatment of HBE or HEK-TRPV4a cells with PAR$_2$-AP enhanced Ca^{2+} signals to 4αPDD and hypotonic stimuli, indicating that PAR$_2$ sensitizes TRPV4. PAR$_2$ also sensitized TRPV4 currents in HEK-FLPTREX-TRPV4a cells. PAR$_1$-AP increased $[Ca^{2+}]_i$ in HEK-TRPV4a cells to a similar level as PAR$_2$-AP, but did not affect the responses to 4αPDD, indicating that PAR$_1$ does not sensitize TRPV4.

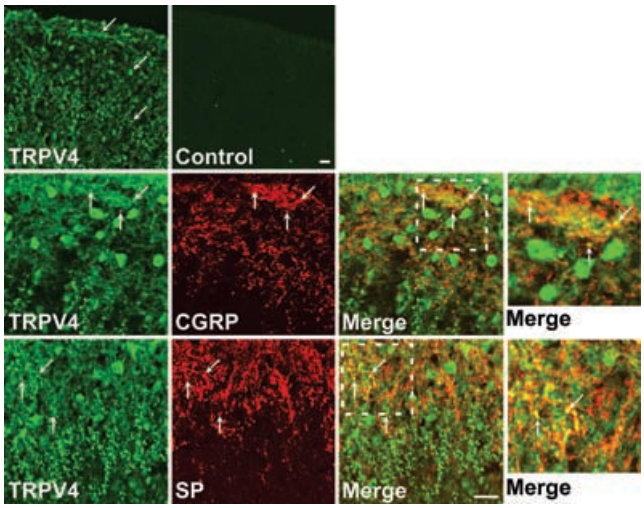

Figure 7. Localization of immunoreactive TRPV4, CGRP or SP in dorsal horn of rat spinal cord
Control shows preabsorption of TRPV4 antibody with antigen used for immunization. White arrows show colocalization of TRPV4 with CGRP and SP in some fibres of superficial laminae I and II. Scale bar = 10 μm.

kinases on PAR$_2$-induced sensitization of Ca^{2+} signals to 4αPDD. Results are expressed as percentage of response to 4αPDD in neurons pretreated with PAR$_2$-AP (100%). H-89 (10 μM) and GF109203X (GFX) 1 μM inhibited PAR$_2$-induced sensitization of Ca^{2+} responses to 4αPDD (*$P < 0.05$, ANOVA and Dunnett's test). *D–F*, whole-cell currents of mouse DRG neurons. *D*, whole-cell currents recorded during voltage ramp (−100 mV to +100 mV every 15 s) before and during application of 4αPDD (10 μM). *E*, current densities at −80 mV and +80 mV. PAR$_2$-AP (10 μM) increased 4αPDD-mediated current density (**$P < 0.01$; *$P < 0.02$, Student's *t* test). *F*, proportion of neurons that responded to 4αPDD (10 μM) by activation of an outwardly rectifying current. *G–H*, whole-cell currents of HEK-FLPTREX-TRPV4a. *G*, whole-cell currents recorded during voltage ramp (−100 mV to +100 mV every 15 s) before and during application of 4αPDD (0.5 μM). *H*, current densities at −80 mV and +80 mV. PAR$_2$-AP (100 μM) increased 4αPDD-mediated current density (**$P < 0.01$; *$P < 0.05$, Student's *t* test). *I*, effects of antagonists of signalling pathways on PAR$_2$-induced sensitization of currents to 4αPDD. H-89 (3 μM) and calphostin C (CaC) 2 μM inhibited sensitization (*$P < 0.05$; Student's *t* test). In *A–I*, numbers in parentheses refer to the number of cells.

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

Unlike PAR_2 agonists, PAR_1 agonists do not cause thermal or mechanical hyperalgesia (Asfaha *et al.* 2002) and do not sensitize TRPV1 (Amadesi *et al.* 2004). Thus, sensitization of TRPV4 is specific to PAR_2, rather than being a general effect of PARs that do not influence nociception.

If PAR_2-mediated sensitization of TRPV4 is relevant to mechanical hyperalgesia, it should occur in the DRG neurons that transduce painful mechanical stimuli. We observed that PAR_2-AP enhanced 4αPDD-induced Ca^{2+} signals and currents in isolated DRG neurons, and also increased the proportion of responsive neurons. These results confirm that DRG neurons coexpress PAR_2 and TRPV4, and demonstrate a functional interaction between these proteins that increases the sensitivity of TRPV4 to its agonist. This interaction occurs at the level of individual neurons, suggesting that it is mediated through activation of intracellular signalling cascades. In a similar manner, PAR_2 agonists also enhance capsaicin Ca^{2+} signals and currents in DRG neurons and increase the proportion of capsaicin-responsive neurons, indicative of sensitization of TRPV1 (Amadesi *et al.* 2004; Amadesi *et al.* 2006; Dai *et al.* 2004).

