Cellular/Molecular

Protease-Activated Receptor 2 Sensitizes the Capsaicin Receptor Transient Receptor Potential Vanilloid Receptor 1 to Induce Hyperalgesia

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Inflammatory proteases (mast cell tryptase and trypsins) cleave protease-activated receptor 2 (PAR_2) on spinal afferent neurons and cause persistent inflammation and hyperalgesia by unknown mechanisms. We determined whether transient receptor potential vanilloid receptor 1 (TRPV1), a cation channel activated by capsaicin, protons, and noxious heat, mediates PAR_2-induced hyperalgesia. PAR_2 was coexpressed with TRPV1 in small- to medium-diameter neurons of the dorsal root ganglia (DRG), as determined by immunofluorescence. PAR_2 agonists increased intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in these neurons in culture, and PAR_2-responsive neurons also responded to the TRPV1 agonist capsaicin, confirming coexpression of PAR_2 and TRPV1. PAR_2 agonists potentiated capsaicin-induced increases in $[Ca^{2+}]_i$ in TRPV1-transfected human embryonic kidney (HEK) cells and DRG neurons and potentiated capsaicin-induced currents in DRG neurons. Inhibitors of phospholipase C and protein kinase C (PKC) suppressed PAR_2-induced sensitization of TRPV1-mediated changes in $[Ca^{2+}]_i$ and TRPV1 currents. Activation of PAR_2 or PKC induced phosphorylation of TRPV1 in HEK cells, suggesting a direct regulation of the channel. Intraplantar injection of a PAR_2 agonist caused persistent thermal hyperalgesia that was prevented by antagonism or deletion of TRPV1. Coinjection of nonhyperalgesic doses of PAR_2 agonist and capsaicin induced hyperalgesia that was inhibited by deletion of TRPV1 or antagonism of PKC. PAR_2 activation also potentiated capsaicin-induced release of substance P and calcitonin gene-related peptide from superfused segments of the dorsal horn of the spinal cord, where they mediate hyperalgesia. We have identified a novel mechanism by which proteases that activate PAR_2 sensitize TRPV1 through PKC. Antagonism of PAR_2, TRPV1, or PKC may abrogate protease-induced thermal hyperalgesia.

Key words: protease-activated receptors; TRPV1; protein kinase C; hyperalgesia; inflammation; substance P

Introduction

Proteases from the circulation, inflammatory cells, epithelial cells, and neurons, which are generated during injury, cleave protease-activated receptors (PARs) to regulate hemostasis, inflammation, pain, and healing (Ossovskaya and Bunnett, 2004). Cleavage exposes tethered ligand domains that bind and activate cleaved receptors. Thrombin activates PAR_1, PAR_3, and PAR_4 (Vu et al., 1991; Ishihara et al., 1997; Kahn et al., 1998), and factors VIIa and Xa activate PAR_2 and PAR_1 (Camerer et al.,

2000). Mast cell tryptase activates PAR_2 (Corvera et al., 1997; Molino et al., 1997), and neutrophil cathepsin G activates PAR_4 (Sambrano et al., 2000). Pancreatic and extrapancreatic trypsins activate PAR_2 and PAR_4 (Nystedt et al., 1994; Kahn et al., 1998; Alm et al., 2000; Sawada et al., 2000; Cottrell et al., 2004).

Many of the effects of proteases that activate PAR_2 are mediated by receptors in the peripheral nervous system. PAR_2 is expressed by dorsal root ganglia (DRG) neurons containing substance P (SP) and calcitonin gene-related peptide (CGRP) (Steinhoff et al., 2000), which control inflammation and pain. PAR_2 agonists stimulate SP and CGRP release from the peripheral projections of DRG neurons in the skin and intestine, causing edema and hyperemia ("neurogenic inflammation"; Steinhoff et al., 2000; Cenac et al., 2002, 2003). They also stimulate airway constriction (Ricciardolo et al., 2000) and gastric mucus secretion (Kawabata et al., 2001b) by neurogenic mechanisms. Intraplantar injection of PAR_2 agonists induces neuropeptide release from the central projections of DRG neurons in the spinal cord to activate nociceptive neurons and cause hyperalgesia

(Kawabata et al., 2001a; Vergnolle et al., 2001). Activation of PAR$_2$ in the pancreas and intestine also excites nociceptive neurons and induces visceral hyperalgesia (Hoogerwerf et al., 2001; Coelho et al., 2002; Kirkup et al., 2003). However, the mechanisms by which PAR$_2$ regulates neuronal function to cause inflammation and hyperalgesia are unknown.

We investigated the contribution of transient receptor potential vanilloid receptor 1 (TRPV1) to PAR$_2$-mediated hyperalgesia. TRPV1 is a nonselective cation channel expressed by nociceptive neurons that makes a major contribution to inflammatory and thermal hyperalgesia (Caterina et al., 1997; Caterina et al., 2000; Davis et al., 2000). Exogenous (capsaicin and ethanol) and endogenous (protons of pH <6.0, noxious heat of >43°C, and anandamide) factors directly activate TRPV1 (Caterina et al., 1997; Zygmunt et al., 1999; Trevisani et al., 2002). Inflammatory agents that activate G-protein-coupled receptors [e.g., bradykinin, ATP, and prostaglandin E$_2$ (PGE$_2$) (Tominaga et al., 2001; Vellani et al., 2001; Hu et al., 2002; Rathee et al., 2002; Mohapatra and Nau, 2003)] and receptor-tyrosine kinases (e.g., NGF; Shu and Mendell, 1999; Chuang et al., 2001) can indirectly sensitize TRPV1 to cause hyperalgesia.

We hypothesized that PAR$_2$ sensitizes TRPV1 to induce hyperalgesia. Sensitization is suggested by the observations that PAR$_2$ agonists enhance capsaicin-evoked CGRP release from DRG and fos expression in spinal neurons (Hoogerwerf et al., 2001). The TRPV1 antagonist capsazepine attenuates PAR$_2$-stimulated thermal hyperalgesia (Kawao et al., 2002) and gastric mucus secretion (Kawabata et al., 2002). However, direct evidence of PAR$_2$-induced sensitization of TRPV1 is lacking and the molecular mechanisms are unknown. Our aims were (1) to determine whether PAR$_2$ activation sensitizes TRPV1 Ca^{2+} signaling and TRPV1 currents to induce thermal hyperalgesia; (2) to elucidate the mechanism of sensitization; (3) to determine whether PAR$_2$ activation phosphorylates TRPV1; and (4) to investigate whether PAR$_2$ activation potentiates TRPV1-induced release of neuropeptides from the central projections of nociceptive DRG neurons.

Materials and Methods

Animals. Sprague Dawley rats (male, 200–250 gm) and C57BL/6 mice (6–8 weeks) were from Charles River Laboratories. TRPV1$^{-/-}$ and TRPV1$^{+/+}$ (C57BL/6J) mice were from The Jackson Laboratory (Bar Harbor, ME). Littermates were used in control experiments. Procedures were approved by Institutional Animal Care and Use Committees.

PAR activators and inhibitors. Synthetic peptides corresponding to the tethered ligands [activating peptides (APs)] can directly activate PAR$_1$, PAR$_2$, and PAR$_4$ and are useful tools for investigating receptor functions. PAR$_2$-AP (SLIGRL-NH$_2$), corresponding to the tethered ligand of the rat receptor, and an analog of PAR$_1$-AP (TFLLR-NH$_2$), which specifically activates PAR$_1$, were synthesized and purified as described (Steinhoff et al., 2000). The reverse peptide sequences (PAR-RP), which do not activate PARs, were used for controls. Tryptase was purified from human lung (Steinhoff et al., 2000). Bovine pancreatic trypsin was from Worthington (Lakewood, NJ). Thrombin, U73122 [1-[6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione], GFX [(2-[1-(3-dimethyl-aminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide], Gö6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole, and phorbol-12-myristate 13-acetate (PMA) were from Calbiochem (La Jolla, CA). We used concentrations of agonists and antagonists that we have previously shown to selectively activate PAR$_1$ and PAR$_2$ in neurons (Steinhoff et al., 2000; de Garavilla et al., 2001) and that effectively and selectively inhibit the targeted enzymes (Bohm et al., 1996).

Cell and neuronal culture. Human embryonic kidney 293 (HEK293) cells stably expressing human TRPV1 were generated and maintained in Eagle's MEM with Earle's BSS medium (MEM), 10% fetal bovine serum, and geniticin (400 mg/l) (Hayes et al., 2000). DRG from thoracic and lumbar spinal cord of rats were minced in cold HBSS and incubated for 60–90 min at 37°C in DMEM containing (in mg/ml): 0.5 trypsin, 1 collagenase type IA, and 0.1 DNase type IV (Sigma, St. Louis, MO) (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 penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine, and 2.5 μg/ml DNase type IV, plated on glass coverslips coated with Matrigel (BD Biosciences, Bedford, MA), and cultured for 2–3 d.

Reverse transcription-PCR. Total RNA from HEK293-TRPV1 (1 μg) was reverse-transcribed with oligo(dT)$_{15}$ and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). PCR reactions used primers specific to the C terminus of human PAR$_2$ (forward, 5′-ccctttg-tatgtcgtgaagcagac-3′; reverse, 5′-ttcctggagtgtttctttgaggtg-3′) and human PAR$_1$ (forward, 5′-agcaaaccatccaggtgc-3′; reverse, 5′-tgttataactgctgggatcg-3′). Control reactions omitted reverse transcriptase. Products were separated by electrophoresis (2% agarose gel), detected using ethidium bromide, and sequenced.

Immunofluorescence. Rats were transcardially perfused with 4% paraformaldehyde in 100 mM PBS, pH 7.4. DRG were fixed overnight and embedded on optimal cutting temperature compound, and 14 μm sections were prepared (Steinhoff et al., 2000). Cultured DRG were fixed with 4% paraformaldehyde for 20 min at 4°C. Sections and cultures were incubated with the primary antibodies for 16 hr at 4°C: PAR$_2$, rabbit antibody (Ab) B5 to the N terminus of rat PAR$_2$ (Dr. M. Hollenberg, University of Calgary; Kong et al., 1997), 1:250–1:500; and TRPV1, guinea pig Ab to the C terminus of rat TRPV1 (Chemicon, Temecula, CA), 1:1000–1:20,000. Sections and cultures were incubated with anti-rabbit or anti-guinea pig IgG conjugated to Texas Red or FITC (Jackson ImmunoResearch, West Grove, PA). In the case of the sections, the TRPV1 signal was amplified using a tyramide signal amplification system (PerkinElmer Life Sciences, Boston, MA). Specimens were observed using a Zeiss (Thornwood, NY) Axiovert and an MRC 1000 laser scanning confocal microscope (Bio-Rad, Hercules, CA). In controls, primary antibodies were preincubated with 10 μM peptides used for immunization for 48 hr at 4°C, which abolished staining.

