Protease-activated Receptor 2 (PAR$_2$) Protein and Transient Receptor Potential Vanilloid 4 (TRPV4) Protein Coupling Is Required for Sustained Inflammatory Signaling*

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Daniel P. Poole,† Silvia Amadesi,‡ Nicholas A. Veldhuis,§ Fe C. Abogadie,*, TinaMarie Lieu†, William Darby‡, Wolfgang Liedtke‡, Michael J. Lew†, Peter McIntyre†§, and Nigel W. Bunnett‡

From the †Monash Institute of Pharmaceutical Sciences, Parkville, Victoria 3052, Australia, the ‡Department of Anatomy and Neuroscience, Section of Pharmacology, University of Melbourne, Parkville, Victoria 3010, Australia, the §School of Pharmacy, Reading University, Reading, RG6 6UR, United Kingdom, the **School of Medical Sciences and Health Innovations Research Institute, Royal Melbourne Institute of Technology University, Bundoora, Victoria 3083, Australia, and the ††Division of Neurology, Department of Medicine, Duke University, Medical Center, Durham, North Carolina 27710

Background: Receptors activate channels of sensory nerves to cause inflammation and pain by unknown mechanisms.

Results: Protease-activated receptor 2 (PAR$_2$) stimulated transient receptor potential vanilloid 4 (TRPV4) by generation of channel agonists. This required a key TRPV4 tyrosine and induced inflammation.

Conclusion: PAR$_2$ opens TRPV4 by functional coupling.

Significance: Antagonism of PAR$_2$-TRPV4 coupling could alleviate inflammation and pain.

G protein-coupled receptors of nociceptive neurons can sensitize transient receptor potential (TRP) ion channels, which amplify neurogenic inflammation and pain. Protease-activated receptor 2 (PAR$_2$), a receptor for inflammatory proteases, is a major mediator of neurogenic inflammation and pain. We investigated the signaling mechanisms by which PAR$_2$ regulates TRPV4 and determined the importance of tyrosine phosphorylation in this process. Human TRPV4 was expressed in HEK293 cells under control of a tetracycline-inducible promoter, allowing controlled and graded channel expression. In cells lacking TRPV4, the PAR$_2$ agonist stimulated a transient increase in [Ca$^{2+}$]i. TRPV4 expression led to a markedly sustained increase in [Ca$^{2+}$]i. Removal of extracellular Ca$^{2+}$ and treatment with the TRPV4 antagonists Ruthenium Red or HC067047 prevented the sustained response. Inhibitors of phospholipase A$_2$ and cytochrome P450 epoxyenase attenuated the sustained response, suggesting that PAR$_2$ generates arachidonic acid-derived lipid mediators, such as 5,6-EET, that activate TRPV4. Src inhibitor 1 suppressed PAR$_2$-induced activation of TRPV4, indicating the importance of tyrosine phosphorylation. The TRPV4 tyrosine mutants Y110F, Y805F, and Y110F/Y805F were expressed normally at the cell surface. However, PAR$_2$ was unable to activate TRPV4 with the Y110F mutation. TRPV4 antagonism suppressed PAR$_2$ signaling to primary nociceptive neurons, and TRPV4 deletion attenuated PAR$_2$-stimulated neurogenic inflammation. Thus, PAR$_2$ activation generates a signal that induces sustained activation of TRPV4, which requires a key tyrosine residue (TRPV4-Tyr-110). This mechanism partly mediates the proinflammatory actions of PAR$_2$.

Injury and inflammation trigger the activation of proteases from the circulation, immune cells, and epithelial tissues that regulate cells by cleaving protease-activated receptors (PARs)$^3$, members of a family of four G protein-coupled receptors (GPCRs) (1, 2). By cleaving PARs at specific sites within the extracellular N-terminal domains, proteases reveal tethered ligands that bind to and activate the cleaved receptors. Synthetic peptides that mimic the tethered ligand domains (PAR-activating peptides, APs) can directly activate PARs and are useful tools to probe receptor functions. Once activated, PARs regulate multiple pathophysiological processes, including inflammation, pain, hemostasis, and healing.

PAR$_2$ is coexpressed with substance P and calcitonin gene-related peptide by a subpopulation of primary spinal-afferent neurons that control neurogenic inflammation and pain transmission (3, 4). Activation of PAR$_2$ on sensory nerve endings evokes the local release of these neuropeptides, which stimulate extravasation of plasma proteins, infiltration of neutrophils, and vasodilation (neurogenic inflammation). PAR$_2$ activation also promotes the central release of neuropeptides that activate second-order spinal neurons that transmit pain. These mechanisms contribute to painful inflammation of the intestine (5, 6), pancreas (7, 8), and joints (9). Therefore, it is of considerable

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† Both authors contributed equally to this work.