We have previously reported that >60% of DRG neurons express immunoreactive PAR_2 and respond to PAR_2 agonists with increased $[Ca^{2+}]_i$ (Steinhoff *et al.* 2000). Although variability in the levels of immunoreactive TRPV4 precluded accurate quantification of the proportion of TRPV4-expressing neurons, >60% of DRG neurons responded to 4αPDD with a detectable current, and this proportion was increased by pretreatment with PAR_2-AP, indicative of sensitization. Thus, similar proportions of neurons express functional PAR_2 and TRPV4, and we also observed that immunoreactive PAR2 and TRPV4 are frequently coexpressed. PAR_2 agonists may directly regulate TRPV4 activity in these neurons. However, some neurons containing immunoreactive TRPV4 did not express detectable PAR_2 (or CGRP or SP), and it is possible that some PAR_2-stained neurons do not express TRPV4. PAR_2 may regulate these neurons by TRPV4-independent mechanisms, which remain to be characterized. Other mechanisms may regulate TRPV4 in neurons that do not express PAR_2. We detected immunoreactive TRPV4 at the plasma membrane, in cytosolic vesicles and sometimes in the nucleus of DRG neurons. TRPV4 at the plasma membrane may be a target for regulation by PAR_2 and other inflammatory agents, which could also control TRPV4 trafficking between the plasma membrane

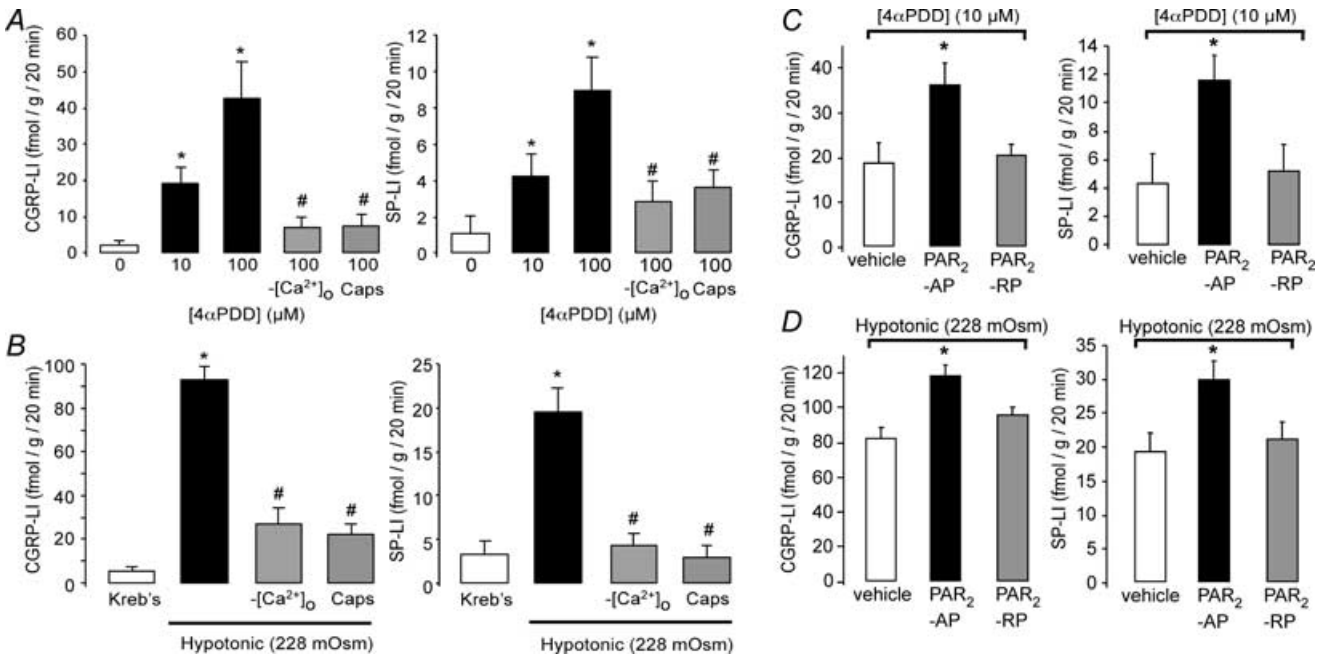

Figure 8. TRPV4-mediated peptide release from rat dorsal horn
Effects of 4αPDD (10–100 μM) (*A*) and hypotonic stimulus (228 mosmol l^{-1}) (*B*) on release of CGRP (left panels) and SP (right panels) from superfused slices of dorsal spinal cord. $-[Ca^{2+}]_o$ denotes Ca^{2+}-free solution and Caps denotes pretreatment with capsaicin. 4αPDD and hypotonic stimulus induced release of CGRP and SP, and release required extracellular Ca^{2+} ions and was prevented by capsaicin pretreatment. $^*P < 0.05$ compared to vehicle; ANOVA and Bonferroni's test. *C* and *D*, PAR_2-induced sensitization of neuropeptide release from dorsal spinal cord. Effects of preincubation with PAR_2-AP, PAR_2-RP (both 10 μM) or vehicle for 20 min on release of immunoreactive CGRP (left panels) and SP (right panels) in response to 4αPDD (*C*) or hypotonic stimulus (*D*). Pretreatment with PAR_2-AP increased release of CGRP and SP in response to 4αPDD and hypotonic stimulus, indicative of TRPV4 sensitization. $^*P < 0.05$ compared to vehicle; ANOVA followed by Bonferroni's test. $n = 6$–9 experiments.