Immunoprecipitation and Western blotting. HEK-TRPV1 cells were maintained overnight in MEM and 0.1% BSA, incubated with agonists for 10 min at 37°C, and lysed with radioimmunoprecipitation assay buffer. Samples (500 μl) were incubated with rabbit anti-human TRPV1 (1 μg/ml, overnight, 4°C; Chan et al., 2003). Protein A/G Plus (Santa Cruz Biotechnology, Santa Cruz, CA; 30 μl) was added and mixed for 2 hr at 4°C. Beads were pelleted, suspended in 2× SDS loading buffer, and boiled for 5 min, and the supernatant was fractionated by SDS-PAGE (8% gel). Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were incubated with Ab to phosphoserine (Zymed, South San Francisco, CA; 1:1000 in PBS, 2% BSA, and 0.1% Tween 20, overnight, 4°C) and then with goat anti-rabbit horseradish peroxidase (Jackson ImmunoResearch; 1:10,000, 1 hr, room temperature). Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). To ensure equal loading, membranes were stripped in 62.5 nM Tris-HCl, pH 6.8, 2% SDS, and 100 mM 2-mercaptoethanol and re-probed with anti-TRPV1 (0.2 μg/ml, overnight, 4°C).

Measurement of intracellular [Ca^{2+}]. HEK293-TRPV1 cells and DRG neurons were incubated in HBSS, 0.1% BSA, and 20 mM HEPES, pH 7.4, containing 2.5–5 μM fura-2 AM (Molecular Probes, Eugene, OR) for 30–45 min at 37°C (Steinhoff et al., 2000). Coverslips were mounted in an open chamber at 37°C. Fluorescence of individual cells was measured at 340 and 380 nm excitation and 510 nm emission using a Zeiss Axiovert microscope, an intensified CCD video camera (Stanford Photonics, Stanford, CA), and a video microscopy acquisition program (Axon Instruments, Union City, CA). Test substances were directly added to the chamber (50 μl injection). Each coverslip received only one treatment with PAR$_2$-AP or -RP followed by capsaicin. DRG preparations were challenged with KCl (50 mM) at the end of each experiment. In some

experiments, cells were pretreated with inhibitors for 30 min at 37°C before the challenge with test compounds. The magnitude of responses to test agents was calculated as the increase above baseline. Results are expressed as the 340:380 nm emission ratio, which is proportional to the intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$). The effects of the inhibitors on sensitization of TRPV1 were calculated as a ratio and are expressed as a percentage of the potentiation observed in positive controls (100%) pretreated with PAR$_2$ agonists or PMA. To determine the source of PAR-induced increases in $[Ca^{2+}]_i$, some experiments were completed in Ca^{2+}-free buffer.

Electrophysiology. DRG neurons were placed in an open recording chamber and perfused with Ringer's solution (in mM: 140 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, and 10 glucose, pH 7.4 at 21°C). Membrane currents and voltages were recorded from small- and medium-diameter neurons under the whole-cell configuration with an Axon Instruments 1D patch-clamp amplifier. Recording pipettes (outer diameter, 1.65 ± 0.05 μm) had resistances of 2–4 MΩ when filled with buffer (in mM: 140 KCl, 5 MgCl$_2$, 4 Na$_2$ATP, 0.3 Na$_3$GTP, 2.5 CaCl$_2$, 5 EGTA, and 10 HEPES, pH 7.2). After establishing the whole-cell configuration, neurons were required to maintain a zero-current potential of less than −45 mV for 5 min. Voltage changes evoked by the injection of current or application of PAR$_2$ agonists (applied by fast perfusion for 3 min) were filtered at 2 kHz and sampled at 20 kHz using pClamp 8 software (Axon Instruments). To assess excitability, the rheobase was measured by step increases of the amount of injected current (40 pA for 200 msec every 30 sec) under the current-clamp mode (Abdulla and Smith, 1997). Capsaicin-induced currents were recorded under the voltage-clamp mode when membrane potential was held at −60 mV (Nicol and Cui, 1994). Whole-cell currents evoked by capsaicin were filtered at 1 kHz and sampled at 1 KHz. Capsaicin was administered by fast perfusion for 150 msec to 1 sec, adjusted according to the amplitude of the current. PAR$_2$-AP, trypsin, PAR$_2$-RP, and inactivated trypsin were also applied by fast perfusion for 3 min. Because of limited quantities, tryptase was "puffed" into the neurons (Picospritzer II; General Valve, Fairfield, NJ). In some experiments, neurons were pretreated with inhibitors for 5 min. Rheobase is expressed as a change from basal (normalized to a basal value of 1) and as a percent change from basal rheobase. Membrane potential is expressed as absolute values (millivolts). TRPV1 currents are expressed as absolute values (nanoamperes) or as fold change from basal.

Paw withdrawal latency. PAR$_2$-AP, PAR$_2$-RP (in sterile 0.9% saline), and capsaicin (in 80% sterile saline, 10% ethanol, and 10% Tween 80) were administered by intraplantar injection in mice (final, 10 μl/paw) under light halothane (5%) anesthesia (Vergnolle et al., 2001). Capsazepine (35 mg/kg, s.c.) was administered 30 min before the intraplantar injections. The protein kinase C (PKC) inhibitor GFX (1 μg in 5 μl) or vehicle (saline) was administered by intraplantar injection immediately after the intraplantar injection of distilled water (2 μl) to osmotically shock cells. These animals received

PAR$_2$-AP (1 ng) and capsaicin (1 μg) 15 min later (final, 5 μl). The latency of paw withdrawal to a radiant heat stimulus was measured before and after the intraplantar injections of test substances using a plantar test apparatus (Ugo Basile, Milan, Italy). Thermal hyperalgesia was defined as a decrease in the withdrawal latency compared with the basal measurement. The investigator was unaware of the genotype of the TRPV1$^{+/+}$ and TRPV1$^{-/-}$ mice before the experiments.

Neuropeptide release from the dorsal horn of the spinal cord. Slices (0.4 mm) of the dorsal spinal cord of rats were superfused at 0.4 ml/min with Krebs' solution (in mM: 119 NaCl, 25 NaHCO$_3$, 1.2 KH$_2$PO$_4$, 1.5 MgSO$_4$,
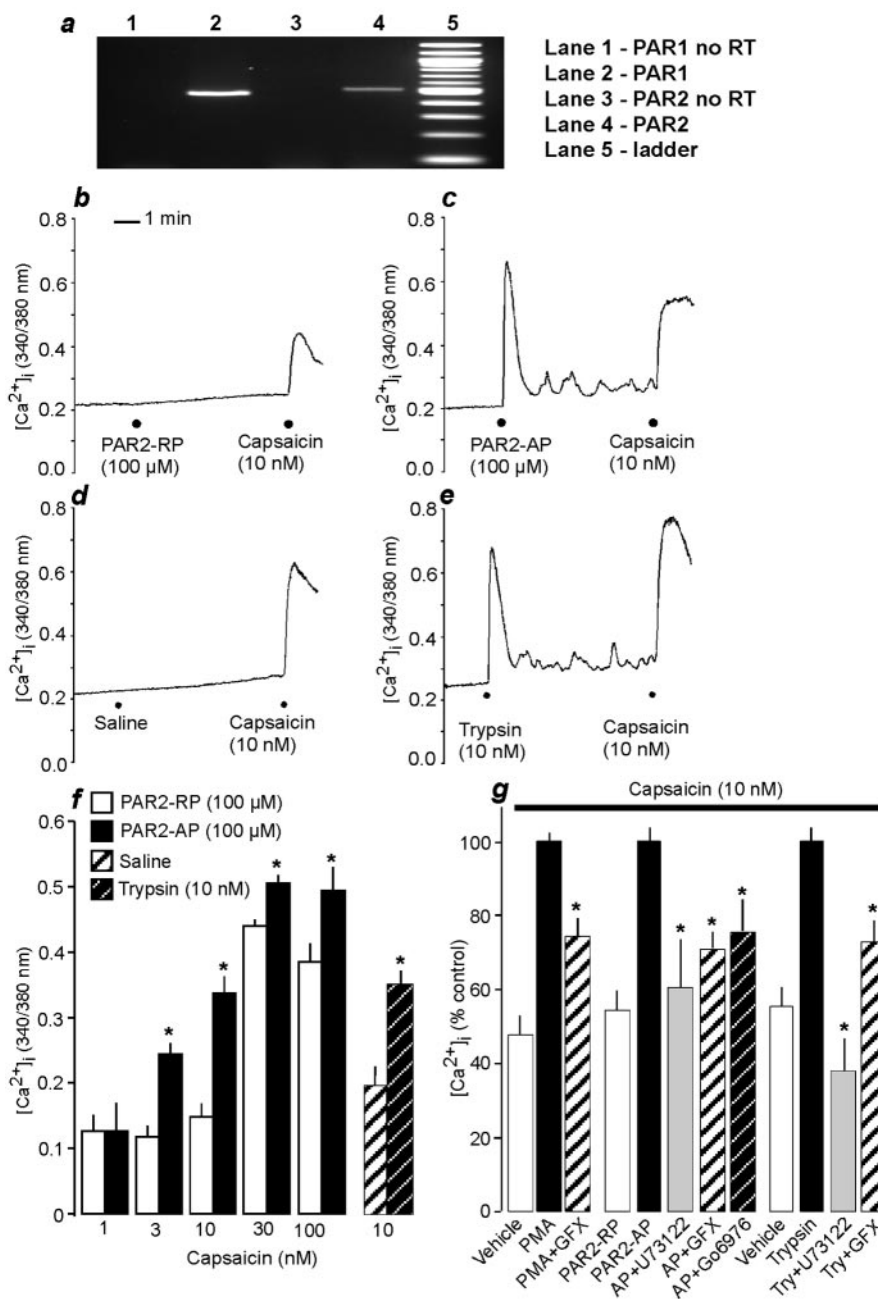

Figure 1. PAR$_2$ activation sensitizes TRPV1 in transfected cells. *a*, HEK-TRPV1 cells express PAR$_1$ and PAR$_2$. mRNA for PAR$_1$ (lane 2) and PAR$_2$ (lane 4) was detected in HEK-TRPV1 cells by RT-PCR. Lanes 1 and 3 are controls (no RT). Results are representative of three experiments. *b–g*, Cells were exposed to PAR$_2$-AP or -RP (100 μM), trypsin (10 nM), PMA (5 μM), or vehicle for 5 min and were then challenged with capsaicin. PAR$_2$ agonists (*c, e–g*) and PMA (*g*) potentiated responses to capsaicin. Inhibition of PLC (U73122, 5 μM) and PKC (GFX and Gö6976, 1 and 0.1 μM, respectively) suppressed the potentiation (*g*). Results in *g* are expressed as a percentage of the positive control (cells treated with PAR$_2$ agonists or PMA). *$p < 0.05$ compared with PAR$_2$-RP or saline (*f*), PMA, PAR$_2$-AP, or trypsin (*g*). Try, Trypsin; AP, PAR$_2$-AP; $n = 120$–1040 cells.