‡ To whom correspondence should be addressed: Monash Institute of Pharmaceutical Sciences, 381 Royal Parade, Parkville, VIC 3052, Australia. Tel: office, 61-3-9903-9136 and mobile, 61-407-392-619; Fax: 61-3-9903-9581; E-mail: nigel.bunnett@monash.edu.

§ School of Medical Sciences and Health Innovations Research Institute, Royal Melbourne Institute of Technology University, Bundoora, Victoria 3083, Australia, and the ††Division of Neurology, Department of Medicine, Duke University, Medical Center, Durham, North Carolina 27710.
interest to understand the mechanisms by which PARs regulate the activity of nociceptive neurons.

Members of the transient receptor potential (TRP) family of ion channels, including TRP vanilloids 1 and 4 (TRPV1 and TRPV4) and TRP ankyrin 1 (TRPA1), mediate neurogenic inflammation and pain (10, 11) and are major downstream targets of PAR2 (12–19). Activation of these non-selective cation channels stimulates the influx of extracellular Ca2+ ions and the release of neuromodulators in peripheral tissues and the spinal cord, which induces neurogenic inflammation and pain. During injury and inflammation, several factors are generated that can directly activate these channels (10, 11). Elevated temperatures, protons, and lipid mediators activate TRPV1 (20–22); mechanical shear stress, osmotic stimuli, and lipid mediators activate TRPV4 (23–25); and products of reactive oxygen species and reactive prostaglandin metabolites activate TRPA1 (26, 27). However, indirect mechanisms, particularly those triggered by GPCRs, play a prominent role in TRP channel activation. Many GPCRs that induce neurogenic inflammation and pain indirectly regulate TRP channels, which mediate their proinflammatory and pronociceptive actions. Sensitization, whereby pre-treatment with a GPCR agonist amplifies responses to a TRP channel agonist, is a well recognized mechanism of indirect regulation. For example, agonists of PAR2 (12–19), bradykinin-B2 (22, 28), histamine H3 (16, 29), neurokinin NK2 (30) and NK3 (31), prostaglandin E2 (32, 33), prokineticin PKR1 and PKR2 (34), and purinergic P2Y1 and P2Y2 receptors (35, 36) sensitize TRP channels. The mechanisms by which GPCRs sensitize TRPs are not fully understood. However, in a manner that is reminiscent of the process by which rhodopsin sensitizes ancestral TRP channels in the Drosophila eye (37), a major mechanism depends on phospholipase C-mediated cleavage of phosphatidylinositol 4,5 bisphosphate in the plasma membrane (17, 38, 39). This mechanism relieves tonic TRP inhibition and activates protein kinases A and C, which phosphorylate TRPs and modify channel gating (12, 22, 40, 41).

In addition to sensitization, emerging evidence suggests that GPCR signaling can directly activate TRP channels. Responses of dorsal root ganglion (DRG) neurons to bradykinin and histamine are largely dependent on Ca2+ influx through TRPV1 (28, 42). Products of phospholipase A2 (PLA2) and lipoxygenase can directly activate TRPV1, including N-arachidonoylethanalamide, which is derived from membrane phospholipids, and hydroperoxy or hydroxy eicosatetraenoic acids and leukotriene B4 products of the lipoxygenase-dependent metabolism of arachidonic acid (43). Diacylglycerol directly activates TRPV1 through a kinase-independent mechanism that underlies cellular responses to Mn2+ muscarinic and glutamate mGlur5 agonists (44, 45). 5-Hydroxytryptamine and acetylcholine activate TRPV4 by a mechanism that is dependent on generation of epoxygenase-dependent catalysis of arachidonic acid (46–48).

