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Protease-activated receptor 2 sensitizes TRPV1 by protein kinase Cε- and A-dependent mechanisms in rats and mice

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Proteases that are released during inflammation and injury cleave protease-activated receptor 2 (PAR_2) on primary afferent neurons to cause neurogenic inflammation and hyperalgesia. PAR_2-induced thermal hyperalgesia depends on sensitization of transient receptor potential vanilloid receptor 1 (TRPV1), which is gated by capsaicin, protons and noxious heat. However, the signalling mechanisms by which PAR_2 sensitizes TRPV1 are not fully characterized. Using immunofluorescence and confocal microscopy, we observed that PAR_2 was colocalized with protein kinase (PK) Cε and PKA in a subset of dorsal root ganglia neurons in rats, and that PAR_2 agonists promoted translocation of PKCε and PKA catalytic subunits from the cytosol to the plasma membrane of cultured neurons and HEK 293 cells. Subcellular fractionation and Western blotting confirmed this redistribution of kinases, which is indicative of activation. Although PAR_2 couples to phospholipase Cβ, leading to stimulation of PKC, we also observed that PAR_2 agonists increased cAMP generation in neurons and HEK 293 cells, which would activate PKA. PAR_2 agonists enhanced capsaicin-stimulated increases in $[Ca^{2+}]_i$ and whole-cell currents in HEK 293 cells, indicating TRPV1 sensitization. The combined intraplantar injection of non-algesic doses of PAR_2 agonist and capsaicin decreased the latency of paw withdrawal to radiant heat in mice, indicative of thermal hyperalgesia. Antagonists of PKCε and PKA prevented sensitization of TRPV1 Ca^{2+} signals and currents in HEK 293 cells, and suppressed thermal hyperalgesia in mice. Thus, PAR_2 activates PKCε and PKA in sensory neurons, and thereby sensitizes TRPV1 to cause thermal hyperalgesia. These mechanisms may underlie inflammatory pain, where multiple proteases are generated and released.

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Protease-activated receptor 2 (PAR_2) is widely expressed in the nervous system where it mediates the actions of proteases on diverse neuronal processes (reviewed in Ossovskaya & Bunnett, 2004). Proteases from the circulation (coagulation factors VIIa, Xa; Camerer *et al.* 2000), inflammatory cells (tryptase; Corvera *et al.* 1997; Molino *et al.* 1997), epithelial cells and neurons (trypsins I, II, IV; Cottrell *et al.* 2004) can cleave PAR_2 to expose a tethered ligand domain that binds to and activates the cleaved receptor. Activated PAR_2 controls neurogenic inflammation, pain and neuronal excitability. PAR_2 is expressed by primary spinal afferent neurons, where activation stimulates release of substance P and calcitonin gene-related peptide in peripheral tissues to cause neurogenic inflammation (Steinhoff *et al.* 2000; Cenac *et al.* 2002, 2003; Nguyen *et al.* 2003). Similar mechanisms mediate the effects of PAR_2 agonists on airway constriction (Ricciardolo *et al.* 2000) and gastric mucus secretion (Kawabata *et al.* 2001*b*). PAR_2 agonists also cause thermal and mechanical somatic hyperalgesia (Kawabata *et al.* 2001*a*; Vergnolle *et al.* 2001), and excite mesenteric sensory neurons to induce visceral hyperalgesia (Hoogerwerf *et al.* 2001; Coelho *et al.* 2002; Kirkup *et al.* 2003). However, the molecular mechanisms by which PAR_2 regulates neuronal functions are incompletely understood.

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PAR_2-induced thermal hyperalgesia depends on sensitization of transient potential receptor vanilloid 1 (TRPV1) (Amadesi *et al.* 2004; Dai *et al.* 2004). TRPV1 is a non-selective cation channel expressed by nociceptive neurons, that mediates inflammatory and thermal hyperalgesia (Caterina *et al.* 1997, 2000; Davis *et al.* 2000). Exogenous (capsaicin, ethanol) and endogenous (protons pH < 6.0, heat $> 43^{\circ}$C, anandamide) factors directly activate TRPV1 (Caterina *et al.* 1997; Zygmunt *et al.* 1999; Trevisani *et al.* 2002). In addition, PAR_2 agonists (Amadesi *et al.* 2004; Dai *et al.* 2004) and other inflammatory agents, including bradykinin, ATP, prostaglandin E_2 (PGE_2) and nerve growth factor (NGF) (Lopshire & Nicol, 1997; Chuang *et al.* 2001; Tominaga *et al.* 2001; Vellani *et al.* 2001) indirectly sensitize TRPV1, causing hyperalgesia. The mechanisms of this sensitization include activation of protein kinase (PK) C and PKA, which phosphorylate TRPV1 to modify channel gating (Lopshire & Nicol, 1998; Premkumar & Ahern, 2000; Bhave *et al.* 2002; Mohapatra & Nau, 2003). The PKCε isozyme, which plays a major role in mechanical and thermal hyperalgesia (Khasar *et al.* 1999), phosphorylates TRPV1 (Numazaki *et al.* 2002) to mediate bradykinin-induced sensitization of TRPV1 currents (Cesare *et al.* 1999). PKA, a mediator of injury-induced hyperalgesia (Malmberg *et al.* 1997), also phosphorylates TRPV1 to regulate its desensitization (Bhave *et al.* 2002; Mohapatra & Nau, 2003), and thereby mediates PGE_2-induced sensitization of TRPV1 (Lopshire & Nicol, 1998; Rathee *et al.* 2002). Additional mechanisms of TRPV1 sensitization include altered interactions of TRPV1 with the endogenous inhibitor phosphatidylinositol-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).

The purpose of the present investigation was to define the mechanisms by which PAR_2 sensitizes TRPV1 to induce thermal hyperalgesia. Although PKCε contributes to PAR_2-induced sensitization of TRPV1 currents (Dai *et al.* 2004), the role of PKCε in PAR_2-induced thermal hyperalgesia has not been examined, and the contributions of other second messenger kinases, such as PKA, are unknown. Our objectives were to determine whether (1) primary spinal afferent neurons that express PAR_2 also contain PKCε and PKA, using immunofluorescence and confocal microscopy; (2) PAR_2 agonists activate and cause membrane translocation of PKCε and PKA in neurons and cell lines, using microscopy, subcellular fractionation and Western blotting; (3) PKCε and PKA mediate PAR_2-induced sensitization of TRPV1 in cell lines, by measuring TRPV1 Ca^{2+} signals and currents; and (4) PKCε and PKA mediate PAR_2-induced sensitization of TRPV1-dependent thermal hyperalgesia.

Methods

Animals

Sprague-Dawley rats (male, 200–250 g) and C57Bl6 mice (male, 6–8 weeks) were obtained from Charles River Laboratories (CA, USA, and Canada). The study was approved by Institutional Animal Care and Use Committees of the University of California, San Francisco and the University of Calgary. At the end of the experiments animals were humanely killed using sodium pentobarbital (200 mg kg^{-1} I.P.) and bilateral thoracotomy.

Agonists and antagonists

PAR_2-activating peptide (PAR_2-AP, SLIGRL-NH_2), corresponding to the tethered ligand of rat and mouse PAR_2, and the inactive reverse peptide sequence (PAR_2-RP), which was used as the control, were obtained from Sigma Genosys (The Woodlands, TX, USA). Bovine pancreatic trypsin was from Worthington Biochemical Corporation (Lakewood, NJ, USA). The selective PKCε translocation inhibitor peptide (EAVSLKPT, PKCεI), inactive scrambled sequence (PKCεI-sc), the PKA inhibitor H-89 (*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulphonamide), cell-permeable myristoylated PKA inhibitor 14–22 amide ($PKAI_{14-22}$, Myr-GRTGRRNAI-NH_2), and the PKA activator forskolin were from Calbiochem (La Jolla, CA, USA). The PKC activators phorbol 12-myristate 13-acetate (PMA) and phorbol dibutyrate, and indomethacin, a cyclo-oxygenase inhibitor, were from Sigma (St Louis, MO, USA). The PKA inhibitor WIPTIDE (TTYADFIASGRTGRRNAI-NH_2) was from Peninsula Laboratories (San Carlos, CA, USA). The PKCεI conjugated to TAT protein to promote membrane permeability (Schwarze *et al.* 1999) (TAT-PKCεI, YGRKKRRQRRRC-disulphide bond-CEAVSLKPT-COOH) and the inactive scrambled sequence (TAT-PKCεI-sc) were from SynPep Corp (Dublin, CA, USA). The selection of inhibitors and doses used in this study was based on previous studies (Aley *et al.* 2000, 2001; Hu *et al.* 2002; Rathee *et al.* 2002).

Antibodies

Rabbit anti-human PKCε, rabbit anti-human PKAα catalytic subunit (PKA_C), mouse anti-human PAR_2 (SAM11) and mouse anti-EGFP (enhanced green fluorescent protein) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Goat anti-rabbit and anti-mouse IgG conjugated to FITC, Rhodamine Red X or horseradish peroxidase were from Jackson ImmunoResearch (West Grove, PA, USA). In control experiments,

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primary antibodies were incubated before use with peptides used for immunization (1 μM, 24 h, 4°C).

Cell lines

Human embryonic kidney (HEK) 293 cells were maintained in modified Eagle's with Earle's BSS medium (MEM), 10% fetal bovine serum (FBS) and 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin. HEK 293 cells stably expressing human TRPV1 (HEK-TRPV1, a gift from Dr J. Davis, GSK Harlow, UK) were generated and maintained in MEM, 10% FBS and geneticin (400 mg l^{-1}) (Hayes *et al.* 2000). HEK-FLP cells (Invitrogen, Carlsbad, CA, USA) stably expressing human PAR$_2$ with N-terminal Flag and C-terminal T7 epitopes (HEK-PAR$_2$) were generated and maintained as described (Jacob *et al.* 2005). HEK 293 cells were transiently transfected with 1 μg of EGFP-human PKCε (a gift from Dr Daria Mochly Rosen, Stanford University) using Lipofectamine 2000 (Invitrogen) to generate HEK-PKCε-EGFP cells (Schechtman *et al.* 2004). These cells were studied 48 h after transfection.