2.5 $CaCl_2$, 4.7 KCl, and 11 D-glucose containing 0.1% BSA, 1 μM phosphoramidon, and 1 μM captopril; 37°C, 95% O_2 and 5% CO_2). Slices were pretreated with PAR₂-AP, PAR₂-RP, or vehicle for 20 min. The superfusate was then collected at 10 min intervals. After two basal collections, slices were perfused with capsaicin for 10 min. Superfusate was analyzed for SP-like immunoreactivity (LI) and CGRP-LI by ELISA (Steinhoff et al., 2000). Peptide release was determined by subtracting basal levels from concentrations measured during and immediately after exposure to capsaicin. Results are expressed as femtomoles of peptide per gram of weighed tissue per 20 min period. The highest concentration of capsaicin, PAR₂-AP, and PAR₂-RP did not interfere with the assays.

Statistical analysis. Results are expressed as mean ± SEM and were compared by ANOVA and Student's *t* test with Bonferroni correction or by Dunnett's test. Differences were considered significant at $p < 0.05$.

Results

PAR₂ agonists sensitized TRPV1 Ca^{2+} responses in HEK-TRPV1 cells by activation of phospholipase C and PKC

We determined whether PAR₂ activation sensitizes TRPV1 expressed in HEK293 cells. By using reverse transcription (RT)-PCR, we found that these cells endogenously expressed PAR₂ mRNA (Fig. 1a). We measured $[Ca^{2+}]_i$ to assess activation of PAR₂, which couples to mobilization of Ca^{2+}, and TRPV1, a nonselective cation channel with preference for Ca^{2+} ions. PAR₂-AP (100 μM) and trypsin (10 nM) rapidly increased $[Ca^{2+}]_i$, confirming expression of functional PAR₂ (Fig. 1c,e). This rapid increase in $[Ca^{2+}]_i$ was fully maintained in Ca^{2+}-free medium and is thus attributable to mobilization of intracellular Ca^{2+} (data not shown). PAR₂-RP, which does not activate PAR₂, had no effect on $[Ca^{2+}]_i$ (Fig. 1b). Exposure to PAR₂-AP or trypsin potentiated Ca^{2+} responses to the selective TRPV1 agonist capsaicin applied 5 min later (1–100 nM; Fig. 1b–f). The response to 10 nM capsaicin was increased 127 ± 12% by PAR₂-AP (100 μM) and 79 ± 10% by trypsin (10 nM), compared with cells treated with PAR₂-RP or vehicle. PAR₂-AP increased both the potency and the efficacy of the response to capsaicin (Fig. 1f). Thus, PAR₂ activation sensitizes TRPV1. Conversely, pretreatment with capsaicin (10 nM) did not affect the increase in $[Ca^{2+}]_i$ to PAR₂-AP applied 5 min later (10–100 μM; data not shown), indicating that TRPV1 activation does not sensitize PAR₂.

The membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2) and phospholipase Cβ (PLCβ), which catalyzes the hydrolysis of PIP_2 to 1,4,5-inositol trisphosphate ($InsP_3$) and diacylglycerol (DAG), regulate TRPV1 (Chuang et al., 2001; Prescott and Julius, 2003). We investigated whether PAR₂-induced potentiation of TRPV1 was mediated by PLC. Pretreatment with the PLC inhibitor U73122 (5 μM, 30 min) attenuated the magnitude of the response to capsaicin (10 nM) by 31 ± 4% when compared with cells treated with vehicle (data not shown). These results are consistent with the observation that the inhibitory action of PIP_2 on TRPV1 is enhanced by suppression of tonic PLC activity (Chuang et al., 2001; Hu et al., 2002; Prescott and Julius, 2003). Nonetheless, inhibition of PLC suppressed the potentiation of capsaicin responses induced by PAR₂-AP by >80% and eliminated potentiation by trypsin, reducing the response below levels in vehicle-treated cells. Thus, when compared with the positive control (capsaicin response in cells treated with PAR₂-AP or trypsin, 100%), the response to capsaicin was 61 ± 13% after U73122 and PAR₂-AP and 38 ± 9% after U73122 and trypsin (Fig. 1g).

DAG generated by PLCβ from membrane phospholipids activates PKC. Because PKC can directly phosphorylate TRPV1 and thereby sensitize responses to TRPV1 agonists (Premkumar and Ahern, 2000; Vellani et al., 2001; Crandall et al., 2002; Numazaki

et al., 2002), we hypothesized that PKC mediates PAR₂-induced sensitization of TRPV1. The PKC activator PMA (5 μM, 5 min) enhanced the response to 10 nM capsaicin (Fig. 1g). This effect was reduced by ~50% by the general PKC inhibitor GFX (1 μM, 30 min). Similarly, GFX inhibited PAR₂-induced potentiation of responses to capsaicin >60%. The effects of capsaicin after GFX and PAR₂-AP and GFX and trypsin were 71 ± 5% and 73 ± 6%, respectively, when compared with the positive control (Fig. 1g). To characterize the isozymes of PKC that mediate PAR₂-induced sensitization of TRPV1, we used Gö6976, an inhibitor of conventional PKC isoforms (α, β_1, β_2, and γ) but not the PKCϵ isoform. Pretreatment with Gö6976 (0.1 μM, 30 min) inhibited PAR₂-induced sensitization of responses to 10 nM capsaicin by ~50%. The effect of capsaicin after Gö6976 and PAR₂-AP was 77 ± 7% of the positive control (Fig. 1g). Pretreatment with GFX or Gö6976 did not affect the responses of capsaicin in cells treated with vehicle and PAR₂-RP (data not shown).

PAR₂ agonists sensitized TRPV1 Ca^{2+} responses in DRG neurons by activation of PLC and PKC

To determine whether PAR₂ and TRPV1 are coexpressed in DRG neurons, we localized the proteins by immunofluorescence in tissue sections and cultures and measured $[Ca^{2+}]_i$ in DRG cultures to selective agonists. Immunoreactive PAR₂ and TRPV1 were colocalized in small- and medium-diameter ($\leq$30 μm) neurons in sections of DRG from adult rat (Fig. 2a, top panels), and expression was retained in culture (Fig. 2a, bottom panels). Capsaicin (100 nM) increased $[Ca^{2+}]_i$ in 52 ± 5% of neurons responding to KCl (50 mM; n = 103 total neurons). PAR₂-AP (100 μM) increased $[Ca^{2+}]_i$ in 32 ± 2% (n = 439) of neurons, and trypsin (10 nM) increased it in 44 ± 9% (n = 34) of neurons, whereas PAR₂-RP was ineffective (Fig. 2b–e). We have previously shown that tryptase mobilizes Ca^{2+} in a similar proportion of neurons by activating PAR₂ (Steinhoff et al., 2000). The rapid increase in $[Ca^{2+}]_i$ was fully maintained in Ca^{2+}-free medium and is thus attributable to mobilization of intracellular Ca^{2+} (data not shown). Most of the neurons responding to PAR₂-AP (79 ± 3%) and all of the neurons responding to trypsin were capsaicin-sensitive (Fig. 2d,e). Thus, PAR₂ and TRPV1 are mostly coexpressed in DRG neurons.

We examined whether PAR₂ activation sensitizes TRPV1-mediated increases in $[Ca^{2+}]_i$ in neurons. Exposure to PAR₂-AP (100 μM) and trypsin (10 nM) potentiated the response to capsaicin (100 nM) applied 5 min later by 53 ± 7% (n = 231 capsaicin-responsive neurons) and 88 ± 24% (n = 21), respectively, compared with control cells pretreated with PAR₂-RP (100 μM; n = 140) or vehicle (PBS, 1:100; n = 17; Fig. 2b–g). PAR₂ activation also increased the proportion of neurons responding to capsaicin (Fig. 2h). After pretreatment with PAR₂-RP and vehicle, 46 ± 3% (n = 304 total neurons) and 53 ± 9% (n = 32), respectively, of neurons were capsaicin-sensitive. However, after pretreatment with PAR₂-AP and trypsin, 65 ± 3% (n = 357) and 62 ± 8% (n = 34), respectively, of neurons responded to capsaicin. Pretreatment with capsaicin (10 nM) did not affect the response to PAR₂-AP applied 5 min later (10–100 μM; data not shown). Thus, PAR₂ activation potentiates the magnitude of responses to capsaicin and increases the proportion of capsaicin-responsive neurons, indicating sensitization of TRPV1.

Inhibition of PLC with U73122 (5 μM, 30 min) had no effect on the magnitude of the Ca^{2+} response to capsaicin (100 nM) but inhibited the PAR₂-AP-induced potentiation of the response to capsaicin by >90%; the effect of capsaicin after U73122 and PAR₂-AP was 65 ± 6% compared with the positive control of

vehicle and PAR$_2$-AP (100%; $n = 72$ total neurons; Fig. 2g). Activation of PKC with PMA (5 μM) potentiated the increase in $[Ca^{2+}]_i$ to capsaicin ($n = 45$) compared with cells pretreated with the vehicle ($n = 35$). This effect was abolished by GFX (1 μM, 30 min; $n = 22$; Fig. 2g). GFX also inhibited PAR$_2$-AP-induced potentiation of the response to capsaicin by >80%; the effect of capsaicin after GFX and PAR$_2$-AP was $73 \pm 7\%$ compared with the positive control ($n = 60$; Fig. 2g). Pretreatment with Gö6976 (0.1 μM, 30 min) inhibited PAR$_2$-induced sensitization of the response to capsaicin by >80%; the effect of capsaicin after Gö6976 and PAR$_2$-AP was $73 \pm 16\%$ compared with the positive control ($n = 37$; Fig. 2g).