We have identified a new mechanism by which PAR2 activators TRPV4 channels. Although TRPV1, TRPV4, and TRPA1 mediate the pronociceptive actions of PAR2 (12–19), the mechanisms underlying the functional interactions between TRPs and PARs are not fully understood. HEK293 cells endogenously express PAR2 (49) and respond robustly to PAR2 stimuli, including trypsin or the tethered ligand mimetic peptide, SLIGRL-NH2, with a rapid, transient increase in intracellular calcium ([Ca2+]i). By modulating the expression of TRP channels, we report the novel finding that TRPV4, but not TRPA1, contributes to the cellular response to PAR2 agonists in HEK cells. This mechanism involves PLA2- and cytochrome P450 epoxyenase-dependent catalysis of arachidonic acid and requires the phosphorylation of a key TRPV4 tyrosine residue (Tyr-110) that has been implicated in sensitization of TRPV4 (50). We propose that the activation of TRPV4 serves to prolong PAR2 signaling and to amplify its proinflammatory and pronociceptive actions. These findings have implications for the consequences of protease-dependent PAR2 activation during injury and inflammation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The following reagents were used: mouse/rat PAR2-AP (SLIGRL-NH2) (CPC Scientific, San Jose, CA); 4α-phorbol didecanoate (4α-PDD) and bisindolylmaleimide I (BIM-1) (Calbiochem, La Jolla, CA); 17-octadecenoic acid (17-OYA) and methyl arachidonyl fluorophosphonate (MAFP) (Enzo Life Sciences, Waterloo, Australia); HC067047 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and capsaicin, GSK1016790A, indomethacin, Ruthenium Red, trypsin, Src inhibitor 1, and ATP (Sigma-Aldrich, St. Louis, MO).

**Site-directed Mutagenesis**—Tyrosines 110, 805, and 110/805 of human TRPV4 were mutated to phenylalanine using the QuikChange™ site-directed mutagenesis kit (Stratagene, Santa Clara, CA) according to the directions of the manufacturer. The following primer sequences were used: hTRPV4 Y110F, ATGGACCTCAGTTGACCTATGCTACACT (forward) and AGTGTTGAGATTGTTGCC- AAAGTCAAACAGTGAGTCCAT (reverse); hTRPV4 Y805F, TCACCACT (forward) and AGTGGTGACGATAGGTGCC- TCACACT (reverse); hTRPV4 Y110/805F, AGTGGTGACGATAGGTGCC-TCACACT (forward) and GAAGCCATAATCTGGTAGGTCTCATTCTT (reverse). Sequences were confirmed by automated DNA sequencing.

**Cell Lines**—HEK293 cells stably expressing human TRPV4 or rat TRPA1 were generated using a tacrycline-inducible system as described (18, 26, 51). Briefly, Flp-In™ T-Rex™ HEK293 cells were transfected with pcDNAs/FRT/TO containing human TRPV4 (HEK-TRPV4 cells, TRPV4), GSK1016790A, indomethacin, Ruthenium Red, trypsin, Src inhibitor 1, and ATP (Sigma-Aldrich, St. Louis, MO).

**Animals**—Rats (Sprague-Dawley, 200–250 g, male) and mice (C57Bl/6, male) were used. Procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco or the University of Melbourne Animal Experimentation Ethics Committee.
**PAR2 and TRPV4 Coupling**

**Immunofluorescence**—HEK-TRPV4-HA cells (2.5 × 10⁵/35-mm dish) were plated onto poly-D-lysine-coated coverslips (100 μg/ml). Cells were incubated with graded concentrations of tetracycline (0–0.1 μg/ml, 16 h). Cells were washed with PBS (pH 7.4) and fixed (4% paraformaldehyde, 100 mM PBS (pH 7.4), 20 min on ice). Cells were incubated in blocking buffer (1% normal horse serum in PBS with 0.1% saponin, 3 × 10 min) and then incubated with rat anti-hemagglutinin (1:1,000, overnight, 4 °C; clone 3F10, Roche). Cells were washed and incubated with donkey anti-rat IgG coupled to Rhodamine Red-X (1:200, 2 h, 4 °C; clone 3F10, Roche). Cells were washed and incubated with donkey anti-rat IgG coupled to Rhodamine Red-X (1:200, 2 h, room temperature; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Slides were examined using a Zeiss LSM510 Meta confocal microscope (Carl Zeiss Inc., Thornwood, NY). Images were acquired using Zeiss Neofluar ×40 (numerical aperture 1.3) or Fluar Plan Apochromat ×63 (numerical aperture 1.4) objectives.