Neuronal culture

Rats were anaesthetized with sodium pentobarbital (200 mg kg^{-1} I.P.). Dorsal root ganglia (DRG) from the thoracic and lumbar spinal cord were removed and minced in cold Hank's balanced salt solution (HBSS) and incubated for 60–90 min at 37°C in Dulbecco's modified Eagle's medium (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% FBS, 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.

Localization of PKCε and PKA$_C$ in cultured cells

HEK 293 cells and cultured DRG neurons were incubated in HBSS, 0.1% BSA, 20 mM Hepes, pH 7.4 at 37°C and treated with test substances. Cells were fixed with 4% paraformaldehyde, 100 mM PBS, pH 7.4 for 20 min at 4°C. Cultured cells were incubated with PBS containing 5–10% normal goat serum and 0.1–0.3% Triton X-100 for 30 min, and incubated with primary antibodies to PKCε (1 : 250) or PKA$_C$ (1: 400) for 16 h at 4°C. After washing, cells were incubated with goat anti-rabbit IgG conjugated to FITC (1 : 300) for 2 h at room temperature. Cells expressing PKCε-EGFP were similarly treated and fixed.

Localization of PKCε, PKA$_C$ and PAR$_2$ in DRG sections

Rats (male, 200 g) were anaesthetized with sodium pentobarbital (200 mg kg^{-1} I.P.) and transcardially perfused with PBS containing 100 U of heparin, followed by 4% paraformaldehyde in 100 mM PBS, pH 7.4. DRG (L4–L6) were immersion fixed for 4 h at 4°C. DRG were incubated in 20% sucrose in PBS for 24 h at 4°C, embedded in OCT compound (Miles, Elkhart, IN, USA), and frozen sections of 16 μm were prepared. Sections were incubated with PBS containing 5–10% normal goat serum and 0.1–0.3% Triton X-100 for 30 min, and incubated with primary antibodies: PKCε (1 : 750), PKA$_C$ (1 : 750), PAR$_2$ (1 : 250) for 16 h at 4°C. After washing, sections were incubated with goat anti-rabbit IgG conjugated to FITC or goat anti-mouse IgG conjugated to Rhodamine Red X (1 : 200) for 2 h at room temperature. Slides were washed and mounted in Prolong (Molecular Probes, Eugene, OR, USA).

Confocal microscopy

Specimens were observed using Zeiss Axiovert and Bio-Rad MRC1000 confocal microscopes with Zeiss Plan Apo ×40 (NA 1.4) or ×100 (NA 1.3) objectives. Images were collected at zoom of 1–2, iris of < 3 μm and typically 5–10 optical sections were taken at intervals of 0.5 μm. Images were coloured to represent the appropriate fluorophores, and processed using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA) to adjust contrast and brightness. Images of stained and control slides were collected and processed identically. To quantify the effects of agonists on the subcellular distribution of PKCε and PKA$_C$ in cultured neurons, the fluorescence intensity of the cells was measured by selecting a straight line across the neuronal soma and using the plot profile function of ImageJ software (version 10.2, NIH image). The averaged fluorescence intensities between peripheral and central regions of the cell were compared. The number of neurons showing redistribution of these kinases between the cytosol and periphery of the cells was also determined and expressed as a percentage of the total number of neurons analysed.

Subcellular fractionation and Western blotting

HEK 293 cells were maintained overnight in MEM and 0.1% BSA. Cells were incubated in HBSS, 0.1% BSA, 20 mM Hepes, pH 7.4 and treated at 37°C for 0–10 min with PAR$_2$ agonists. Cells were washed with ice-cold PBS, scraped into 150 μl of homogenization buffer (20 mM Tris-HCl, pH 7.4 and protease inhibitors) and disrupted with 20 strokes in a glass homogenizer. The lysate was centrifuged at 500 *g* for 10 min, and the resulting supernatant was centrifuged at 100 000 *g* for 1 h. The

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supernatant was collected as the cytosolic fraction. The pellet containing the membrane fraction was treated with lysis buffer (1% SDS, 20 mM Tris-HCl, pH 7.4 and protease inhibitor cocktail, Roche Diagnostics, Indianapolis, IN, USA). Both cytosolic and membrane fractions were boiled for 5 min and clarified by centrifugation at 15 000 *g* for 15 min. Protein concentration was measured by BCA assay. Samples (5 μg protein) were separated by SDS-PAGE (8% for PKCε, 12% for PKA_C). Proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were incubated overnight at 4°C with antibodies to PKCε (1 : 10 000), PKA_C (1 : 10 000) or EGFP (1 : 10 000) in PBS containing 5% milk powder, 2% BSA, 0.1% Tween-20. Membranes were washed and incubated with goat anti-rabbit or anti-mouse horseradish peroxidase (1 : 10 000) for 1 h at room temperature. Immunoreactive proteins were detected by Enhanced Chemiluminescence (Pierce, Rockford, IL, USA). To ensure equal loading, membranes were stained with amido-black to detect all proteins.

Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

The experimental procedure has been previously described (Amadesi *et al.* 2004). HEK 293 cells were incubated in HBSS, 0.1% BSA, 20 mM Hepes, pH 7.4, containing 2.5 μM of fura-2/AM (Invitrogen) for 30–45 min at 37°C. Coverslips were mounted in an open chamber (350 μl) at 37°C. 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 injection). Each coverslip received only one treatment with PAR_2-AP or RP followed by capsaicin. In some experiments cells were pretreated for 30 min at 37°C with inhibitors before challenging with test substances. The magnitude of capsaicin responses was calculated as the increase above baseline. Results were first calculated as the 340 nm/380 nm emission ratio, which is proportional to the $[Ca^{2+}]_i$, and then expressed as a percentage of the potentiation observed in cells pretreated with PAR_2-AP and antagonist vehicle (100%).

Measurement of intracellular cAMP

HEK 293 cells were maintained overnight in MEM-0.1% BSA. HEK 293 cells and DRG neurons in culture were incubated in HBSS, 0.1% BSA, 20 mM Hepes, pH 7.4 and treated with PAR_2 agonists for 0–5 min at 37°C. Intracellular cAMP levels were measured using the cAMP enzyme immunoassay system following the non-acetylation protocol according to the manufacturer's directions (Amersham Bioscience, Bucks, UK).

Electrophysiology

The experimental procedure has been previously described (Amadesi *et al.* 2004) with slight modifications. Membrane currents of HEK 293 cells were recorded using an Axopatch 1D amplifier (Axon Instruments). Patch pipette resistance ranged from 1 to 3 MΩ, and the holding potential was −60 mV. The pipette solution was (mM): KCl 140, $MgCl_2$ 1, EGTA 5, Hepes 5, and $ATPNa_2$ 5. The external solution was (mM): NaCl 140, $CaCl_2$ 2, KCl 4, $MgCl_2$ 1, glucose 11, Hepes 5 and CsCl 3. The pH was adjusted with either KOH (pipette solution, pH 7.2) or NaOH (external solution, pH 7.4). In these standard solutions the input resistance of the HEK 293 cells was 1–10 GΩ. Whole-cell currents were recorded using Axotape software and analysed using Axograph software (Axon Instruments). Membrane potentials were corrected for the liquid junction potential (pipette −11 mV). The recording chamber was continuously superfused with the external solution at approximately 2 ml min^{-1}. Rapid application of agonists in the external solution was made using an eight-barrelled fast-flow device (Barajas-Lopez *et al.* 1994). Experiments were at room temperature (~23°C). Control TRPV1 responses were obtained by repeated application of capsaicin (300 nM) for ~20 s. Only neurons that did not exhibit desensitization to two consecutive capsaicin applications were selected for the study. PAR_2-AP (100 μM) or, in a small series of experiments, the PKC agonist phorbol dibutyrate (300 nM) was then applied for 2 min and TRPV1 responses were examined at 30 s to 18 min following PAR_2 activation. Results were expressed as a percentage of the control TRPV1 current. In some experiments, PKCε translocation inhibitor peptide or H89 was added to the pipette solution.

Paw withdrawal latency

Test substances were administered by intraplantar injection in mice under light halothane (5%) anaesthesia (Vergnolle *et al.* 2001). PAR_2-AP, PAR_2-RP, PKCε and PKA inhibitors were dissolved in physiological saline (0.9% NaCl); capsaicin was dissolved in 80% physiological saline, 10% ethanol, and 10% Tween 80. To allow the entry of the membrane-impermeant inhibitors into cells, injection of PKCεI, PKCεI-sc and WIPTIDE (all 2.5 μl paw^{-1}) was preceded by injection of distilled water (2.5 μl paw^{-1}) to cause a transient hyposmotic permeabilization. Mice received an intraplantar injection of PAR_2-AP and capsaicin (5 μl paw^{-1}) 15–30 min later (final volume 10 μl paw^{-1}). The latency of paw withdrawal to a radiant heat stimulus was measured before and after

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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 to the basal measurement.

Statistical analysis

Results are expressed as mean ± S.E.M. Comparisons between groups were made by ANOVA and the Bonferroni test, Student's *t* test, or a paired Student's *t* test to evaluate differences between the same cells. Comparisons between proportions were made using Primer of Biostatistics software (3.1 version, Macintosh version, McGraw Hill, 1992) and the 'standard error of proportion' and the 'compare two proportions' functions. Differences were considered significant when $P \leq 0.05$.