Thus, observations in both HEK-TRPV1 cells and DRG neurons indicate that PAR$_2$ agonists sensitize TRPV1 by a mechanism that is dependent on PLC and the conventional isoforms of PKC.

PAR$_1$ agonists did not sensitize TRPV1 Ca^{2+} responses in HEK-TRPV1 or DRG neurons

To evaluate the specificity of PAR$_2$-mediated sensitization of TRPV1, we investigated whether agonists of PAR$_1$ sensitize this channel. PAR$_1$ was endogenously expressed in HEK-TRPV1 cells (Fig. 1a) and is also present in small-diameter DRG neurons (de Garavilla et al., 2001). PAR$_1$-AP (100 μM) rapidly increased $[Ca^{2+}]_i$ in HEK-TRPV1 cells (Fig. 3b) and DRG neurons (Fig. 3d), whereas PAR$_1$-RP was ineffective. PAR$_1$-AP increased $[Ca^{2+}]_i$ in $56 \pm 5\%$ of DRG neurons ($n = 117$ total neurons), $54 \pm 6\%$ of which also responded to capsaicin. The rapid increase in $[Ca^{2+}]_i$ in HEK-TRPV1 cells and DRG neurons was still observed in Ca^{2+}-free medium and is thus attributable to mobilization of intracellular Ca^{2+} (data not shown). PAR$_1$-AP did not affect the magnitude of the Ca^{2+} response to 3 or 10 nM capsaicin in HEK-TRPV1 cells (Fig. 3e) or to 100 nM capsaicin in DRG neurons ($n = 53$ capsaicin-responsive neurons; Fig. 3f). PAR$_1$-AP also did not influence the number of capsaicin-responsive neurons (Fig, 3g). Thus, activation of PAR$_2$ selectively sensitizes TRPV1.

PAR$_2$ agonists increased excitability of DRG neurons

Activation of PAR$_2$ on enteric neurons results in depolarization and sustained hyperexcitability (Linden et al., 2001; Gao et al., 2002; Reed et al., 2003). We therefore determined whether PAR$_2$ activation depolarized small- and medium-diameter DRG neurons in culture and lowered their firing threshold, thereby increasing their excitability to other stimuli, by measuring the
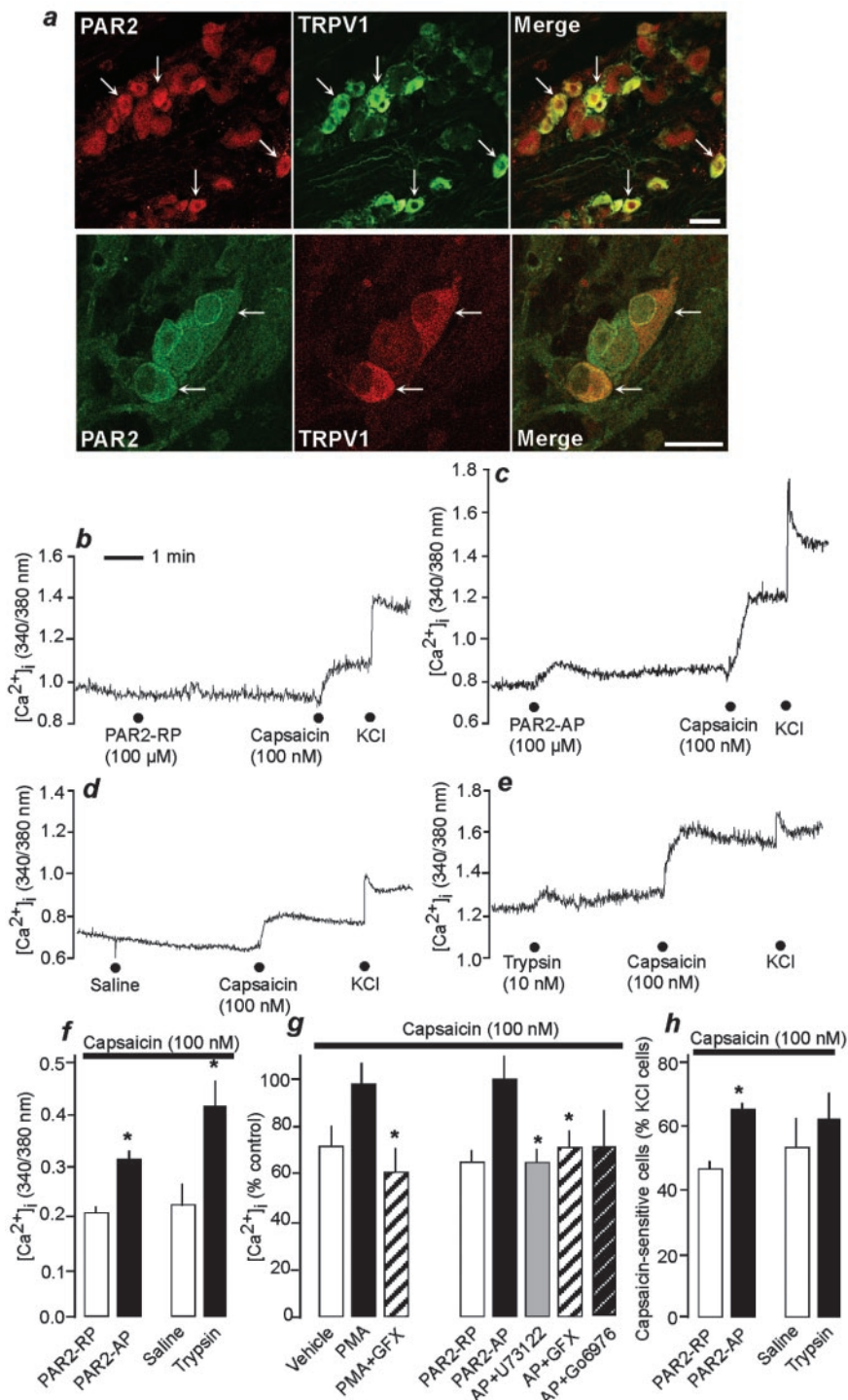

Figure 2. PAR$_2$ activation sensitizes TRPV1 in DRG neurons. a, Colocalization (arrows) of immunoreactive PAR$_2$ and TRPV1 in tissue sections of DRG neurons (top panels) and in cultured DRG neurons (bottom panels). Scale bars: top panels, 50 μm; bottom panels, 25 μm. b–h, Neurons were exposed to PAR$_2$-AP or -RP (100 μM), trypsin (10 nM), PMA (5 μM) or vehicle for 5 min, and were then challenged with capsaicin (100 nM). PAR$_2$ agonists (c, e–h) and PMA (g) potentiated responses to capsaicin. Inhibition of PLC (U73122, 5 μM) and PKC (GFX and Gö6976, 1 and 0.1 μM, respectively) suppressed the potentiation (g). PAR$_2$ activation also increased the proportion of neurons that gave detectable responses to capsaicin (h). Results in g are expressed as a percentage of the positive control (magnitude of capsaicin response in cells after PAR$_2$ activation). *$p < 0.05$ compared with PAR$_2$-RP or saline (f, h) and PMA or PAR$_2$-AP (g). AP, PAR$_2$-AP; $n = 17$–231 neurons.

rheobase (minimum current to evoke one action potential in the current-clamp mode). The average initial rheobase was 0.83 ± 0.11 nA and was not statistically different between any of the experimental groups of neurons. Trypsin (10 nM, 3 min) and
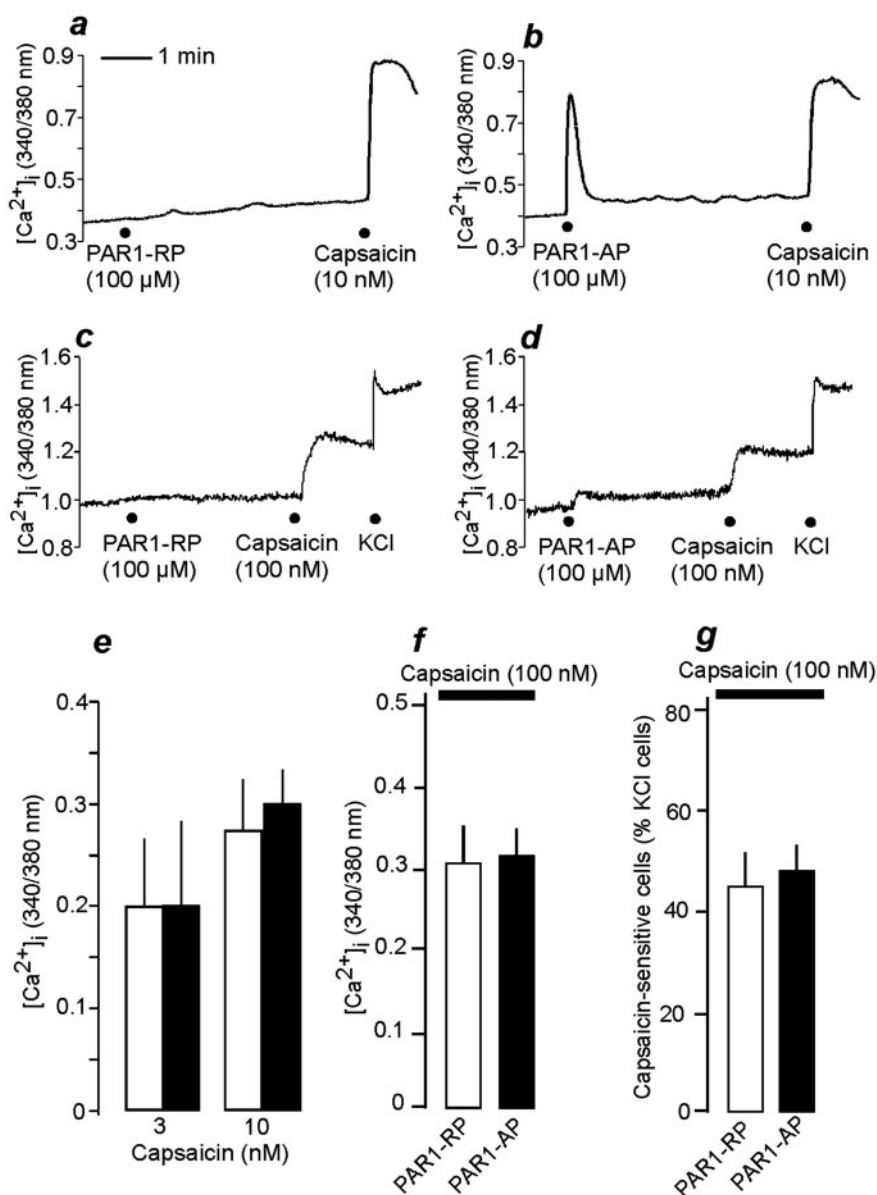
Figure 3. PAR₁ activation does not sensitize TRPV1 in transfected cells (a, b, e) or DRG neurons (c, d, f, g). HEK-TRPV1 cells (a, b, e) or DRG neurons (c, d, f, g) were exposed to PAR₁-AP or -RP (100 μM) for 5 min and were then challenged with capsaicin. In HEK-TRPV1 cells, PAR₁-AP failed to potentiate the response to 3 or 10 nM capsaicin (b, e). In DRG, PAR₁-AP failed to potentiate the magnitude of the response to 100 nM capsaicin (d, f) or affect the proportion of neurons that gave a detectable response to capsaicin (g); n = 160–320 cells, 28–53 neurons.