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

and vesicles. A function of TRPV4 in the nucleus is unknown.

Mechanisms of PAR_2-induced sensitization of TRPV4

PAR_2 couples through $G\alpha_{q/11}$ to PLCβ and the release of inositol trisphosphate and diacylglycerol (Bohm *et al.* 1996*a*), and in afferent neurons PAR_2 activates PKA, PKC (Amadesi *et al.* 2006) and PKD (N.W. Burnett, unpublished observation). We examined the contribution of these signalling pathways to PAR_2-induced sensitization of TRPV4 Ca^{2+} signals and currents in cell lines and neurons. As expected, the PLCβ inhibitor U73122 abolished PAR_2-induced sensitization of responses to 4αPDD in HBE and HEK-TRPV4a cells. The PKA inhibitor H-89, the broad-spectrum PKC inhibitors GF109203X and calphostin C, and Gö6976, which blocks PKCα, β and PKD (Martiny-Baron *et al.* 1993; Gschwendt *et al.* 1996), also inhibited PAR_2-induced sensitization of responses to 4αPDD. The lack of effect of Gö6983, which blocks PKCα, β, γ, δ and ζ but not PKD (Gschwendt *et al.* 1996), suggests that the inhibitory effects of Gö6976 are due to an action on PKD, which requires additional investigation. Together, these data suggest that PAR_2-induced sensitization of TRPV4 responses to 4αPDD requires activation of PLCβ, followed by PKA, PKC and PKD activity. It remains to be determined whether these kinases act sequentially or in parallel. It is also unknown whether PKA, PKC and PKD phosphorylate and thereby sensitize TRPV4, as they do for TRPV1 (Lopshire & Nicol, 1998; Premkumar & Ahern, 2000; Vellani *et al.* 2001; Bhave *et al.* 2002; Mohapatra & Nau, 2003; Wang *et al.* 2004). In support of our results, PLCβ, PKA and PKC mediate PAR_2-induced sensitization of TRPV1 (Amadesi *et al.* 2004; Dai *et al.* 2004; Amadesi *et al.* 2006).

Slightly different mechanisms mediate PAR_2-induced sensitization of TRPV4 responses to hypotonic stimulus. Although antagonism of PLCβ and PKA prevented sensitization of responses to hypotonic stimulus in HBE cells, the broad-spectrum PKC inhibitor GF109203X was without effect. Further investigations, using selective approaches to antagonize activity or expression of second messenger systems, are required to elucidate the precise mechanisms of this sensitization. However, our results showing differences in the mechanisms of PAR_2-induced sensitization of TRPV4 responses to hypotonic stimuli and 4αPDD are consistent with the view that these agonists activate TRPV4 by different mechanisms (Vriens *et al.* 2004).

PAR2-induced sensitization of TRPV4-mediated peptide release and mechanical hyperalgesia

Release of SP and CGRP from the central projections of nociceptive DRG neurons within the dorsal horn correlates with nociceptive behaviour, and agonists of both TRPV1 (Mantyh *et al.* 1995; Marvizon *et al.* 1997, 2003; Amadesi *et al.* 2004) and PAR_2 (Steinhoff *et al.* 2000) promote SP and CGRP release from these fibres. We detected immunoreactive TRPV4 in nerve fibres of superficial laminae of the dorsal horn that also contained immunoreactive SP and CGRP. These fibres probably represent the central projections of primary spinal afferent neurons, although additional experiments are required to