Results

DRG neurons that express PAR_2 also contain PKCε and PKA_C

PAR_2 is present in DRG neurons that express substance P, calcitonin gene-related peptide and TRPV1, and which therefore mediate neurogenic inflammation and nociception (Steinhoff *et al.* 2000; Amadesi *et al.* 2004). To determine if PAR_2-expressing neurons also contain the potential regulatory kinases PKCε and PKA_C, we used immunofluorescence. Immunoreactive PAR_2 was present in small- and medium-diameter neurons that are typically considered nociceptive neurons (Fig. 1*A* and *D*). These neurons also contained immunoreactive PKCε and PKA_C (Fig. 1*B*, *C*, *E* and *F*, arrows). Staining for PKCε and PKA_C was abolished by preabsorption of the antibodies with the peptides used for immunization, suggesting specificity

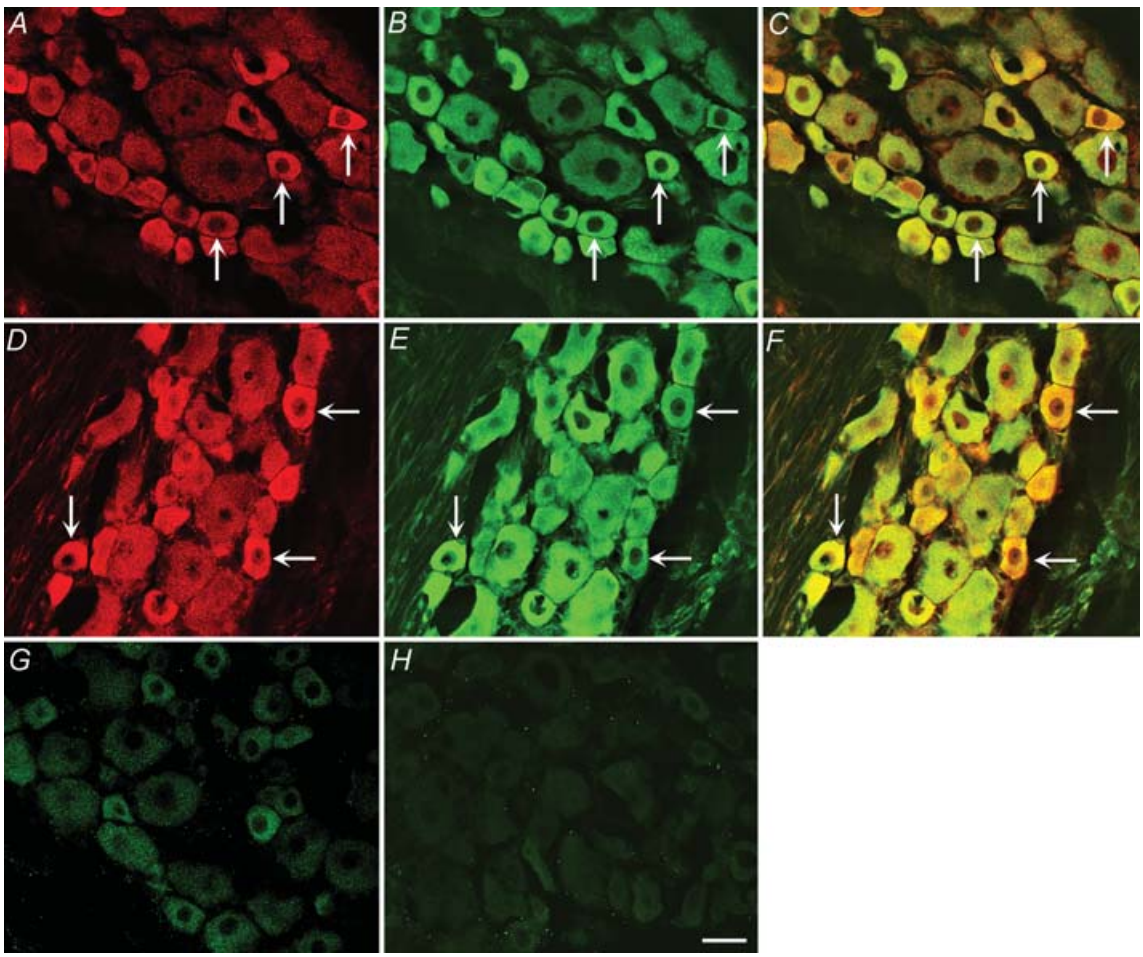

Figure 1. Localization of immunoreactive PAR_2, PKCε and PKA_C in DRG neurons
A and *D*, PAR_2; *B*, PKCε; *E*, PKA_C; *C* and *F*, merge. *G*, PKCε preabsorption control; *H*, PKA_C preabsorption control. Neurons expressing PAR_2 also expressed PKCε and PKA_C (arrows). Scale bar = 20 μm.

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(Fig. 1*G* and *H*). Since PAR_2 and TRPV1 are coexpressed in nociceptive neurons (Amadesi *et al.* 2004), PKCε and PKA_C are appropriately localized to mediate PAR_2-induced regulation of TRPV1.

PAR_2 agonists activate PKCε in HEK 293 cells and DRG neurons

PKCε phosphorylates and sensitizes TRPV1 (Numazaki *et al.* 2002). We investigated the effects of PAR_2 agonists on translocation of PKCε from the cytosol to the plasma membrane, since membrane translocation of PKCε is an index of activation of this kinase (Dorn & Mochly Rosen, 2002). We first studied trafficking of PKCε-EGFP expressed in HEK 293 cells, which can be detected with high sensitivity and specificity. PAR_2 agonists sensitize TRPV1 similarly in HEK 293 cells and DRG neurons (Amadesi *et al.* 2004). Thus, HEK 293 cells, which naturally express PAR_2, are a useful model system to study regulation of TRPV1. In unstimulated HEK 293 cells, PKCε-EGFP was detected in the cytosol (Fig. 2*A*). PMA (1 μM), a PKC activator, induced a prominent redistribution of PKCε-EGFP from the cytosol to the plasma membrane at 5 min (Fig. 2*A*). In a similar manner, PAR_2-AP (100 μM) induced trafficking of PKCε-EGFP to the membrane within 0.5 min and 1 min (Fig. 2*A*), and after 15 min PKCε-EGFP returned to the cytosol (results not shown). PAR_2-RP (100 μM, 1 min) did not affect the subcellular distribution of PKCε-EGFP (Fig. 2*A*). PMA and PAR_2-AP also stimulated membrane translocation of immunoreactive PKCε in HEK-TRPV1 cells (results not shown). Thus, EGFP does not interfere with the redistribution of PKCε to the plasma membrane. Of note, PAR_2-AP also caused translocation of PKCε-EGFP to the nucleus after 1 min (Fig. 2*A*). However, we did not detect nuclear translocation of endogenous PKCε after exposure to PAR_2-AP (not shown). Thus, the physiological relevance of this redistribution is unknown.

We also examined the effects of PMA and PAR_2 agonists on the subcellular localization of immunoreactive PKCε in rat DRG neurons in culture. Since the effects of agonists on PKCε trafficking in neurons were less prominent than in HEK 293 cells, we measured the pixel intensity of signals in the central (cytosol) and peripheral (membrane) regions of the soma, and also determined the proportion of neurons in which there was detectable redistribution of PKCε. In unstimulated neurons, immunoreactive PKCε was predominantly diffusely localized in the cytosol (Fig. 2*B*). The PKCε signal was most intense in the cytosol, with minimal signal at the plasma membrane (Fig. 2*C*). Indeed, PKCε was detected at the plasma membrane of only $3 \pm 5\%$ of the unstimulated neurons ($n = 318$ total neurons) (Fig. 2*D*). PMA (1 μM, 5 min) induced a prominent redistribution of PKCε from the cytosol to the plasma membrane of neurons (Fig. 2*B* and *C*), and now membrane staining was detected in $99 \pm 2\%$ of neurons ($n = 176$) (Fig. 2*D*). PAR_2-AP (100 μM) also induced trafficking of PKCε to the plasma membrane within 0.5 min ($14 \pm 4\%$ of $n = 196$), with a maximal effect at 1 min ($62 \pm 3\%$ of $n = 125$) (Fig. 2*B–D*). At 5 min, PKCε was still localized at the membrane ($77 \pm 5\%$ of $n = 142$), but the staining was more diffuse, suggesting return to the cytosol (not shown). PAR_2-RP (100 μM) had no effect on the localization of PKCε, with $< 15\%$ of neurons having detectable PKCε at the plasma membrane ($9 \pm 3\%$ of $n = 104$ at 0.5 min and $12 \pm 3\%$ of $n = 152$ at 1 min) (Fig. 2*B–D*).

To confirm that PAR_2 activation induces membrane translocation of PKCε in HEK 293 cells, and to determine the kinetics of this process, we quantified PKCε in cytosolic and membrane fractions by Western blotting. In unstimulated HEK-PKCε-EGFP cells, PKCε-EGFP was predominantly in cytosolic fractions (Fig. 3*A* and *B*). After incubation with PAR_2-AP (100 μM), the level of PKCε-EGFP increased in membrane fractions and diminished in cytosolic fractions within 0.5–1 min (Fig. 3*A* and *B*). PAR_2-AP also caused translocation of endogenous immunoreactive PKCε from cytosolic to membrane fractions of HEK-TRPV1 cells within 1 min (Fig. 3*C* and *D*). PKCε-EGFP was still detected in membrane fractions 10 min after PAR_2 activation, when endogenous PKCε had returned to the cytosol. The more persistent presence of PKCε-EGFP in membrane fractions may reflect the higher sensitivity of detection of PKCε-EGFP, or be due to the over-expression of PKCε, or be related to an effect of EGFP on association of PKCε with the membrane. Trypsin (10 nM) caused a similar membrane translocation of PKCε-EGFP and immunoreactive PKCε in HEK 293 cells (not shown). We did not quantify membrane translocation of PKCε in DRG neurons by Western blotting, due to the limited number of cells and the low levels of PKCε expression. Together, our results show that agonists of PAR_2 induce redistribution of PKCε to the plasma membrane in both HEK 293 cells and DRG neurons, suggesting activation of PKCε.