tryptase (3.8 μM pipette concentration, puffed onto cells) induced a sustained decrease in the rheobase that was detected at 10 min and maintained for at least 50 min (50 min: trypsin, 59.6 ± 9.6% decrease; n = 7 total neurons; tryptase, 50.4 ± 3.5% decrease; n = 5; Fig. 4a,b). Inactivated trypsin [10 nM, 3 min, pretreated with soybean trypsin inhibitor (SBTI), 10 mg/ml, 30 min, 37°C] had no effect on the rheobase (Fig. 4b). Surprisingly, PAR₂-AP (50 μM, 3 min) increased rheobase at 10 min by 31% (Fig. 4a). However, PAR₂-RP (50 μM, 3 min) also increased the rheobase at this time to a similar extent (44%), suggesting that the increase in rheobase to PAR₂-AP is nonspecific. At later time points, PAR₂-AP caused a sustained decrease in the rheobase (50 min: 40.2 ± 5.9%; n = 7), whereas PAR₂-RP had no effect (Fig. 4a,b). The resting potential of neurons was 57.2 ± 1.6 mV. Trypsin, tryptase, and PAR₂-AP depolarized neurons after 10 min

(trypsin, 11.0 ± 2.1 mV; n = 7; tryptase, 13.2 ± 2.5 mV; n = 5; PAR₂-AP, 4.9 ± 2.3 mV; n = 7), but within 20 min, the membrane potential had repolarized (Fig. 4c,d). Inactivated trypsin and PAR₂-RP had no effect on membrane potential (Fig. 4d). Thus, brief activation of PAR₂ induces a sustained hyperexcitability of DRG neurons.

PAR₂ agonists sensitized TRPV1 currents in DRG neurons by activating PKC

To study the effect of activation of PAR₂ on the activity of TRPV1, we measured whole-cell currents induced by capsaicin before and after PAR₂ activation in DRG neurons. Approximately 50% of small and medium DRG neurons responded to brief (150 msec–1 sec) application of capsaicin (500 nM). Stable inward currents to repeated application (3 min interstimulus interval) of capsaicin were recorded in 40–50% of responsive neurons, whereas capsaicin currents desensitized in other neurons. Only neurons that did not exhibit desensitization to two consecutive capsaicin applications were selected for further study. PAR₂-AP (50 μM, 3 min) and trypsin (10 nM, 3 min) increased the inward current induced by capsaicin (500 nM; Fig. 5a–c). This potentiation was observed within 10 min of PAR₂ activation and was sustained for at least 60 min. For example, at 30 min after application of PAR₂-AP or trypsin, the capsaicin-induced TRPV1 current was significantly increased by a 3.1- ± 0.6-fold change over basal (n = 6 neurons) and 2.0- ± 0.1-fold (n = 10), respectively, compared with controls treated with PAR₂-RP (50 μM, 3 min) or vehicle (Fig. 5a–c). The PKC inhibitor GFX (1 μM, 5 min) abolished the potentiation of the TRPV1 current induced by PAR₂-AP (1.1- ± 0.2-fold after GFX and PAR₂-AP; n = 4; compared with 4.5- ± 1.2-fold after vehicle and PAR₂-AP; Fig. 6a,b). However, GFX had minimal affect on the basal TRPV1 current (Fig. 6c,d). Thus, transient activation of PAR₂ causes sustained sensitization of TRPV1 by a PKC-dependent process. In contrast, PAR₁-AP (50 μM, 3 min) did not sensitize the capsaicin current (data not shown).

PAR₂ agonists induced phosphorylation of TRPV1 in HEK-TRPV1 cells

PKC can directly phosphorylate TRPV1 and thereby sensitize responses to TRPV1 agonists (Numazaki et al., 2002). Because PAR₂-induced sensitization of TRPV1 requires PKC activation, we hypothesized that PAR₂ activation can modify phosphorylation of TRPV1. To assess phosphorylation of TRPV1, we immunoprecipitated the channel and probed Western blots for phosphoserine. PAR₂-AP (100 μM, 10 min; Fig. 7) or trypsin (10 nM, 10 min; data not shown) induced phosphorylation of TRPV1 in
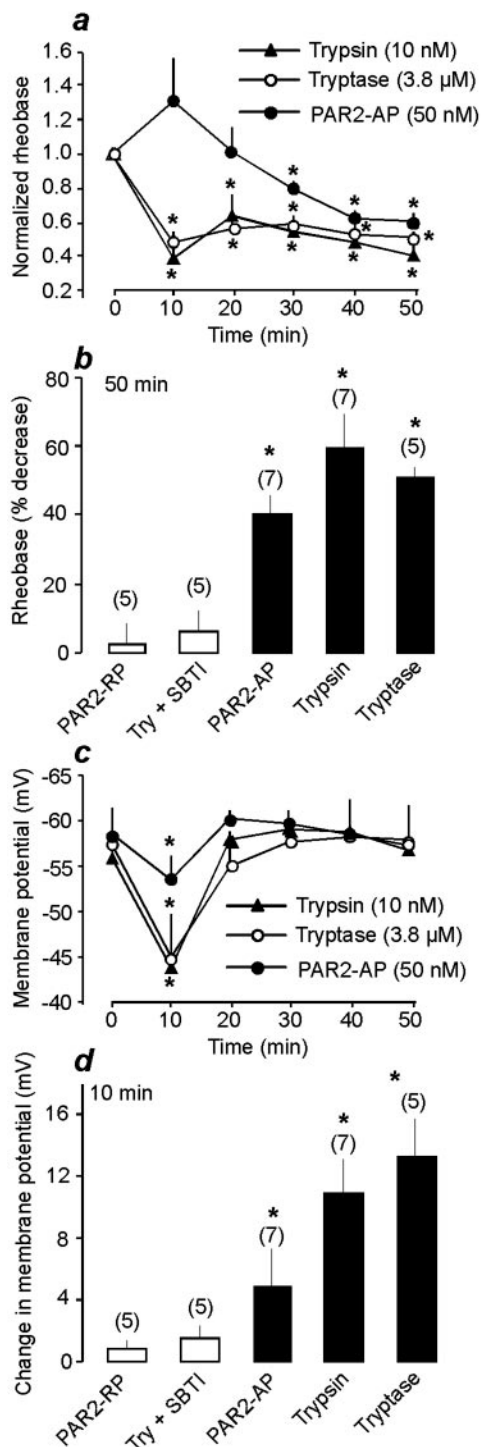
Figure 4. PAR₂ activation excites DRG neurons in culture. Neurons were superfused for 3 min with PAR₂-AP or -RP (50 μM) or trypsin or SBTI-treated trypsin (10 nM) or puffed with tryptase (3.8 μM). The rheobase (*a, b*) and membrane potential (*c, d*) were measured at the indicated times. Trypsin and tryptase caused a sustained decrease in rheobase that was maintained for at least 50 min. PAR₂-AP caused a transient increase in rheobase at 10 min followed by a sustained decrease (*a*). This effect was nonspecific because PAR₂-RP produced an identical transient increase (data not shown) without a sustained effect on rheobase (*b*) or a transient decrease in membrane potential (*d*). PAR₂ agonists caused a transient decrease in membrane potential (*c, d*). *$p < 0.01$ compared with basal (*a, b*) or PAR₂-RP and trypsin (Try) + SBTI (*c, d*). Numbers in parentheses are numbers of neurons.
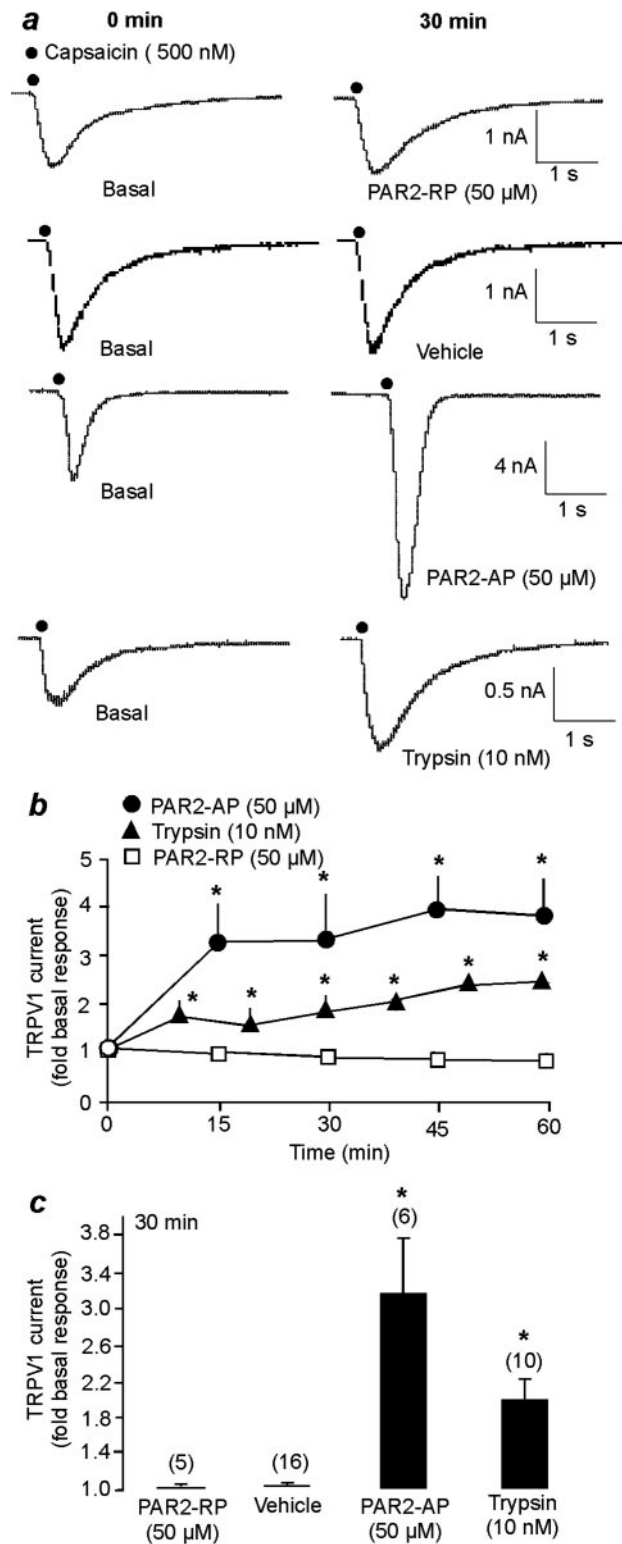