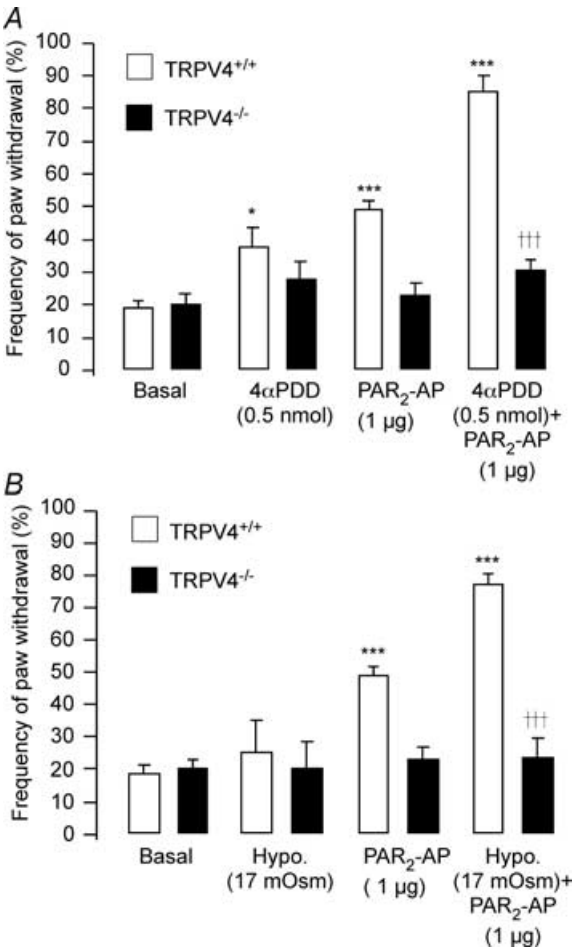

Figure 9. Role of TRPV4 in PAR_2-induced mechanical hyperalgesia
Frequency of paw withdrawal to stimulation with a von Frey hair under basal conditions, after intraplantar injection of 4αPDD or PAR_2-AP alone, or after injection of PAR_2-AP followed 5 min later by 4αPDD (*A*), and distilled water or PAR_2-AP alone, or after injection of PAR_2-AP followed 5 min later by distilled water (*B*). When given alone, 4αPDD (10 μl of 50 μM solution) and PAR_2-AP (1 μg in 10 μl saline), but not hypotonic stimulus (10 μl of 17 mosmol l^{-1}), increased the frequency of paw withdrawal in $TRPV4^{+/+}$ but not $TRPV4^{-/-}$ mice, indicating that TRPV4 mediates 4αPDD- and PAR_2-AP-induced mechanical hyperalgesia. Preinjection of PAR_2-AP enhanced responses to 4αPDD and hypotonic stimulus in $TRPV4^{+/+}$ but not $TRPV4^{-/-}$ mice, indicating that PAR_2 sensitizes TRPV4 to cause mechanical hyperalgesia. $^{***}P < 0.001$; $^{*}P < 0.05$ compared to vehicle; $\dagger\dagger\dagger P < 0.001$ compared to $TRPV4^{+/+}$. ANOVA and Bonferroni's test. $N = 6$–9 animals.

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

confirm this hypothesis. The TRPV4 agonists 4αPDD and hypotonic solutions stimulated release of both neuropeptides from slices of the dorsal spinal cord, consistent with a role of TRPV4 in nociception. This release required extracellular Ca^{2+} ions, indicative of regulated exocytosis from spinal nociceptive afferent fibres. Pretreatment with capsaicin prevented the effects of TRPV4 agonists on peptide release, confirming that TRPV4 agonists stimulate peptide secretion from nociceptive nerve fibres that express TRPV1. We also detected immunoreactive TRPV4 on unidentified neurons of the dorsal horn, and thus cannot exclude the possibility that TRPV4 agonists stimulated peptide release by indirect mechanisms. The identity of these TRPV4-expressing neurons and the role of TRPV4 in these cells remains to de determined.

PAR_2-AP increased the release of SP and CGRP in response to 4αPDD or hypotonic saline. Thus, PAR_2 not only sensitizes TRPV4 Ca^{2+} signals and currents in DRG neurons, but also sensitizes TRPV4-dependent peptide release from intact nerve fibres. Increased peptide release following PAR_2-mediated TRPV4 sensitization may contribute to enhanced mechanical nociception by facilitating central nociceptive transmission. In support of these results, PAR_2 agonists also sensitize TRPV1-mediated SP and CGRP release from the dorsal horn (Amadesi *et al.* 2004).

Intraplantar injection of PAR_2-AP alone increased paw withdrawal responses to a mechanical stimulus, and enhanced responses to 4αPDD and hypotonic solution, indicating that PAR_2 induces mechanical allodynia and hyperalgesia. These responses were not present in TRPV4-deficient mice and thus depend on the presence of this channel. Hypotonic solution alone had no effect on paw withdrawal in the absence of a sensitizing stimulus, in agreement with our previous finding that pretreatment with prostaglandin E_2 is necessary to induce nocifensive responses to a hypotonic stimulus (Alessandri-Haber *et al.* 2003). In contrast, injection of 4αPDD alone was sufficient to cause increased paw withdrawal in wild-type but not TRPV4-deficient mice. These data provide novel evidence that 4αPDD selectively activates TRPV4 *in vivo*, and possibly that hypotonic stimuli and 4αPDD activate TRPV4 by distinct mechanisms in live animals, as previously suggested in heterologous expression systems (Vriens *et al.* 2004).

Although our results suggest that PAR_2 agonists can sensitize TRPV4 on primary spinal afferent neurons to enhance peptide release and cause mechanical hyperalgesia, we cannot exclude the possibility that PAR_2 also sensitizes TRPV4 on other cell types to enhance nociception. Keratinocytes highly express PAR_2 and TRPV4 (Steinhoff *et al.* 1999; Chung *et al.* 2003, 2004). These cells receive a rich sensory innervation and have been proposed to participate in TRPV4-dependent thermosensation (Lee *et al.* 2005). Indeed, our results show that PAR_2 agonist strongly sensitizes TRPV4 in airway epithelial cells, and similar sensitization in keratinocytes or enterocytes could contribute to mechanical hyperalgesia in the skin and intestine. Additional experiments are necessary to investigate this possibility.