PAR_2 agonists activate PKA in DRG neurons and HEK 293 cells

TRPV1 has been proposed as a target for the cAMP/PKA cascade, and PKA phosphorylates TRPV1 to regulate its desensitization (Bhave *et al.* 2002; Rathee *et al.* 2002). Exposure of sensory neurons to forskolin, which activates the cAMP/PKA cascade, induces translocation of the PKA_C to the cell periphery, which is indicative of activation (Rathee *et al.* 2002). To determine if PAR_2 agonists activate PKA, we examined their effects on the subcellular distribution of PKA_C in DRG neurons. In most

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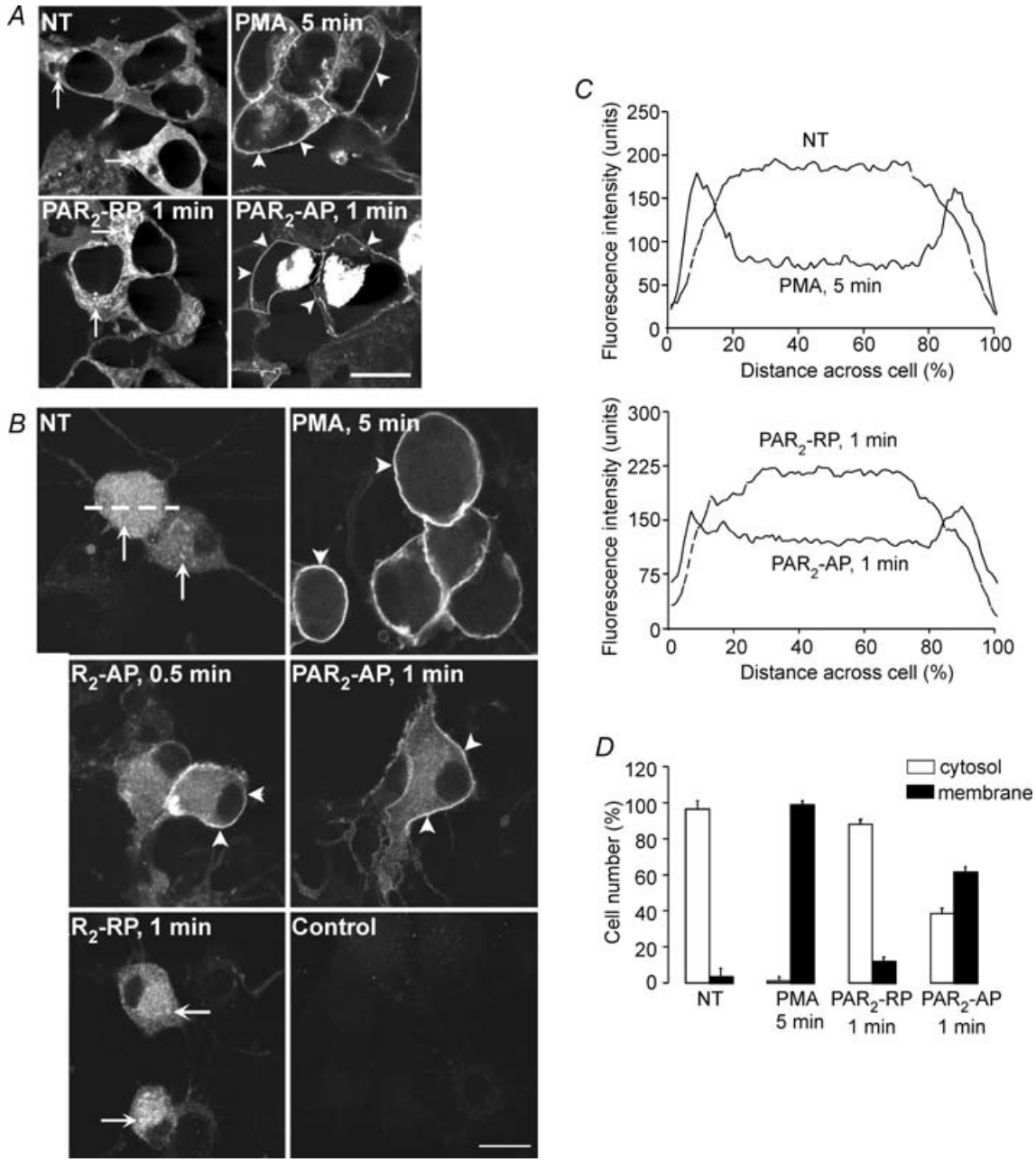

Figure 2. Effects of PAR$_2$ agonist on the subcellular distribution of PKCε determined by confocal microscopy

A, HEK 293 cells expressing PKCε-EGFP (enhanced green fluorescent protein). In unstimulated cells (non-treated, NT), PKCε-EGFP was cytosolic and in vesicles (arrows). PMA (phorbol 12-myristate 13-acetate) induced translocation to the plasma membrane at 5 min (arrowheads). PAR$_2$-AP induced translocation to the plasma membrane at 1 min (arrowheads). PAR$_2$-RP did not affect the subcellular location of PKCε-EGFP, which remained in the cytosol and vesicles (arrows). Scale bar = 10 μm. *B*, rat DRG neurons in culture. In unstimulated neurons (NT, non-treated), PKCε was cytosolic and in vesicles (arrows). PMA induced translocation of PKCε to the plasma membrane at 5 min (arrowheads). PAR$_2$-AP also induced translocation of PKCε to the plasma membrane at 0.5 and 1 min (arrowheads). PKCε was in the cytosol and vesicles of cells treated with PAR$_2$-RP (arrows). Control shows staining with preabsorbed PKCε antibody. Scale bar = 10 μm. *C*, fluorescence intensity (in arbitrary units) measured in a line bisecting the neuronal soma (e.g. see dashed line in *B*). In untreated cells (NT) or cells incubated with PAR$_2$-RP (1 min), the signal was mostly cytosolic. In cells treated with PMA (5 min) or PAR$_2$-AP (1 min), the signal in the cytosol diminished and was more prominent at the plasma membrane. Each trace is an average of 4–6 cells. *D*, the effect of PMA, PAR$_2$-AP and PAR$_2$-RP on the percentage of the total number of observed neurons expressing PKCε with PKCε either in the cytosol or at the plasma membrane of the soma. The proportion of cells with PKCε at the plasma membrane was increased after treatment with PMA and PAR$_2$-AP. Observations from $n > 100$ cells.

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unstimulated neurons, immunoreactive PKA_C was cytosolic or present in vesicles that were uniformly distributed throughout the cytoplasm (Fig. 4*A*). This uniform cytosolic distribution of PKA_C was confirmed by measuring the pixel intensity (Fig. 4*B*). Indeed, PKA_C was detected in the vicinity of the plasma membrane in only $22 \pm 5\%$ of unstimulated neurons (of $n = 270$ total neurons) (Fig. 4*C*). Forskolin (10 μM, 0.5 min), which activates PKA, stimulated the redistribution of PKA_C from the central to the peripheral region of the cell (Fig. 4*A*). This redistribution was confirmed by measurement of the pixel intensity of immunoreactive PKA_c (Fig. 4*B*). This peripheral staining of PKA_c was detected in $92 \pm 2\%$ of neurons ($n = 141$) (Fig. 4*C*). PAR_2-AP (100 μM) also stimulated the redistribution of PKA_C from the central to the peripheral regions of the soma, and this effect was observed in $70 \pm 4\%$ of neurons at 1 min ($n = 143$), and $76 \pm 3\%$ of neurons at 5 min ($n = 205$) after stimulation (Fig. 4*A*–*C*). After 10–15 min, PKA_C returned to cytosolic vesicles (not shown). In contrast, PAR_2-RP had no effect on the subcellular distribution of immunoreactive PKA_C, with only $15 \pm 4\%$ of neurons showing localization of PKA_C close to the plasma membrane at 5 min ($n = 106$) (Fig. 4*A*–*C*).

To confirm that PAR_2 activation induces membrane translocation of PKA_C in HEK-TRPV1 cells, which endogenously express PKA regulatory and catalytic subunits (Rathee *et al.* 2002), we quantified PKA_C in cytosolic and membrane fractions by Western blotting. After 1 min of incubation with PAR_2-AP (100 μM), there were elevated levels of PKA_C in membrane fractions, and diminished levels in cytosolic fractions (Fig. 5*A* and *B*). Thus, PAR_2 agonists cause translocation of PKA_C from the cytosol to membrane fractions, which is indicative of activation of PKA.