Figure 5. PAR₂ activation sensitizes TRPV1 currents. Neurons were superfused for 3 min with PAR₂-AP or -RP (50 μM), trypsin (10 nM), or vehicle. They were challenged at the indicated times with capsaicin (500 nM), and currents were measured. *a*, Representative currents under basal conditions and at 30 min after activation of PAR₂. PAR₂ activation caused a marked and sustained increase in the capsaicin-induced current (*a, b*), and the increase observed 30 min after activation is shown in *c*. *$p < 0.01$ compared with basal (*b*) or vehicle and PAR₂-RP (*c*). Numbers in parentheses are numbers of neurons.
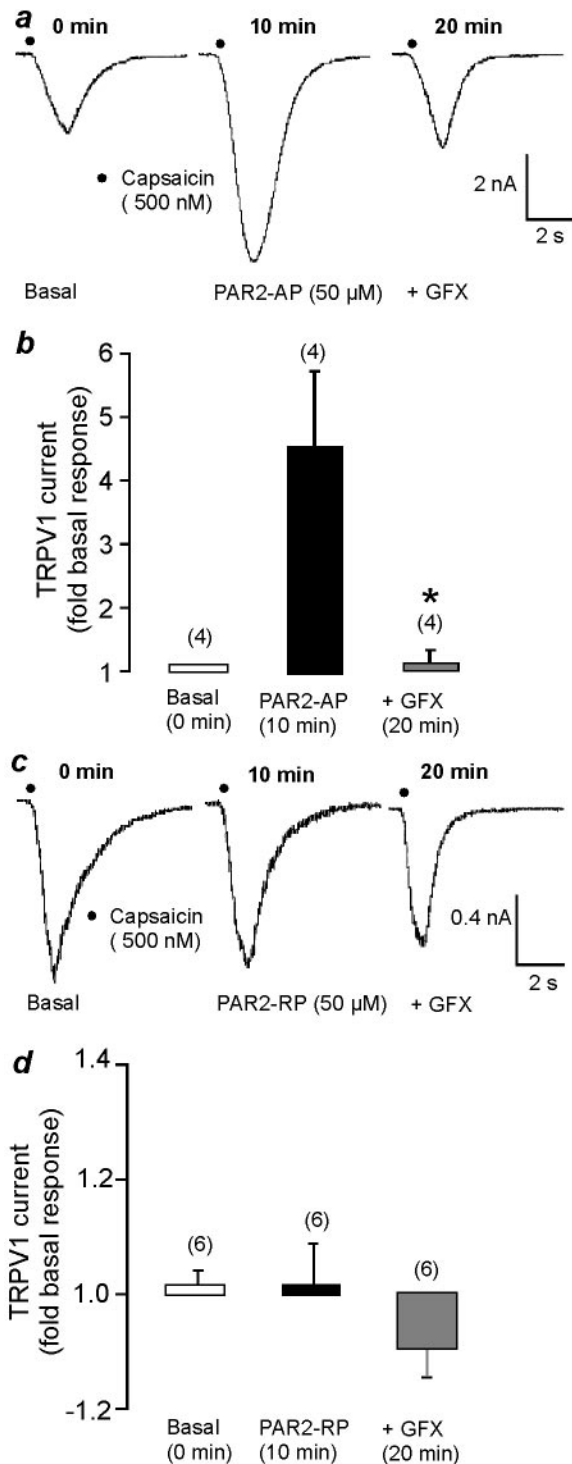
Figure 6. PAR₂ activation sensitizes TRPV1 currents by a PKC-dependent mechanism. Neurons were superfused for 3 min with PAR₂-AP or -RP (50 μM) and then challenged with capsaicin (500 nM). PAR₂ activation caused a marked increase in the capsaicin current, and the increase was abolished by GFX (1 μM). GFX had no effect in neurons treated with PAR₂-RP. Representative traces are shown in a and c, and pooled data are shown in b and d. *p < 0.01 compared with vehicle and PAR₂-AP. Numbers in parentheses are numbers of neurons.

Lane 1- PAR2-RP (100 μM, 10 min)
Lane 2 - PAR2-AP (100 μM, 10 min)
Lane 3 - DMSO (1:250, 10 min)
Lane 4 - PMA (10 μM, 10 min)

Figure 7. Agonist of PAR₂ induced phosphorylation of TRPV1. HEK-TRPV1 cells were exposed to PAR₂-AP or -RP (100 μM), PMA (10 μM), or DMSO (1:250) for 10 min. TRPV1 was immunoprecipitated (IP), and Western blots (WB) were probed for phosphoserine (a). Blots were then stripped and reprobed to TRPV1 to ensure equal loading (b). Activation of PAR₂ and PKC induced serine phosphorylation of TRPV1. Representative blots are shown.

PAR₂ agonists induced thermal hyperalgesia by a mechanism that was mediated by TRPV1 and protein kinase C

The intraplantar injection of trypsin, tryptase, and PAR₂-AP, at doses that do not cause detectable inflammation of the paw, induces sustained thermal and mechanical hyperalgesia (Vergnolle et al., 2001). Because TRPV1 is an important mediator of thermal hyperalgesia and inflammatory pain (Caterina et al., 1997; Tominaga et al., 1998; Zygmunt et al., 1999; Caterina et al., 2000; Davis et al., 2000), we evaluated the role of TRPV1 in PAR₂-induced thermal hyperalgesia using pharmacological and genetic approaches. Intraplantar injection of a subinflammatory dose of PAR₂-AP in C57BL/6 mice (1 μg/paw; Vergnolle et al., 2001), decreased the latency of paw withdrawal from a thermal stimulus for 30 min to at least 4 hr, indicative of sustained thermal hyperalgesia (Fig. 8a). Intraplantar PAR₂-RP (1 μg/paw) did not affect withdrawal latency. Pretreatment with the TRPV1 antagonist capsazepine (35 mg/kg, s.c.) markedly inhibited PAR₂-induced hyperalgesia compared with animals treated with vehicle (Fig. 8a). In TRPV1$^{+/+}$ mice, intraplantar injection PAR₂-AP but not PAR₂-RP (1 μg/paw) also caused a sustained decrease in latency of paw withdrawal (Fig. 8b). In contrast, PAR₂-AP did not affect the latency of withdrawal in TRPV1$^{-/-}$ mice. Thus, TRPV1 is required for PAR₂-induced thermal hyperalgesia.

To examine the capacity of PAR₂-AP to sensitize TRPV1, we

HEK-TRPV1 cells, whereas PAR₂-RP (100 μM) or vehicle had no effect. PMA (10 μM, 10 min) also increased the phosphorylation of TRPV1, whereas vehicle (DMSO, 1:250) had no effect (Fig. 7). Thus, stimulation of PAR₂ directly phosphorylates TRPV1.
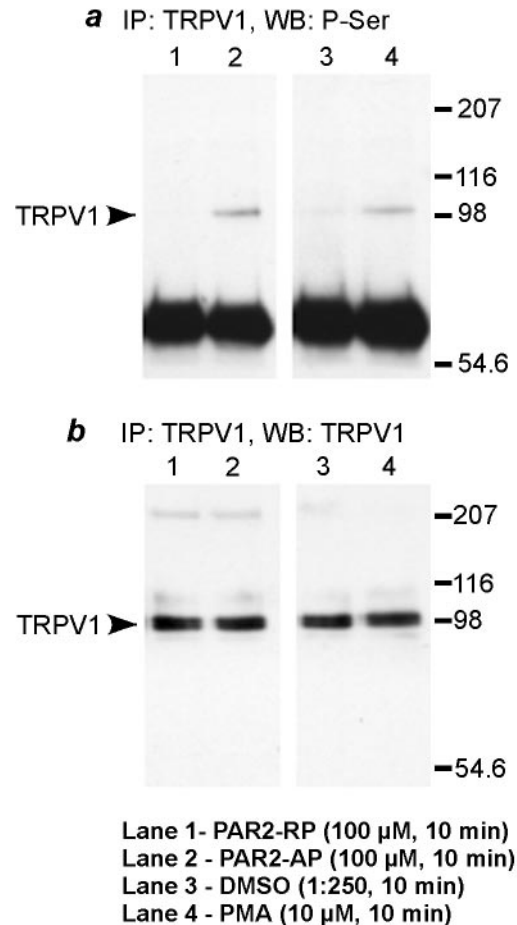
simultaneously administered doses of PAR$_2$-AP and capsaicin that alone did not cause hyperalgesia. By administration of graded doses of PAR$_2$-AP (1–500 ng/paw) to C57BL/6 mice, we determined that 1 ng PAR$_2$-AP had no effect on thermal hyperalgesia (Fig. 8c). Capsaicin (1 μg/paw) also did not cause thermal hyperalgesia (Fig. 8d). However, coinjection of PAR$_2$-AP (1 ng/paw) and capsaicin (1 μg/paw) decreased the latency of paw withdrawal, suggesting that PAR$_2$ sensitizes TRPV1 to capsaicin (Fig. 8d). Similarly, in TRPV1$^{+/+}$ mice, coinjection of PAR$_2$-AP (1 ng/paw) and capsaicin (1 μg/paw) decreased the latency of paw withdrawal for 30 min to 2 hr (Fig. 8e). In TRPV1$^{-/-}$ mice, coinjection of PAR$_2$-AP and capsaicin had no effect of withdrawal (Fig. 8e). Thus, PAR$_2$ sensitizes responses to capsaicin by a mechanism that depends on TRPV1.