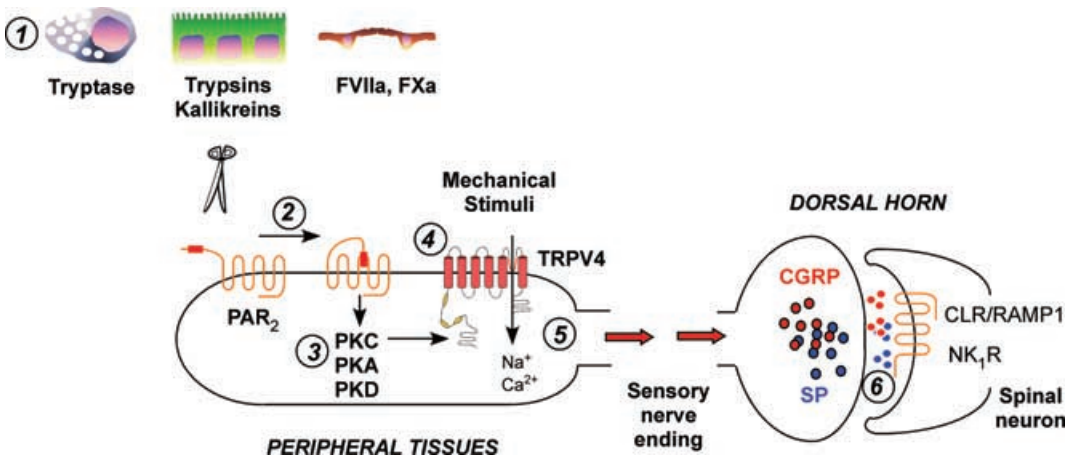

Figure 10. Proposed model of protease-induced mechanical hyperalgesia
Proteases that are generated during inflammation (1) activate PAR_2 on afferent nerve endings in peripheral tissues (2). PAR_2 couples to activation of second messenger kinases (3) that may phosphorylate and sensitize TRPV4 (4), resulting in enhanced influx of Na^+ and Ca^{2+} ions (5) and elevated release of CGRP and SP in the dorsal horn in response to mechanical stimuli (6). CGRP and SP activate their receptors on spinal neurons (CGRP, calcitonin-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1); SP, neurokinin 1 receptor (NK_1R)), resulting in enhanced transmission of nociceptive signals and mechanical hyperalgesia.

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

General mechanisms by which PAR_2 regulates afferent neurons

PAR_2 may regulate the activity of primary spinal afferent neurons by several mechanisms. The mechanism that we describe, by which PAR_2 activates second messenger kinases that sensitize TRPV4 Ca^{2+} signals and currents to enhance release of nociceptive peptides in the spinal cord and induce mechanical hyperalgesia and allodynia *in vivo* (Fig. 10), resembles the mechanism by which PAR_2 sensitizes TRPV1 to cause hyperalgesia to thermal stimuli (Amadesi *et al.* 2004, 2006; Dai *et al.* 2004). Agonists of PAR_2 sensitize TRPV1 Ca^{2+} signals and currents and enhance release of SP and CGRP from the dorsal horn of the spinal cord (Amadesi *et al.* 2004; Dai *et al.* 2004). Antagonism or deletion of TRPV1 inhibits PAR_2-induced hyperalgesia to thermal stimuli, indicating an essential role for TRPV1 in this process (Amadesi *et al.* 2004). PAR_2 agonists promote phosphorylation of TRPV1, and sensitization of TRPV1 depends on activity of PLCβ, PKA and PKCϵ (Amadesi *et al.* 2004; Amadesi *et al.* 2006; Dai *et al.* 2004).

In addition to PAR_2 agonists, other inflammatory agents sensitize TRPV1 and TRPV4, including bradykinin, ATP, prostaglandin E_2 and nerve growth factor (Lopshire & Nicol, 1997; Chuang *et al.* 2001; Tominaga *et al.* 2001; Vellani *et al.* 2001; Alessandri-Haber *et al.* 2003, 2005; Alessandri-Haber *et al.* 2006). Mechanisms of sensitization include PKC- and PKA-dependent phosphorylation of TRPV1, which modify channel gating and desensitization (Lopshire & Nicol, 1998; Cesare *et al.* 1999; Premkumar & Ahern, 2000; Bhave *et al.* 2002; Numazaki *et al.* 2002; Rathee *et al.* 2002; Mohapatra & Nau, 2003). Additional mechanisms of TRPV1 sensitization include altered interactions of TRPV1 with the endogenous inhibitor phospatidylinositol-4,5-bisphosphate (Chuang *et al.* 2001; Prescott & Julius, 2003), and PKC- and Src kinase-dependent trafficking of TRPV1 to the plasma membrane (Morenilla-Palao *et al.* 2004; Zhang *et al.* 2005). It remains to be determined whether alterations in phosphorylation and membrane translocation mediate PAR_2-induced sensitization of TRPV4, and whether PAR_2 agonists also sensitize other TRP channels of afferent neurons.

PAR_2 agonists also transiently depolarize primary spinal afferent neurons and induce a sustained hyperexcitability to depolarizing stimuli (Amadesi *et al.* 2004). This hyperexcitability does not involve sensitization of TRPV channels, but rather involves suppression of delayed rectifier IK currents (N.W. Burnett and S. Vanner unpublished observation). Thus, PAR_2 can regulate activity of afferent neurons by several mechanisms.