It is well established that PAR_2 couples to $G_{\alpha q/11}$, resulting in activation of phospholipase Cβ and formation of inositol trisphosphate and diacylglycerol, which mobilize intracellular Ca^{2+} ions and activate PKC. However, PAR_2 agonists elevate cAMP levels in keratinocytes (Scott *et al.* 2003), which suggests that PAR_2 may activate the PKA cascade. Therefore, we determined the effects of PAR_2 agonists on cAMP levels in HEK-PAR_2 cells and DRG neurons. PAR_2-AP (100 μM) and trypsin (10 nM) increased the cAMP levels in HEK-PAR_2 cells by ~35% within 1 and 5 min, whereas PAR_2-RP (100 μM) had no effect (Fig. 5*C*). Similarly, PAR_2-AP (100 μM) and trypsin (10 nM) increased the cAMP levels in rat DRG neurons by 40–60% after 5 min, whereas PAR_2-RP was inactive (Fig. 5*C*). PAR_2 agonists caused a similar increase of cAMP level in HEK-TRPV1 cells (not shown). Since PAR_2 agonists can stimulate the generation of prostaglandins (Kong *et al.* 1997), which could activate receptors that couple to form cAMP, we determined

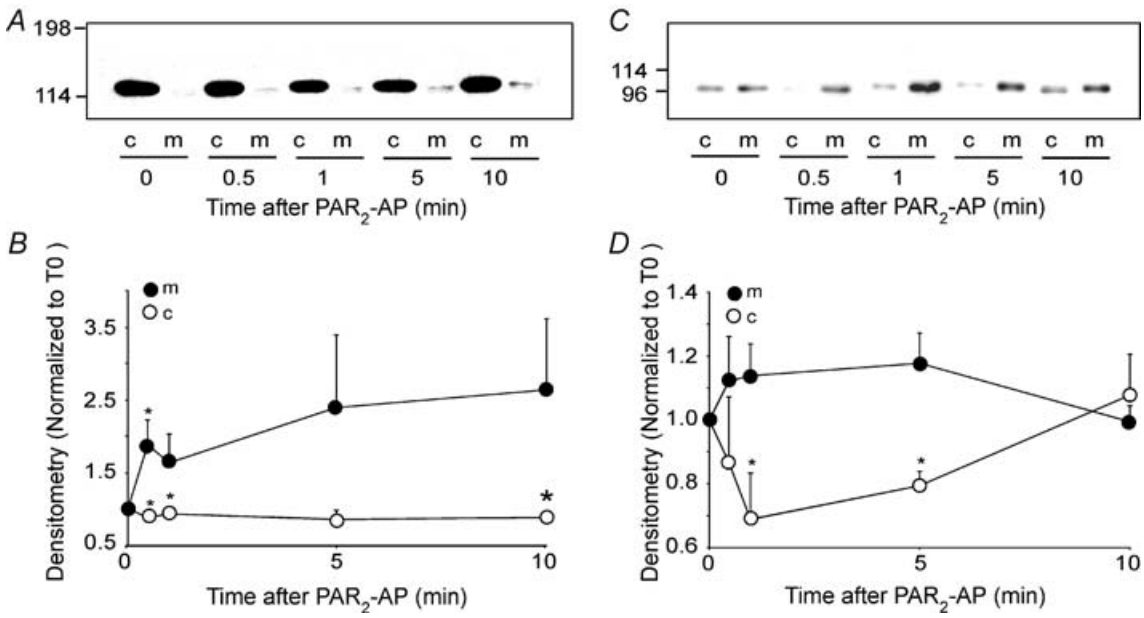

Figure 3. Effects of PAR_2 agonist on the subcellular distribution of PKCε in HEK 293 cells determined by subcellular fractionation and Western blotting
A, Western blot, and *B*, densitometric analysis of PKCε-EGFP (enhanced green fluorescent protein) in cytosolic (c) and membrane (m) fractions of HEK-PKCε-EGFP cells. PAR_2-AP increased PKCε-EGFP in membrane fractions and decreased PKCε-EGFP in cytosolic fractions. *C*, Western blot, and *D*, densitometric analysis of immunoreactive PKCε in cytosolic and membrane fractions of HEK-TRPV1 cells. PAR_2-AP increased PKCε in membrane fractions and decreased PKCε in cytosolic fractions. $^*P < 0.05$ compared to 0 min, $n = 4$ experiments.

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whether inhibitors of cyclo-oxygenase prevented the effects of PAR_2 agonists on cAMP levels. Indomethacin (5 μM) did not affect PAR_2-AP or trypsin-stimulated formation of cAMP in HEK 293 cells (not shown). Thus, agonists of PAR_2 induce redistribution of PKA_C and increase cAMP levels in neurons and cells, suggesting an activation of the cAMP/PKA cascade.

PAR_2 agonists sensitize TRPV1-mediated Ca^{2+} responses and TRPV1 currents by PKCε- and PKA-dependent mechanisms

We have previously reported that PAR_2 agonists sensitize TRPV1 Ca^{2+} signals and currents in HEK 293 cells and DRG neurons by mechanisms that require activation of

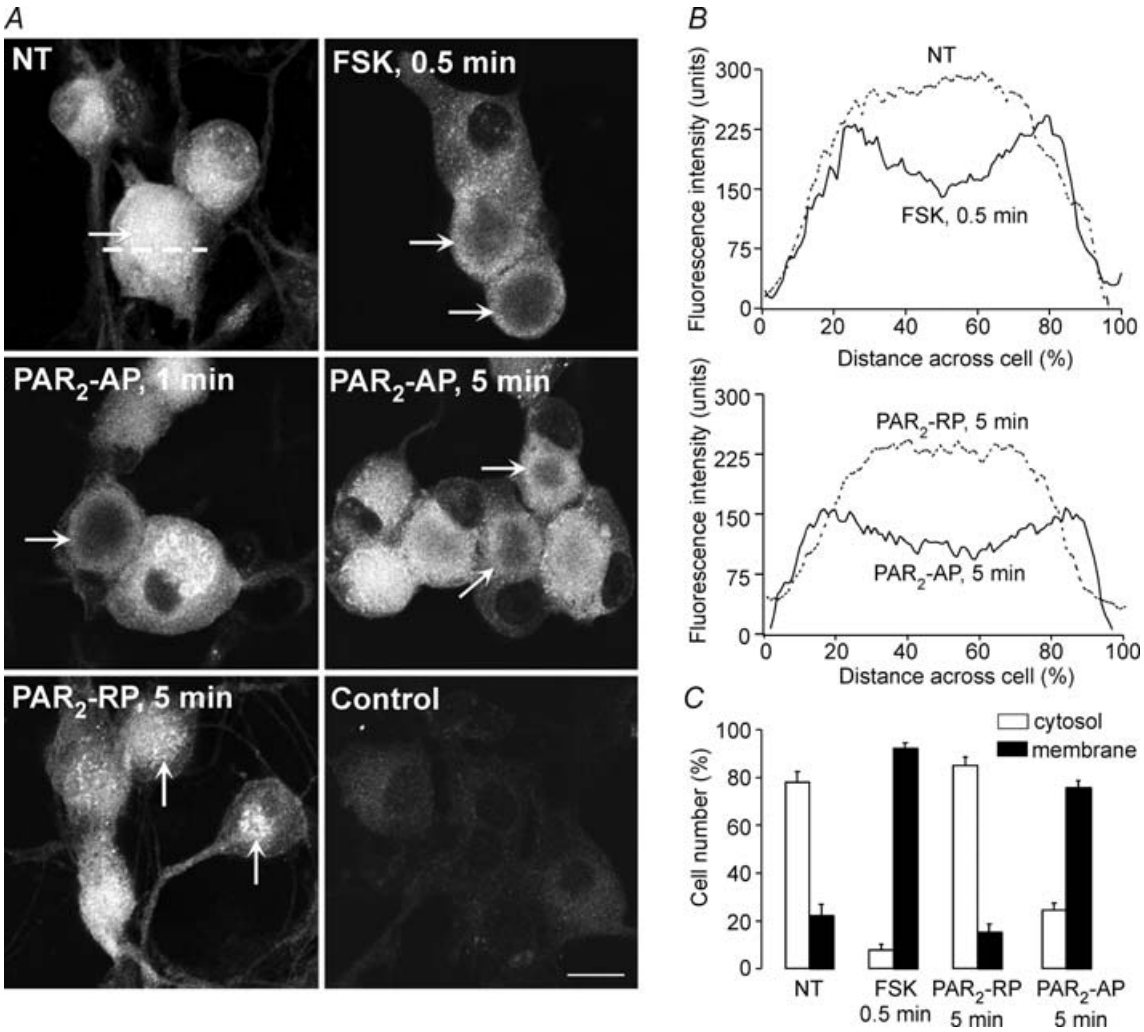

Figure 4. Effects of PAR_2 agonist on the subcellular distribution of PKA_C determined by confocal microscopy in rat DRG neurons in culture

A, unstimulated neuron (non-treated, NT), PKA_C (PKA catalytic subunit) was in vesicles and uniformly distributed throughout the cytosol (arrows). Forskolin (FSK) induced redistribution of PKA_C from the central to the peripheral region of the soma at 0.5 min (arrows). PAR_2-AP also induced redistribution of PKA_C to the peripheral region of the cell at 1 min and 5 min (arrows). PKA_C was uniformly distributed in the cytosol and in vesicles of cells treated with PAR_2-RP (arrows). Control shows staining with preabsorbed PKA_C antibody. Scale bar = 10 μm. *B*, fluorescence intensity (in arbitrary units) measured in a line bisecting the neuronal soma (e.g. see dashed line in *A*). In untreated cells (NT) or cells incubated with PAR_2-RP (5 min), the signal was mostly cytosolic. In cells treated with FSK (0.5 min) or PAR_2-AP (5 min), the signal in the cytosol diminished and was more prominent in superficial regions of the soma. Each trace is an average of 4–6 cells. *C*, the effects of FSK, PAR_2-AP and PAR_2-RP on the percentage of the total number of observed neurons expressing PKA_C with PKA_C either in the cytosol or near the plasma membrane of the soma. The proportion of cells with PKA in a superficial region of the cytosol was increased after treatment with FSK and PAR_2-AP. Observations from $n > 100$ cells.

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phospholipase Cβ and PKC (Amadesi *et al.* 2004). We now used selective inhibitors of PKCε and of PKA to determine their contributions to PAR_2-induced sensitization of TRPV1. HEK-TRPV1 cells were used for these experiments in view of the similar effects of PAR_2 agonists on activation of PKCε and PKA and sensitization of TRPV1 in both HEK 293 cells and DRG neurons (Amadesi *et al.* 2004).