To determine whether PKC mediates this sensitization, we injected GFX locally into the paw. In C57BL/6 mice treated with GFX vehicle, coinjection of PAR$_2$-AP (1 ng/paw) and capsaicin (1 μg/paw) decreased the latency of paw withdrawal (Fig. 8e). Pretreatment with GFX (1 μg/paw) completely inhibited the synergic effect of capsaicin and PAR$_2$-AP on decreasing the withdrawal latency at all times. After 3 hr, animals pretreated with GFX showed an increased nociceptive threshold. GFX injected alone did not affect the latency of paw withdrawal at 0–2 hr but slightly decreased withdrawal latency at 3 and 4 hr. Thus, PAR$_2$ activation sensitizes TRPV1 to induce thermal hyperalgesia by a mechanism that is dependent on PKC.

PAR$_2$ activation enhanced capsaicin-stimulated release of SP and CGRP in the spinal cord

The intraplantar injection of PAR$_2$ agonists causes thermal hyperalgesia in part by stimulating the release of neuropeptides from the central projections of DRG in the dorsal horn of the spinal cord (Vergnolle et al., 2001). To determine whether PAR$_2$ activation sensitizes TRPV1-stimulated neuropeptide release from the central projections of DRG neurons, we measured secretion of SP and CGRP from superfused slices of the rat spinal cord. Superfusion with capsaicin (0.5 or 1 μM) stimulated release of SP- and CGRP-LI from the spinal cord (Fig. 9a,b). Pretreatment with PAR$_2$-AP (10 μM, 20 min) enhanced capsaicin-stimulated release of SP-LI by >2-fold and CGRP-LI by >1.6-fold compared with vehicle, whereas PAR$_2$-RP (10 μM, 20 min) was ineffective. Thus, activation of PAR$_2$ sensitizes TRPV1-mediated release of neuropeptides from the central projections of DRG neurons in the dorsal horn of the spinal cord, where they mediate nociceptive transmission.
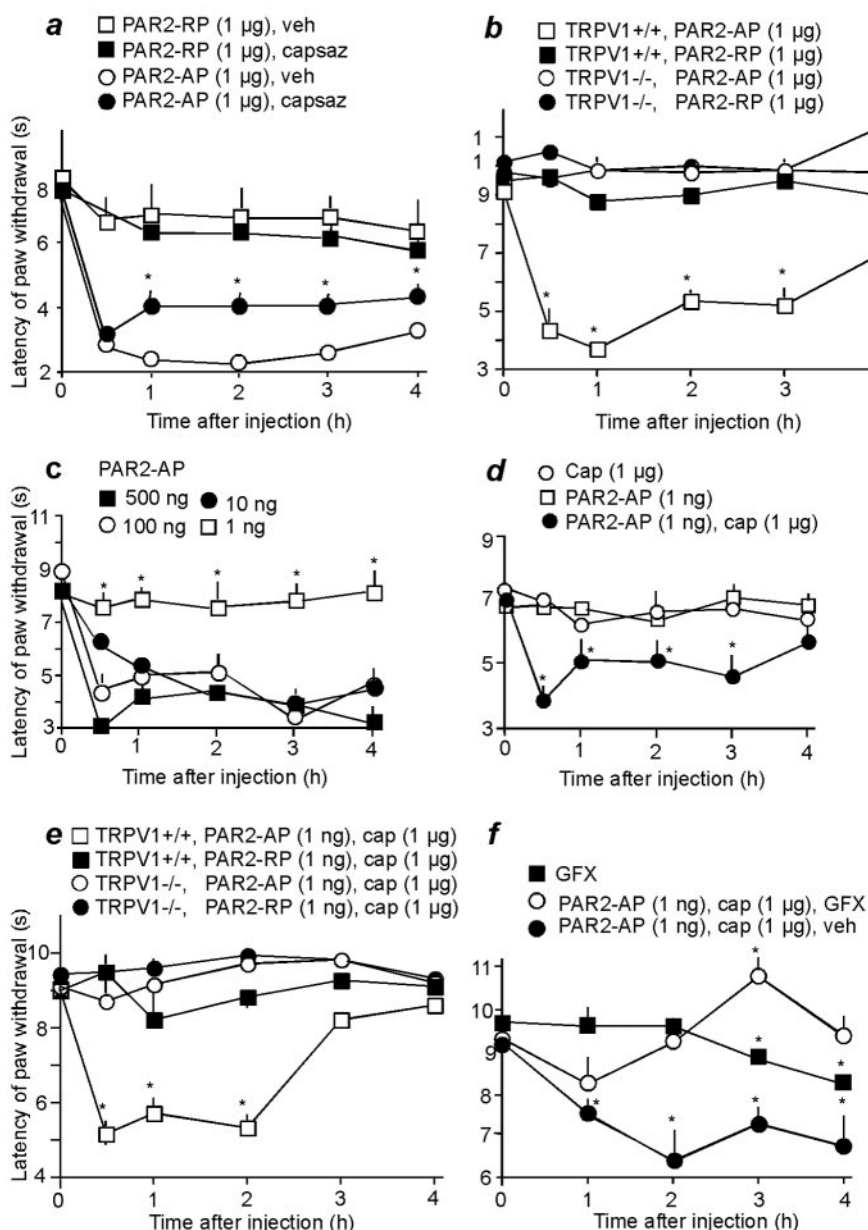

Figure 8. Mechanisms of PAR$_2$-induced thermal hyperalgesia in mice. PAR$_2$-AP or PAR$_2$-RP was administered by intraplantar injection, and latency of paw withdrawal was measured in C57BL/6 mice (a, c, d, f) or TRPV1$^{+/+}$ and TRPV1$^{-/-}$ mice (b, e). a, PAR$_2$-AP decreased the latency of withdrawal, which was inhibited by systemic administration of capsazepine (capsaz; 35 mg/kg, s.c.). b, PAR$_2$-AP also decreased the latency of withdrawal in TRPV1$^{+/+}$ mice but not TRPV1$^{-/-}$ mice. c, The effect of PAR$_2$-AP on withdrawal latency was dose-related. d, Coinjection of PAR$_2$-AP and capsaicin (Cap) at doses that alone did not cause hyperalgesia decreased the latency of paw withdrawal. e, The synergistic effects of coinjected PAR$_2$-AP and capsaicin were observed in TRPV1$^{+/+}$ but not TRPV1$^{-/-}$ mice. f, Intraplantar injection of GFX (1 μg/paw) inhibited the synergistic effects of PAR$_2$-AP and capsaicin. veh, Vehicle. *$p < 0.05$ compared with basal withdrawal latency (b, d–f) or PAR$_2$-AP-induced hyperalgesia (a, c); n = 6–8 mice per group.

Discussion

PAR$_2$ has been recently suggested to play a role in nociceptive signaling (Hoogerwerf et al., 2001; Kawabata et al., 2001a; Vergnolle et al., 2001; Coelho et al., 2002). However, the mechanism by which proteases that activate PAR$_2$ induce hyperalgesia is unknown. From a combination of observations in transfected cell lines, cultured DRG, spinal cord slices, and intact animals, we conclude that PAR$_2$ sensitizes TRPV1 through a PKC-dependent mechanism, and TRPV1 is required for PAR$_2$-induced thermal hyperalgesia.
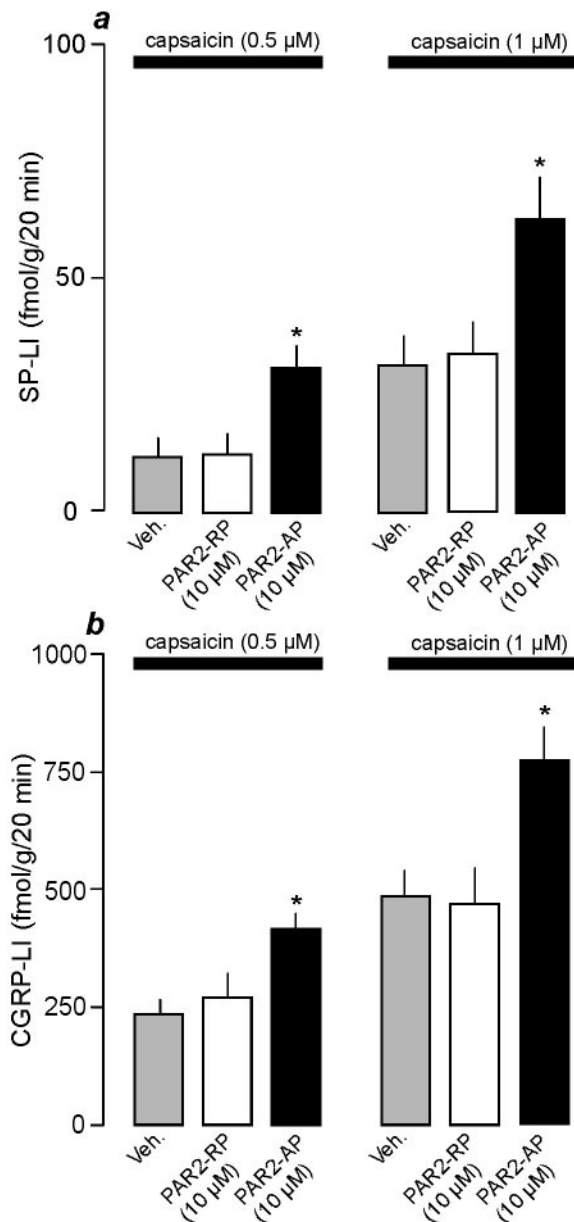
Figure 9. PAR$_2$ activation sensitizes TRPV1-mediated release of SP (*a*) and CGRP (*b*). Slices of dorsal horn of the spinal cord were pretreated with vehicle (Veh.) or PAR$_2$-AP or -RP (10 μM) for 20 min and then challenged with capsaicin (0.5 or 1 μM). Pretreatment with PAR$_2$-AP enhances capsaicin-stimulated release of SP-LI and CGRP-LI. *$p < 0.05$ compared with vehicle or PAR$_2$-RP; $n = 6 – 8$ rats.