These mechanisms, by which PAR_2 agonists signal to primary spinal afferent neurons to sensitize TRPV1 and TRPV4 and suppress IK currents, may be relevant in painful inflammatory diseases such as arthritis, inflammatory bowel disease and irritable bowel syndrome, where multiple PAR_2-activating proteases are produced (Nakano *et al.* 1999; Tarlton *et al.* 2000; Mohammed *et al.* 2003; Barbara *et al.* 2004; Hansen *et al.* 2005). Thus, antagonists of PAR_2, second messenger kinases and TRPV channels may be promising therapies for inflammatory hyperalgesia.

References

Alessandri-Haber N, Dina OA, Joseph EK, Reichling D & Levine JD (2006). A transient receptor potential vanilloid 4-dependent mechanism of hyperalgesia is engaged by concerted action of inflammatory mediators. *J Neurosci* **26**, 3864–3874.

Alessandri-Haber N, Joseph E, Dina OA, Liedtke W & Levine JD (2005). TRPV4 mediates pain-related behavior induced by mild hypertonic stimuli in the presence of inflammatory mediator. *Pain* **118**, 70–79.

Alessandri-Haber N, Yeh JJ, Boyd AE, Parada CA, Chen X, Reichling DB & Levine JD (2003). Hypotonicity induces TRPV4-mediated nociception in rat. *Neuron* **39**, 497–511.

Amadesi S, Cottrell GS, Divino L, Chapman K, Grady EF, Bautista F, Karanjia R, Barajas-Lopez C, Vanner S, Vergnolle N & Bunnett NW (2006). Protease-activated receptor 2 sensitizes TRPV1 by protein kinase Cϵ- and A-dependent mechanisms in rats and mice. *J Physiol* **575**, 555–571.

Amadesi S, Nie J, Vergnolle N, Cottrell GS, Grady EF, Trevisani M, Manni C, Geppetti P, McRoberts JA, Ennes H, Davis JB, Mayer EA & Bunnett NW (2004). Protease-activated receptor 2 sensitizes the capsaicin receptor transient receptor potential vanilloid receptor 1 to induce hyperalgesia. *J Neurosci* **24**, 4300–4312.

Arniges M, Fernandez-Fernandez JM, Albrecht N, Schaefer M & Valverde MA (2006). Human TRPV4 channel splice variants revealed a key role of ankyrin domains in multimerization and trafficking. *J Biol Chem* **281**, 1580–1586.

Asfaha S, Brussee V, Chapman K, Zochodne DW & Vergnolle N (2002). Proteinase-activated receptor-1 agonists attenuate nociception in response to noxious stimuli. *Br J Pharmacol* **135**, 1101–1106.

Barbara G, Stanghellini V, De Giorgio R, Cremon C, Cottrell GS, Santini D, Pasquinelli G, Morselli-Labate AM, Grady EF, Bunnett NW, Collins SM & Corinaldesi R (2004). Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology* **126**, 693–702.

Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau R & Wt. (2002). cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. *Neuron* **35**, 721–731.

Bohm SK, Khitin LM, Grady EF, Aponte G, Payan DG & Bunnett NW (1996*a*). Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. *J Biol Chem* **271**, 22003–22016.

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

Bohm SK, Kong W, Bromme D, Smeekens SP, Anderson DC, Connolly A, Kahn M, Nelken NA, Coughlin SR, Payan DG & Bunnett NW (1996*b*). Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. *Biochem J* **314**, 1009–1016.

Camerer E, Huang W & Coughlin SR (2000). Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proc Natl Acad Sci U S A* **97**, 5255–5260.

Cenac N, Garcia-Villar R, Ferrier L, Larauche M, Vergnolle N, Bunnett NW, Coelho AM, Fioramonti J & Bueno L (2003). Proteinase-activated receptor-2-induced colonic inflammation in mice: possible involvement of afferent neurons, nitric oxide, and paracellular permeability. *J Immunol* **170**, 4296–4300.

Cesare P, Dekker LV, Sardini A, Parker PJ & McNaughton PA (1999). Specific involvement of PKC-epsilon in sensitization of the neuronal response to painful heat. *Neuron* **23**, 617–624.

Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV & Julius D (2001). Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns (4,5),P2-mediated inhibition. *Nature* **411**, 957–962.

Chung MK, Lee H & Caterina MJ (2003). Warm temperatures activate TRPV4 in mouse 308 keratinocytes. *J Biol Chem* **278**, 32037–32046.

Chung MK, Lee H, Mizuno A, Suzuki M & Caterina MJ (2004). TRPV3 and TRPV4 mediate warmth-evoked currents in primary mouse keratinocytes. *J Biol Chem* **279**, 21569–21575.

Coelho AM, Vergnolle N, Guiard B, Fioramonti J & Bueno L (2002). Proteinases and proteinase-activated receptor 2: a possible role to promote visceral hyperalgesia in rats. *Gastroenterology* **122**, 1035–1047.

Corvera CU, Dery O, McConalogue K, Bohm SK, Khitin LM, Caughey GH, Payan DG & Bunnett NW (1997). Mast cell tryptase regulates rat colonic myocytes through proteinase-activated receptor 2. *J Clin Invest* **100**, 1383–1393.