Exposure of HEK-TRPV1 cells to PAR_2-AP (100 μM, 5 min) potentiated the effects of capsaicin (10 nM) on $[Ca^{2+}]_i$ by ~70% ($n = 131$ cells), indicative of TRPV1 sensitization (Fig. 6*A* and *B*). In contrast, PAR_2-RP (100 μM, 5 min) had no effect. Pretreatment with TAT-PKCεI (10 μM) prevented this sensitization (Fig. 6*B*). Thus, the effect of capsaicin in cells treated with TAT-PKCεI and PAR_2-AP was $56 \pm 7\%$ ($n = 116$ cells) of the responses in cells treated with PAR_2-AP and vehicle (100%). Pretreatment with inactive TAT-PKCεI-sc (10 μM) had no effect on PAR_2-AP-induced sensitization of TRPV1 ($n = 110$ cells). Pretreatment with two selective inhibitors of PKA, H-89 (10 μM) and the cell-permeable $PKAI_{14\text{-}22}$ (0.1 μM), also prevented PAR_2-AP-mediated sensitization of TRPV1 (Fig. 6*B*). The effect of capsaicin in cells treated with H-89 and PAR_2-AP was $62 \pm 11\%$, and in cells treated with $PKAI_{14\text{–}22}$ and PAR_2-AP was $53 \pm 4\%$ of the responses of cells treated with PAR_2-AP and vehicle (100%) (both $n > 100$ cells). Together, these results suggest that activation of PAR_2 sensitizes TRPV1-mediated increases in $[Ca^{2+}]_i$ by PKCε-and PKA-dependent mechanisms.

By recording whole-cell currents in HEK-TRPV1 cells, we found that pretreatment with PAR_2-AP (100 μM) resulted in a threefold increase in the current induced by capsaicin (300 nM) applied 30 s later, indicative of TRPV1 sensitization (Fig. 7*A*). This sensitization was maximal when capsaicin was applied 3 min after PAR_2-AP when the capsaicin current was increased by 440% ($n = 10$), and sensitization was sustained for at least 18 min (Fig. 7*B*). Repeated applications of capsaicin alone over the same time period resulted in reproducible responses, with no significant change in magnitude of TRPV1 currents ($n = 5$; data not shown). Thus, TRPV1 did not desensitize in response to repeated challenges with this concentration of capsaicin. When PKCεI (200 μM) or H-89 (3 μM) were included in the pipette, they markedly inhibited the PAR_2-mediated sensitization (Fig. 7*C*). Pretreatment with the PKC agonist phorbol dibutyrate (300 nM) also sensitized TRPV1 currents, causing a fourfold increase in capsaicin-evoked current at 3 min. However, this sensitization was not inhibited by H-89, demonstrating that the effect of H-89 did not result from non-selective inhibition of PKC pathways (phorbol dibutyrate = $429 \pm 126\%$ increase; phorbol dibutyrate + H-89 = $685 \pm 126\%$ increase; $n = 8$). Thus, PAR_2 sensitizes TRPV1 currents in HEK-TRPV1 cells by PKCε and PKA-dependent mechanisms.

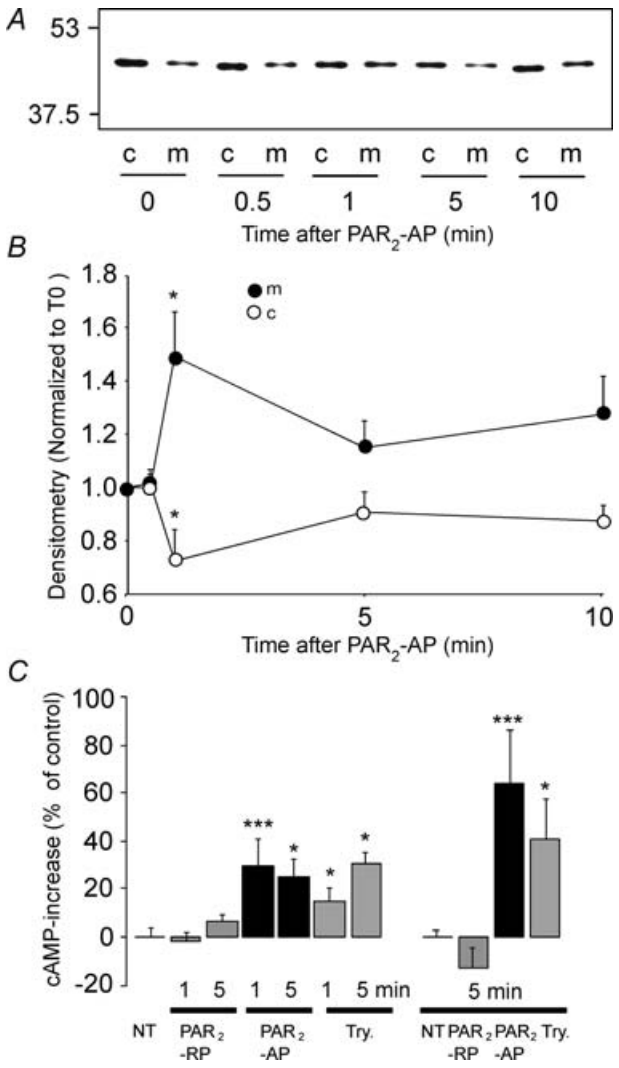

Figure 5. Effects of PAR_2 agonists on the subcellular distribution of PKA_C (catalytic subunit) in HEK 293 cells and on cAMP levels
A, Western blot, and *B*, densitometric analysis of PKA_C in cytosolic (c) and membrane (m) fractions of HEK-TRPV1 cells. PAR_2-AP caused an increase in PKA_C in membrane fractions and a decrease in cytosolic fractions. $^*P < 0.05$ compared to 0 min, $n = 4$ experiments. *C*, effects of PAR_2 agonists on cAMP level in HEK-PAR_2 cells (left) and DRG neurons (right). Try. = trypsin. PAR_2-AP and trypsin caused an increase in cAMP levels after 1 and 5 min. $n = 3$ experiments. $^*P < 0.05$ compared to non-treated cells (NT), $^{***}P < 0.05$ compared to PAR_2-RP.

PAR_2 agonists sensitize TRPV1 to induce thermal hyperalgesia by PKCε- and PKA-dependent mechanisms

We have previously reported that the co-administration of non-algesic doses of PAR_2-AP and capsaicin induces thermal hyperalgesia (Amadesi *et al.* 2004). Antagonism or deletion of TRPV1 prevents this response, indicating that PAR_2 sensitizes TRPV1 to cause thermal hyperalgesia.

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Using the same experimental approach, we now sought to investigate the role of PKCε and PKA in PAR$_2$-induced sensitization of TRPV1-dependent thermal hyperalgesia. By measuring the paw withdrawal latency to radiant heat in mice, we found that co-injection of non-algesic doses of PAR$_2$-AP (1 ng paw^{-1}) and capsaicin (1 μg paw^{-1}) caused hyperalgesia, as indicated by a decrease in the paw withdrawal latency (Fig. 8*A* and *B*). This hyperalgesia was detected within 15 min and sustained for at least 120 min. Thus, as previously reported (Amadesi *et al.* 2004), PAR$_2$-AP can sensitize TRPV1-induced thermal hyperalgesia. To determine the contribution of PKCε and PKA to this effect, we administered selective inhibitors (Aley *et al.* 2000). Intraplantar injection of PKCεI (1 μg paw^{-1}), a selective inhibitor of PKCε translocation, alone did not affect the basal withdrawal latency (Fig. 8*A*). However, PKCεI strongly inhibited the hyperalgesic effect of PAR$_2$-AP and capsaicin at 15 and 30 min, and abolished hyperalgesia at 60 and 120 min (Fig. 8*A*). The inactive PKCεI-sc (1 μg paw^{-1}) had no effect on the response to co-injection of PAR$_2$-AP and capsaicin (Fig. 8*A*). The intraplantar injection of WIPTIDE (1 μg paw^{-1}), a selective PKA inhibitor, alone had no significant effect on the basal withdrawal latency (Fig. 8*B*). However, WIPTIDE abolished the hyperalgesic effect of PAR$_2$-AP and capsaicin at 30–120 min (Fig. 8*B*). Thus, PAR$_2$ sensitizes TRPV1 to induce thermal hyperalgesia by a mechanism that requires activation of both PKCε and PKA.

Discussion

It is well established that certain proteases can cleave PAR$_2$ on sensory neurons to stimulate the release of substance P and calcitonin gene-related peptide, thereby causing neurogenic inflammation and both thermal and mechanical hyperalgesia (Steinhoff *et al.* 2000; Vergnolle *et al.* 2001). It is also known that PAR$_2$-induced thermal hyperalgesia is dependent on sensitization of TRPV1 (Amadesi *et al.* 2004; Dai *et al.* 2004). However, the molecular mechanisms by which PAR$_2$ sensitizes TRPV1 to cause thermal hyperalgesia are not fully understood. Our results show, for the first time, that (1) PAR$_2$ is co-expressed