PAR$_2$ sensitizes TRPV1 to cause thermal hyperalgesia
Our conclusion that PAR$_2$ sensitizes TRPV1 is based on several observations. First, in HEK-TRPV1 cells and DRG neurons, PAR$_2$ agonists increased the magnitude of the Ca^{2+} response to capsaicin and the proportion of capsaicin-responsive neurons. Thus, activation of PAR$_2$ both magnifies TRPV1 responses and recruits additional neurons that were previously unresponsive to capsaicin. Second, PAR$_2$ agonists induced a sustained potentiation of TRPV1 currents in DRG neurons. Third, intraplantar injection of PAR$_2$-AP induced a persistent thermal hyperalgesia that was mediated by TRPV1 because it was inhibited by antagonism or deletion of this channel. The finding that coinjection of nonhyperalgesic doses of PAR$_2$-AP and capsaicin caused hyperalgesia that was also entirely dependent on TRPV1 confirms that

PAR$_2$ sensitizes TRPV1 *in vivo*. Finally, superfusion of spinal cord slices with PAR$_2$-AP potentiated TRPV1-mediated release of SP and CGRP. These neuropeptides derive from the central projections of DRG neurons in the spinal cord, where they mediate PAR$_2$-induced hyperalgesia (Hoogerwerf et al., 2001; Kawabata et al., 2001a; Vergnolle et al., 2001; Coelho et al., 2002). In experiments with transfected cells, cultured neurons, spinal cord slices, and intact animals, we used a synthetic peptide and proteases to activate PAR$_2$. PAR$_2$-AP (SLIGRL-NH$_2$) selectively activates this receptor and does not activate other known PARs. PAR$_2$-RP, which does not activate PAR$_2$, did not sensitize TRPV1-induced Ca^{2+} signals or currents and had no effect on capsaicin-induced neuropeptide release or thermal hyperalgesia, confirming specificity. We have previously reported that the effects of trypsin and tryptase on DRG neurons are desensitized by PAR$_2$-AP, and their hyperalgesic actions are not detected in PAR$_2$-deficient animals, suggesting that they induce hyperalgesia by activation of this receptor (Steinhoff et al., 2000; Vergnolle et al., 2001).

PAR$_1$ is also coexpressed with CGRP in DRG neurons, and PAR$_1$ agonists induce neurogenic inflammation (de Garavilla et al., 2001). Although PAR$_1$ was coexpressed with TRPV1 in HEK-TRPV1 cells and DRG neurons, PAR$_1$-AP did not sensitize TRPV1 Ca^{2+} responses. This finding is consistent with the report that PAR$_1$ activation induces thermal and mechanical analgesia rather than hyperalgesia (Asfaha et al., 2002). The mechanism of the differential effects of PAR$_1$ and PAR$_2$ agonists on TRPV1 agonists is unknown, although activation of divergent signaling pathways is a probable explanation. Although both PAR$_1$ and PAR$_2$ couple to PLCβ and similarly mobilized intracellular Ca^{2+} in HEK-TRPV1 and DRG neurons, PAR$_1$ more prominently regulates adenylyl cyclase, and PAR$_1$ and PAR$_2$ regulate mitogen-activated protein kinases by different mechanisms (for review, see Ossovskaya and Bunnett, 2004). Further investigation is required to define the mechanism of the different effects of PAR$_1$ and PAR$_2$ on TRPV1 in neurons.

PLC and PKC mediate PAR$_2$-induced sensitization of TRPV1
Inflammatory agents (e.g., bradykinin, ATP, PGE$_2$, and NGF) can indirectly sensitize TRPV1 to cause hyperalgesia. It is important to understand the mechanisms of this sensitization because it may explain the hyperalgesic effects of diverse inflammatory mediators. Our observation that an inhibitor of PLC prevented PAR$_2$-induced sensitization of TRPV1 is expected because PAR$_2$ couples to PLCβ with subsequent formation of InsP$_3$ and DAG, followed by mobilization of Ca^{2+} and activation of PKC. PLCβ can regulate TRPV1 by controlling the levels of its substrate, PIP$_2$, which directly binds and inhibits TRPV1 (Chuang et al., 2001; Prescott and Julius, 2003). Thus, bradykinin and NGF, which activate PLCβ, release TRPV1 from PIP$_2$-mediated inhibition. PAR$_2$ could similarly sensitize TRPV1 by reducing PIP$_2$ levels. However, PLCβ also regulates TRPV1 by DAG formation and subsequent activation of PKC, which in turn phosphorylates and sensitizes TRPV1 (Tominaga et al., 2001; Vellani et al., 2001; Crandall et al., 2002; Prescott and Julius, 2003). Our observation that two distinct inhibitors of PKC prevented PAR$_2$-induced sensitization of TRPV1 suggests that the activation of PKC plays a major role in PAR$_2$-induced sensitization of TRPV1.

We do not know which isozyme of PKC is responsible for PAR$_2$-induced sensitization of TRPV1. There are at least 11 PKC isozymes, some of which make major contributions to nociception. An inhibitor of the α, β_1, β_2, and γ PKC isozymes (Gö6976) prevented sensitization in HEK cells and neurons, suggesting an involvement of at least these isozymes, which have been impli-

cated in nociception. PKCα, which is coexpressed with SP and CGRP in DRG neurons (Cesare et al., 1999; Olah et al., 2002), mediates upregulation of CGRP expression during chronic treatment with opioids (Belanger et al., 2002) and may enhance inflammatory pain (Olah et al., 2002). PKCβ participates in inflammatory thermal hyperalgesia (Igwe and Chronwall, 2001) and in persistent pain (Miletic et al., 2000). PKCγ contributes to persistent neuropathic pain but not acute pain (Malmberg et al., 1997). Although Gö6976 does not inhibit PKCϵ, this isoform plays a major role in pain transmission (Khasar et al., 1999) and the regulation of TRPV1 (Cesare et al., 1999). Thus, we cannot exclude the contribution of PKCϵ to PAR2-induced sensitization of TRPV1. Moreover, because PAR$_2$-dependent sensitization of TRPV1 is remarkably sustained, different isoforms of PKC may contribute to sensitization at different times, as reported for the contribution of PKCϵ to mechanical hyperalgesia (Aley et al., 2000).

Additional mechanisms may also regulate TRPV1. For example, PGE$_2$ sensitizes TRPV1 responses by a protein kinase A-mediated mechanism (Hu et al., 2002; Rathee et al., 2002; Mohapatra and Nau, 2003), whereas bradykinin transactivates TRPV1 through the generation of 12-lipoxygenase metabolites of arachidonic acid (Shin et al., 2002). PAR$_2$ stimulation induces release of arachidonic acid and PGE$_2$ from epithelial cells (Kong et al., 1997), and these mechanisms could also contribute to PAR$_2$-induced sensitization of TRPV1. However, inhibitors of cyclooxygenase (indomethacin and ibuprofen) and lipoxygenase (5,8,11,14-eicosatetraynoic acid) do not affect PAR$_2$-mediated sensitization of TRPV1 Ca^{2+} signaling in HEK cells (N. W. Bunnett, unpublished observation).

PAR$_2$ activation induces phosphorylation of TRPV1

TRPV1 is regulated in part by its phosphorylation state. Dephosphorylation of TRPV1 by calcineurin, a Ca^{2+}- and calmodulin-dependent kinase, can desensitize capsaicin responsiveness (Docherty et al., 1996), whereas phosphorylation may sensitize TRPV1 (Numazaki et al., 2002). TRPV1 contains 16 Ser and Thr residues that are potential sites for PKC-dependent phosphorylation. Phorbol esters and ATP, which potentiate the TRPV1 current and reduce the temperature threshold for activation, induce phosphorylation of TRPV1 (Numazaki et al., 2002). This process appears to increase the probability that TRPV1 will open in response to agonists (Vellani et al., 2001). Our results show that PAR$_2$ activation induces phosphorylation of TRPV1. Thus, it is likely that PAR$_2$ agonists similarly induce phosphorylation of TRPV1 by a PKC-dependent mechanism to regulate channel gating.

PAR$_2$ regulates the excitability of nociceptive neurons

We found that PAR$_2$ activation by brief exposure to PAR$_2$-AP, trypsin, or tryptase caused a sustained decrease in the rheobase of nociceptive neurons, indicative of an increase in their electrical excitability. In support of our results, PAR$_2$ activation of S-type neurons in the submucosal plexus also causes a sustained decrease in rheobase and an increase in input resistance (Reed et al., 2003). The mechanism of the increased excitability of DRG neurons remains to be determined, although alterations in activity of ion channels other than TRPV1 may contribute. DRG neurons express multiple voltage-gated channels for Na$^+$, Ca^{2+}, and K$^+$ ions that are regulated directly by G-proteins or by second messenger kinases. For example, PKC regulates a tetrodotoxin-resistant Na$^+$ channel in nociceptive neurons (Gold et al., 1998) and PAR$_2$ may regulate the activity of this channel and thereby

enhance sensitivity. Alterations in membrane potential can also affect TRPV1 activity. The TRPV1 current in DRG neurons increases on membrane depolarization, suggesting that voltage serves as an intrinsic coactivator of this channel (Ahern and Premkumar, 2002). Thus, PAR$_2$ activation increases the excitability of neurons by varying mechanisms and may sensitize their responses to agonists of TRPV1 or other agents to result in exacerbated inflammation and hyperalgesia.

In summary, we have identified a novel mechanism by which proteases cleave PAR$_2$ on sensory nerves and sensitize TRPV1 to induce sustained thermal hyperalgesia. Sensitization requires activation of PLC and PKC and is probably mediated by direct phosphorylation of TRPV1 to enhance channel gating. PAR$_2$-induced sensitization of TRPV1 causes enhanced release of neuropeptides in the spinal cord and exacerbated thermal pain. Proteases that activate PAR$_2$, such as mast cell tryptase, coagulation factors VIIa and Xa, and extrapancreatic trypsins, are released and generated during injury and inflammation, which suggests that this mechanism plays an important role in these states. Although antagonists of PAR$_2$ are not currently available, our results suggest that inhibitors or antagonists of proteases, TRPV1, and PKC may offer new therapies for treatment of sustained inflammatory pain.

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