Cottrell GS, Amadesi S, Grady EF & Bunnett NW (2004). Trypsin IV, a novel agonist of protease-activated receptors 2 and 4. *J Biol Chem* **279**, 13532–13539.

Dai Y, Moriyama T, Higashi T, Togashi K, Kobayashi K, Yamanaka H, Tominaga M & Noguchi K (2004). Proteinase-activated receptor 2-mediated potentiation of transient receptor potential vanilloid subfamily 1 activity reveals a mechanism for proteinase-induced inflammatory pain. *J Neurosci* **24**, 4293–4299.

Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ & Johannes FJ (1996). Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes. *FEBS Lett* **392**, 77–80.

Guler AD, Lee H, Iida T, Shimizu I, Tominaga M & Caterina M (2002). Heat-evoked activation of the ion channel, TRPV4. *J Neurosci* **22**, 6408–6414.

Hansen KK, Sherman PM, Cellars L, Andrade-Gordon P, Pan Z, Baruch A, Wallace JL, Hollenberg MD & Vergnolle N (2005). A major role for proteolytic activity and proteinase-activated receptor-2 in the pathogenesis of infectious colitis. *Proc Natl Acad Sci U S A* **102**, 8363–8368.

Lee H, Iida T, Mizuno A, Suzuki M & Caterina MJ (2005). Altered thermal selection behavior in mice lacking transient receptor potential vanilloid 4. *J Neurosci* **25**, 1304–1310.

Liedtke W, Choe Y, Marti-Renom MA, Bell AM, Denis CS, Sali A, Hudspeth AJ, Friedman JM & Heller S (2000). Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell* **103**, 525–535.

Liedtke W & Friedman JM (2003). Abnormal osmotic regulation in trpv4–/– mice. *Proc Natl Acad Sci U S A* **100**, 13698–13703.

Liedtke W, Tobin DM, Bargmann CI & Friedman JM (2003). Mammalian TRPV4 (VR-OAC) directs behavioral responses to osmotic and mechanical stimuli in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* **100** (Suppl. 2), 14531–14536.

Lopshire JC & Nicol GD (1997). Activation and recovery of the PGE2-mediated sensitization of the capsaicin response in rat sensory neurons. *J Neurophysiol* **78**, 3154–3164.

Lopshire JC & Nicol GD (1998). The cAMP transduction cascade mediates the prostaglandin E2 enhancement of the capsaicin-elicited current in rat sensory neurons: whole-cell and single-channel studies. *J Neurosci* **18**, 6081–6092.

Mantyh PW, DeMaster E, Malhotra A, Ghilardi JR, Rogers SD, Mantyh CR, Liu H, Basbaum AI, Vigna SR, Maggio JE & *et al*. (1995). Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. *Science* **268**, 1629–1632.

Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D & Schachtele C (1993). Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J Biol Chem* **268**, 9194–9197.

Marvizon JC, Martinez V, Grady EF, Bunnett NW & Mayer EA (1997). Neurokinin 1 receptor internalization in spinal cord slices induced by dorsal root stimulation is mediated by NMDA receptors. *J Neurosci* **17**, 8129–8136.

Marvizon JC, Wang X, Matsuka Y, Neubert JK & Spigelman I (2003). Relationship between capsaicin-evoked substance P release and neurokinin 1 receptor internalization in the rat spinal cord. *Neuroscience* **118**, 535–545.

Mohammed FF, Smookler DS & Khokha R (2003). Metalloproteinases, inflammation, and rheumatoid arthritis. *Ann Rheum Dis* **62** (Suppl. 2), ii43–47.

Mohapatra DP & Nau C (2003). Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 Is decreased by the cyclic AMP-dependent protein kinase pathway. *J Biol Chem* **278**, 50080–50090.

Molino M, Barnathan ES, Numerof R, Clark J, Dreyer M, Cumashi A, Hoxie JA, Schechter N, Woolkalis M & Brass LF (1997). Interactions of mast cell tryptase with thrombin receptors and PAR-2. *J Biol Chem* **272**, 4043–4049.

Morenilla-Palao C, Planells-Cases R, Garcia-Sanz N & Ferrer-Montiel A (2004). Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. *J Biol Chem* **279**, 25665–25672.

Nakano S, Ikata T, Kinoshita I, Kanematsu J & Yasuoka S (1999). Characteristics of the protease activity in synovial fluid from patients with rheumatoid arthritis and osteoarthritis. *Clin Exp Rheumatol* **17**, 161–170.

Nguyen C, Coelho AM, Grady E, Compton SJ, Wallace JL, Hollenberg MD, Cenac N, Garcia-Villar R, Bueno L, Steinhoff M, Bunnett NW & Vergnolle N (2003). Colitis induced by proteinase-activated receptor-2 agonists is mediated by a neurogenic mechanism. *Can J Physiol Pharmacol* **81**, 920–927.

© 2007 The Authors. Journal compilation © 2007 The Physiological Society

Numazaki M, Tominaga T, Toyooka H & Tominaga M (2002). Direct phosphorylation of capsaicin receptor VR1 by protein kinase Cepsilon and identification of two target serine residues. *J Biol Chem* **277**, 13375–13378.