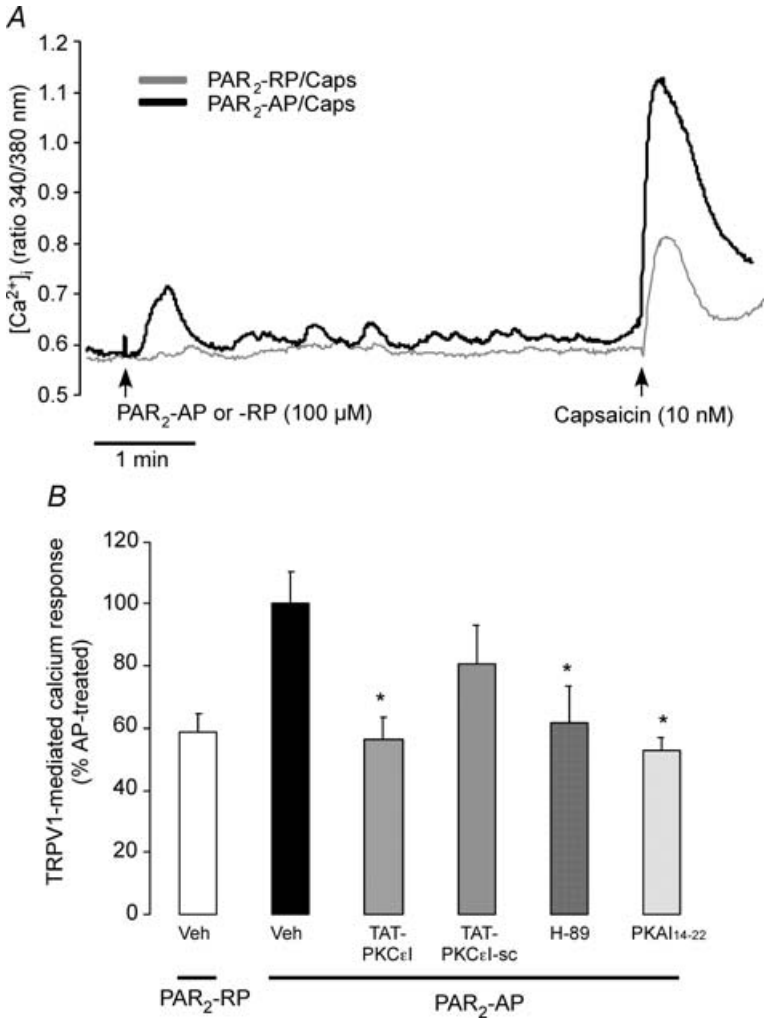

Figure 6. Effects of PAR$_2$ agonists on capsaicin-induced Ca^{2+} signalling in HEK-TRPV1 cells
Cells were exposed to PAR$_2$-AP or PAR$_2$-RP for 5 min, and then challenged with capsaicin. *A*, changes in $[Ca^{2+}]_i$, with each line the average trace from $n = 31$–35 cells. *B*, the effects of antagonists on PAR$_2$-induced sensitization of capsaicin responses (100%). Veh = vehicle control. $^*P < 0.05$ compared to PAR$_2$-AP/vehicle cells. $n = 70$–80 cells from 3 experiments.

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with PKCε and PKA in small- and medium-diameter primary sensory neurons that transmit pain; (2) PAR_2 agonists activate PKCε and PKA, causing their translocation to the plasma membrane in cell lines and DRG neurons and that PAR2 agonists activate the cAMP/PKA cascade in these cells; (3) activation of PKCε and PKA is required for the PAR_2-mediated sensitization of TRPV1 Ca^{2+} signals and currents; and (4) PKCε and PKA mediate PAR_2-induced sensitization of TRPV1 *in vivo,* and are thus responsible for the resultant thermal hyperalgesia.

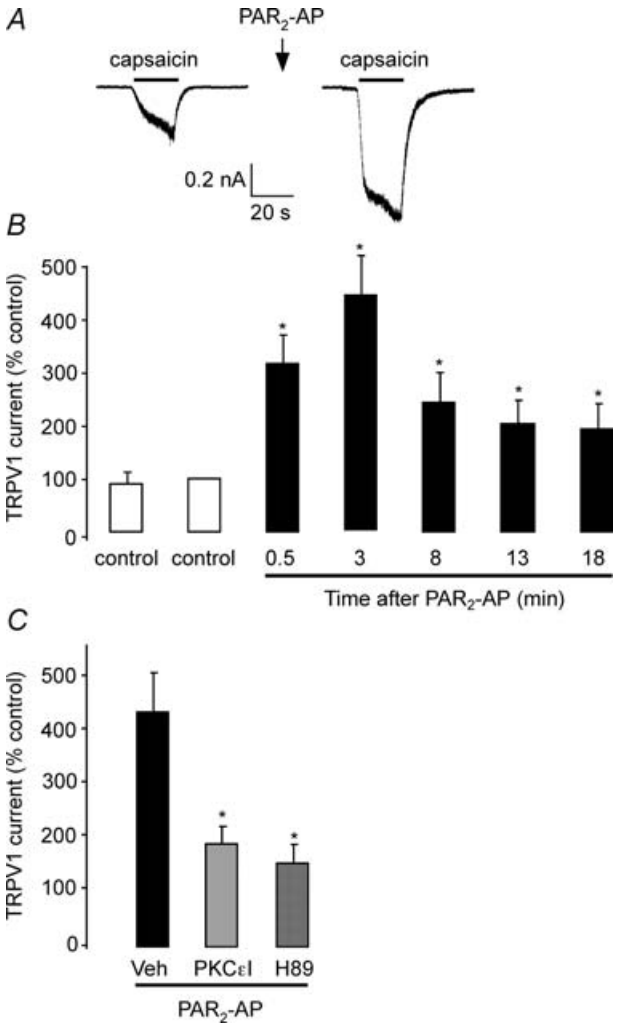

Figure 7. PKCε and PKA antagonists inhibit the PAR_2-mediated sensitization of TRPV1 currents in HEK-TRPV1 cells
A, whole-cell inward currents induced by capsaicin (300 nM) before (left trace) and 3 min after (right trace) application of PAR_2-AP for 2 min. *B*, summary of mean TRPV1 currents over time following activation of PAR_2 with PAR_2-AP (100 μM; $n \geq 5$ for each time point). PAR_2-AP sensitized the capsaicin-induced current in a time-dependent manner; reaching its maximum value 3 min after PAR_2-AP treatment ($P < 0.01$; $n = 10$). Membrane current values are expressed as percentage of the control current recorded prior to PAR_2-AP application (second control bar). In five control cells (first control bar), capsaicin was applied for ~20 s and re-applied 3 min later (second control bar), demonstrating the consistency of the capsaicin response. In another five control cells, capsaicin was only applied once before application of PAR_2-AP. *C*, TRPV1 sensitization by PAR_2-AP (Veh = vehicle control $n = 10$, at 3 min) was prevented by PKCε (PKCεI, 200 μM, $n = 5$) and PKA (H89, 3 μM, $n = 5$) inhibitors. $^*P < 0.05$.

Several observations from the current investigation suggest that PAR_2 agonists activate PKCε and PKA. We found that small- to medium-diameter neurons, which are known to express PAR_2 and TRPV1 (Amadesi *et al.* 2004), also contain PKCε and PKA. By a combination of confocal microscopy, subcellular fractionation and Western blotting, we observed that PAR_2 agonists promoted translocation of PKCε and the catalytic subunit of PKA, which represents the active subunit of the PKA heterotetramer, from the cytosol to the plasma membrane in both DRG neurons and HEK 293 cells. PMA and forskolin, established activators of PKC and PKA, had similar effects on the subcellular localization of these kinases. Thus, translocation of kinases from the cytosol to the plasma membrane can be used in our system as an indicator of kinase activation. Our results show that PKCε and PKA_C are not only appropriately colocalized with PAR_2 and TRPV1 in nociceptive neurons, but that PAR_2 activates these kinases. Our findings are consistent with other reports of the localization of PAR_2, PKCε and PKA_C in primary sensory neurons. PAR_2 is expressed by > 50% of small- to medium-diameter neurons of rat DRG (Steinhoff *et al.* 2000), and PKCε is present in 90% (Khasar *et al.* 1999) and PKA_C in 50% (Rathee *et al.* 2002) of these neurons. We found that PAR_2 agonists caused redistribution of kinases in ~60% of cells expressing PKCε and in ~80% of cells expressing PKA.

It is well established that PAR_2 couples to $G_{\alpha q/11}$, resulting in activation of phospholipase Cβ and generation of 1,4,5-inositol trisphosphate and diacylglycerol, which would be expected to mobilize intracellular Ca^{2+} ions and activate PKC (Ossovskaya & Bunnett, 2004). Therefore, PAR_2-induced activation of PKCε is consistent with the known signalling pathway of this receptor. Moreover, our results are in line with observations that agonists of PAR_2 induce activation and membrane translocation of PKCε in prostate stromal cells (Myatt & Hill, 2005). Given the predominant coupling of PAR_2 to $G_{\alpha q/11}$, the observation that PAR_2 agonists caused membrane translocation of PKA_C was unanticipated. However, we observed that PAR_2 agonists stimulated generation of cAMP in DRG neurons and HEK 293 cells, suggesting that PAR_2 activates the cAMP/PKA cascade. Our results are in agreement with the observations that PAR_2 agonists elevate cAMP levels in keratinocytes (Scott *et al.* 2003), and that PAR_2 activation causes release of von Willebrand factor in endothelial cells by a PKA-dependent mechanism (Cleator *et al.* 2006). Thus, PAR_2, like PAR_1, may couple to different heterotrimeric G proteins and thereby regulate multiple signalling pathways, including mobilization of intracellular Ca^{2+} ions and generation of cAMP

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(Ossovskaya & Bunnett, 2004). Alternatively, PAR$_2$ agonists could induce release of mediators that act in an autocrine manner to generate cAMP and activate PKA. One possible mediator is PGE$_2$, since PAR$_2$ agonists promote PGE$_2$ release from epithelial cells (Kong *et al.* 1997) and PGE$_2$ sensitizes TRPV1 through a PKA-dependent mechanism (Lopshire & Nicol, 1998; Rathee *et al.* 2002). However, we found that indomethacin did not prevent the effects of PAR$_2$ agonists on cAMP generation. Further studies are thus required to determine the mechanism by which PAR$_2$ agonists activate PKA in neurons.