Nystedt S, Emilsson K, Wahlestedt C & Sundelin J (1994). Molecular cloning of a potential proteinase activated receptor [see comments]. *Proc Natl Acad Sci U S A* **91**, 9208–9212.

Oikonomopoulou K, Hansen KK, Saifeddine M, Tea I, Blaber M, Blaber SI, Scarisbrick I, Andrade-Gordon P, Cottrell GS, Bunnett NW, Diamandis EP & Hollenberg MD (2006). Proteinase-activated receptors (PARs): targets for kallikrein signalling. *J Biol Chem* **281**, 32095–32112.

Ossovskaya VS & Bunnett NW (2004). Protease-activated receptors: contribution to physiology and disease. *Physiol Rev* **84**, 579–621.

Pedersen S, Lambert IH, Thoroed SM & Hoffmann EK (2000). Hypotonic cell swelling induces translocation of the alpha isoform of cytosolic phospholipase A2 but not the gamma isoform in Ehrlich ascites tumor cells. *Eur J Biochem* **267**, 5531–5539.

Premkumar LS & Ahern GP (2000). Induction of vanilloid receptor channel activity by protein kinase C. *Nature* **408**, 985–990.

Prescott ED & Julius D (2003). A modular PIP2 binding site as a determinant of capsaicin receptor sensitivity. *Science* **300**, 1284–1288.

Rathee PK, Distler C, Obreja O, Neuhuber W, Wang GK, Wang SY, Nau C & Kress M (2002). PKA/AKAP/VR-1 module: a common link of Gs-mediated signaling to thermal hyperalgesia. *J Neurosci* **22**, 4740–4745.

Steinhoff M, Corvera CU, Thoma MS, Kong W, McAlpine BE, Caughey GH, Ansel JC & Bunnett NW (1999). Proteinase-activated receptor-2 in human skin: tissue distribution and activation of keratinocytes by mast cell tryptase. *Exp Dermatol* **8**, 282–294.

Steinhoff M, Vergnolle N, Young SH, Tognetto M, Amadesi S, Ennes HS, Trevisani M, Hollenberg MD, Wallace JL, Caughey GH, Mitchell SE, Williams LM, Geppetti P, Mayer EA & Bunnett NW (2000). Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. *Nat Med* **6**, 151–158.

Suzuki M, Mizuno A, Kodaira K & Imai M (2003). Impaired pressure sensation in mice lacking TRPV4. *J Biol Chem* **278**, 22664–22668.

Tarlton JF, Whiting CV, Tunmore D, Bregenholt S, Reimann J, Claesson MH & Bland PW (2000). The role of up-regulated serine proteases and matrix metalloproteinases in the pathogenesis of a murine model of colitis. *Am J Pathol* **157**, 1927–1935.

Tominaga M, Wada M & Masu M (2001). Potentiation of capsaicin receptor activity by metabotropic ATP receptors as a possible mechanism for ATP-evoked pain and hyperalgesia. *Proc Natl Acad Sci U S A* **98**, 6951–6956.

Vellani V, Mapplebeck S, Moriondo A, Davis JB & McNaughton PA (2001). Protein kinase C activation potentiates gating of the vanilloid receptor VR1 by capsaicin, protons, heat and anandamide. *J Physiol* **534**, 813–825.

Vergnolle N, Bunnett NW, Sharkey KA, Brussee V, Compton SJ, Grady EF, Cirino G, Gerard N, Basbaum AI, Andrade-Gordon P, Hollenberg MD & Wallace JL (2001). Proteinase-activated receptor-2 and hyperalgesia: a novel pain pathway. *Nat Med* **7**, 821–826.

Vriens J, Watanabe H, Janssens A, Droogmans G, Voets T & Nilius B (2004). Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. *Proc Natl Acad Sci U S A* **101**, 396–401.

Wang Y, Kedei N, Wang M, Wang QJ, Huppler AR, Toth A, Tran R & Blumberg PM (2004). Interaction between protein kinase Cmu and the vanilloid receptor type 1. *J Biol Chem* **279**, 53674–53682.

Watanabe H, Davis JB, Smart D, Jerman JC, Smith GD, Hayes P, Vriens J, Cairns W, Wissenbach U, Prenen J, Flockerzi V, Droogmans G, Benham CD & Nilius B (2002). Activation of TRPV4 channels (hVRL-2/mTRP12) by phorbol derivatives. *J Biol Chem* **277**, 13569–13577.

Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T & Nilius B (2003). Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature* **424**, 434–438.

Zhang X, Huang J & McNaughton PA (2005). NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *EMBO J* **24**, 4211–4223.

Acknowledgements

Grant support: National Institutes of Health DK57840, DK43207 and DK39957 (N.W.B.); Wellcome Trust (A.G., N.W.B.); MIUR-Rome, Italy 2004062950-004 (P.G.); Canadian Institutes of Health Research (N.V., S.V., G.W.Z.); Alberta Heritage Foundation for Medical Research (N.V., G.W.Z., C.A.); Crohn's and Colitis Foundation of Canada (N.C., S.V.); Canadian Association of Gastroenterology (N.C.).

© 2007 The Authors. Journal compilation © 2007 The Physiological Society