Upon translocation to the plasma membrane, activated PKCε and PKA may phosphorylate and thereby sensitize ion channels that participate in nociception, such as TRPV1. Several observations from our investigation support this possibility. Firstly, we observed that PAR$_2$ agonists sensitized capsaicin-induced increases in $[Ca^{2+}]_i$ and capsaicin currents in HEK-TRPV1 cells, and we and others have previously reported similar sensitization in DRG neurons (Amadesi *et al.* 2004; Dai *et al.* 2004). Secondly, the co-injection of non-algesic doses of PAR$_2$ and capsaicin induced sustained thermal hyperalgesia, which we have shown to depend on sensitization of TRPV1 (Amadesi *et al.* 2004). Finally, selective antagonists of PKCε and PKA, including those that prevent membrane translocation of these kinases, prevented the PAR$_2$-induced sensitization of TRPV1-dependent Ca^{2+} signalling, TRPV1 currents and TRPV1 thermal hyperalgesia. These results are supported by the observation that PAR2 agonists phosphorylate TRPV1 in HEK 293 cells (Amadesi *et al.* 2004). Our observation that PKCε makes an important contribution to PAR$_2$-induced sensitization of TRPV1 is in agreement with the report that PKCε antagonists suppress the effects of PAR$_2$ agonists on TRPV1 Ca^{2+} signalling and TRPV1 currents (Amadesi *et al.* 2004; Dai *et al.* 2004). However, our results extend this report by showing that PAR$_2$ activates a specific PKC isozyme, and by demonstrating a major role for both PKCε and PKA in PAR$_2$-induced thermal hyperalgesia.

The precise signal transduction pathway by which PAR$_2$ activates PKCε and PKA to sensitize TRPV1 remains to be elucidated. PKCε and PKA can both directly phosphorylate TRPV1 (Bhave *et al.* 2002; Numazaki *et al.* 2002; Mohapatra & Nau, 2003). However, after activation

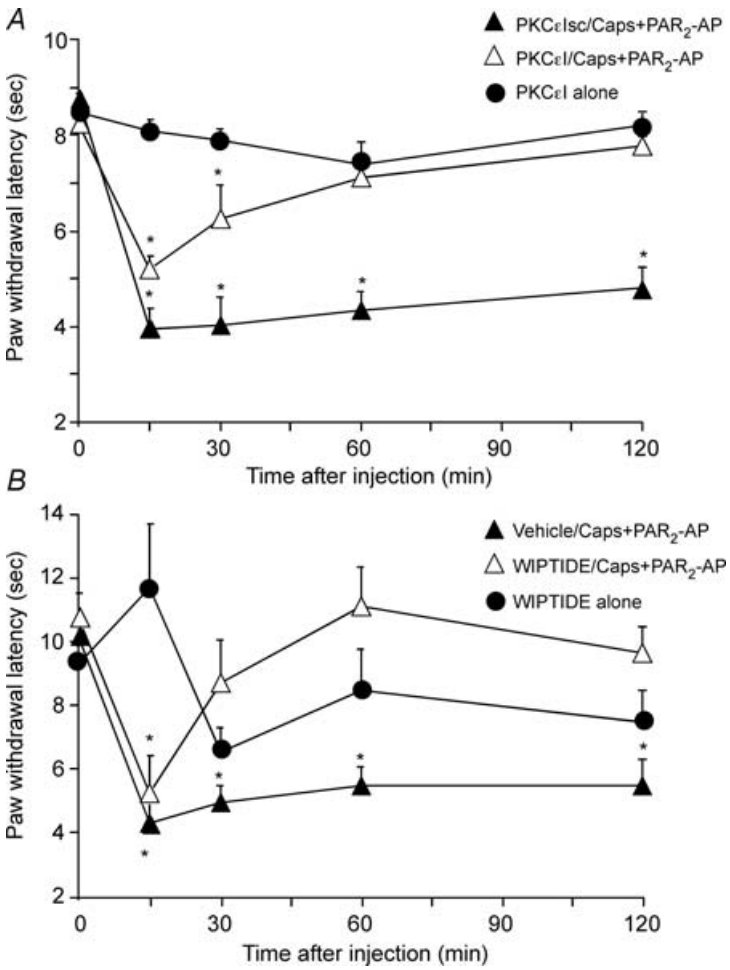

Figure 8. Mechanisms of PAR$_2$-induced potentiation of TRPV1-mediated thermal hyperalgesia
Compounds were injected into the paws of mice, and hyperalgesia was measured as a significant decrease in withdrawal latency in response to a thermal stimulus, compared to basal (time 0). *A*, effects of the PKCε inhibitor (PKCεI) alone or on PAR$_2$-AP and capsaicin-induced hyperalgesia. *B*, effects of the PKA inhibitor (WIPTIDE) alone or on PAR$_2$-AP and capsaicin-induced hyperalgesia. Controls include use of inactive PKCεI-sc or the WIPTIDE vehicle. $n = 8$ mice per group, $^*P < 0.05$ compared to basal values.

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of PAR_2, we do not know if PKCε and PKA regulate TRPV1 in an additive or synergistic manner, or whether one pathway is upstream of the other. When administered separately, we observed that PKCε and PKA inhibitors almost completely prevented PAR_2-induced sensitization of TRPV1 Ca^{2+} signals and currents. Moreover, PKCε and PKA inhibitors both prevented PAR_2-induced thermal hyperalgesia at later times (> 30 min). Together, these results suggest the activation of tandem and possibly redundant PKCε and PKA pathways. However, at earlier times (< 30 min), PKCε and PKA inhibitors, when given separately, did not prevent PAR_2-induced thermal hyperalgesia. Thus, the relative contributions of PKCε and PKA may vary with time after activating PAR_2. This possibility is supported by the identification of a novel cAMP/PKCε signalling pathway that is responsible for the long-lasting latent susceptibility to hyperalgesia induced by an acute inflammatory stimulus (Parada *et al.* 2005) where an upstream release of cAMP and activation of the guanine exchange factor Epac, but not of PKA, are required to activate downstream PKCε (Hucho *et al.* 2005). Although our observations that PKA inhibitors prevented the PAR_2-induced sensitization of TRPV1 suggest a distinct role for PKA, in addition to the contribution of PKCε, further experiments are required to define the relative roles of these kinases at different times after activation of PAR_2.

In addition to the direct phosphorylation of TRPV1 by PKCε and PKA, other mechanisms may account for PAR_2-induced sensitization of this channel. PAR_2 agonists may also activate other second messenger kinases, such as protein kinase D, a substrate for PKCε (Rey *et al.* 2004; Wang *et al.* 2004) that can also phosphorylate and sensitize TRPV1 (Rey *et al.* 2004; Wang *et al.* 2004). Furthermore, PAR_2 agonists could regulate the subcellular distribution of TRPV1 rather than affecting gating of the channel. Indeed, phorbol esters, agonists of the metabotropic glutamate receptor and nerve growth factor sensitize neurons by promoting trafficking of TRPV1 to the plasma membrane (Morenilla-Palao *et al.* 2004; Zhang *et al.* 2005). These possibilities remain to be examined, and the existence of other mechanisms cannot be excluded.

Further studies are required to define the physiological relevance of PAR_2-induced hyperalgesia. In the present investigation, we studied the effects of a synthetic agonist, PAR_2-AP, on thermal hyperalgesia. However, we have previously reported that proteases such as tryptase and trypsin similarly cause hyperalgesia by activating PAR_2 (Vergnolle *et al.* 2001). Agents that degranulate mast cells also induce hyperalgesia by a PAR_2-dependent mechanism (Vergnolle *et al.* 2001). Although tryptase may mediate these effects, experiments with tryptase inhibitors are required to confirm this possibility. Extrapancreatic trypsins, such as trypsin IV, can activate PAR_2 (Cottrell *et al.* 2004), but their role in pain transmission remains to be defined. In the present study, we observed that PAR_2-AP-induced thermal hyperalgesia was sustained for 2 h, and we have previously reported detectable hyperalgesia 24 h after PAR_2 activation (Vergnolle *et al.* 2001). Although our results suggest that PKCε and PKA account for hyperalgesia at up to 120 min after activation, different mechanisms may mediate hyperalgesia at later times. Proteases activate PAR_2 in a catalytic and irreversible manner; once the receptor is cleaved the exposed tethered ligand is always available to activate PAR_2. Receptor phosphorylation by G protein receptor kinases and interaction with β-arrestins serve to uncouple receptors from heterotrimeric G proteins and desensitize G protein signalling (Bohm *et al.* 1996; Dery *et al.* 1999). However, β-arrestins also mediate receptor endocytosis and are scaffolds for mitogen-activated protein kinases, which permits internalized PAR_2 to continue to signal through this pathway (DeFea *et al.* 2000). The contribution of β-arrestins and mitogen-activated protein kinases to sustained hyperalgesia remains to be defined. Eventually, PAR_2 is targeted for degradation in lysosomes by a ubiquitin-mediated mechanism, which irrevocably terminates signalling (Jacob *et al.* 2005). In cell lines, resensitization of responses to proteases requires synthesis of new receptors and mobilization of prominent stores of PAR_2 from the Golgi apparatus. We have shown that PAR_2-AP causes redistribution of β-arrestin1-EGFP from the cytosol to the plasma membrane, followed by translocation of PAR_2-EGFP and β-arrestin1-EGFP from the plasma membrane to endosomes of DRG neurons (S. Amadesi & N. W. Bunnett, unpublished observations). However, the mechanisms of resensitization of PAR_2 in neurons, which may be required for the sustained hyperalgesic effects of PAR_2 agonists, are completely unknown.

Together, our results further clarify the signalling pathways by which PAR_2 regulates TRPV1 to induce hyperalgesia. We used a combination of experimental approaches, including localization of kinases in DRG, analysis of the subcellular distribution of PKCε and PKA_C, measurement of TRPV1 mediated Ca^{2+} signalling and currents, and behavioural studies in conscious animals, to reveal a major role of PKCε and PKA in PAR_2 signalling and PAR_2-induced sensitization of TRPV1. An understanding of the mechanisms by which proteases that activate PAR_2 sensitize ion channels such as TRPV1 is important for the development of novel therapies to treat pain.

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