**Measurement of [Ca²⁺]i in HEK Cells**—For population studies, HEK cells were seeded onto 96-well plates (25,000 cells/well) coated with poly-D-lysine (100 μg/ml) and cultured for 48 h. Cells were loaded with Fura2-AM ester (2.5 μM, 30 min, 37 °C) in Hanks’ balanced salt solution (HBSS) containing 20 mM HEPES and 0.1% BSA. Fluorescence was measured (4-s intervals) at 340 nm and 380 nm excitation and 510 nm emission wavelengths using a FlexStation 3 plate reader (Molecular Devices, Sunnyvale, CA) as described (53). In some experiments, cells were incubated in Ca²⁺-free HBSS containing EDTA (20 mM). Results were expressed as the 340/380-nm fluorescence emission ratio, which is proportional to the [Ca²⁺]i. For measurement of [Ca²⁺]i in individual cells, HEK cells were studied at 37 °C using a Leica DMI-6000B imaging system (Leica, Germany) with ×10 or ×20 objectives. Images were collected (5 s intervals) at 340 nm and 380 nm excitation and 510 nm emission wavelengths. Images were processed using ImageJ software with McMaster Biophotonics Facility plug-ins (vl.46b) as described (53). Cells were challenged with PAR2-AP (50 μM) or trypsin (300 units/ml). In some experiments, cells were preincubated for 30 min with MAFP (1 μM), PLA₂ inhibitor, 17-ODYA (50 μM, cytochrome P450 epoxygenase inhibitor), indomethacin (50 μM, cyclooxygenase inhibitor), BIM-1 (100 nM, PKC inhibitor), or Src inhibitor 1 (Src1, 10 μM, Src family kinase inhibitor) 30 min before stimulation. Cells were also assayed in Ca²⁺-free HBSS containing EDTA (20 mM) or after treatment with Ruthenium Red (10 μM, TRPV antagonist).

**Measurement of [Ca²⁺]i in DRG Neurons**—Rats were anesthetized with isoflurane and killed by bilateral thoracotomy. DRG were collected from all spinal levels and cultured as described (13, 53). Neurons were plated onto glass coverslips coated with poly-D-lysine and laminin (100 μg/ml) and cultured for 48–72 h. Neurons were loaded with Fura2-AM ester (2 μM, 30 min, 37 °C) in HBSS, washed, and incubated in HBSS for 30 min before study. Responses of individual neurons to agonists were measured by microscopy as described for HEK cells. Neurons were challenged sequentially with trypsin (300 units/ml), GSK1016790A (10 nM, TRPV4 agonist), capsaicin (100 nM, TRPV1 agonist), and KCl (50 mM). In some experiments cells were pretreated with HC067047 (10 μM, TRPV4 antagonist) 30 min prior to addition of agonists or were assayed in Ca²⁺-free HBSS.

**Analysis of Ca²⁺ Signals**—The transient Ca²⁺ response induced by PAR2-AP challenge of HEK cells was calculated as the difference between the basal 340/380-nm fluorescence emission ratio (average of four readings immediately prior to application of PAR2-AP) and the maximal fluorescence that was measured 10–20 s after PAR2-AP application. The sustained Ca²⁺ response was calculated as the difference between the basal fluorescence and the fluorescence measured at 50–60 s after PAR2-AP application. The results were expressed as a ratio of the transient to the sustained response. A diagram illustrating these measurements is presented in Fig. 2B. Representative traces were obtained by averaging values recorded from three different wells with paired vehicle and treated groups. At least three technical repeats were performed for each experiment.

For analysis of DRG, Ca²⁺ responses were included of cells that responded to K⁺ stimulation with an increase in 340/380-nm fluorescence emission ratio of > 0.1 units above the initial base line. The trypsin-responsive population was subdivided further on the basis of the magnitude of responses to GSK1016790A, with lower and upper quartiles designated as “low” and “high” responders, respectively. At least 640 neurons were analyzed (n = 5 independent cultures) per treatment group.

**Cell Surface Biotinylation**—Cell surface labeling was performed with EZLink sulfo-NHS-LC-biotin (Pierce) as described in detail (54).

**Western Blotting**—Proteins were resolved in Criterion 4–15% Tris-glycine gels (Bio-Rad), electroblotted onto nitrocellulose membrane (Protran, Whatman, Rydalmere, Australia), and blocked in 5% skim milk/Tris-buffered saline + 0.05% Tween 20 (TBS-T). Membranes were probed with rabbit anti-TRPV4 antibody (1:1,000 in 5% skim milk/TBS-T overnight, 4 °C; Abcam, Waterloo, Australia) and then washed and incubated with IRDye 800 donkey anti-mouse IgG (1:5,000, 1 h, room temperature; Li-Cor Biosciences, Lincoln, NE). Membranes were washed and analyzed using an Odyssey infrared imager (Li-Cor Biosciences). Signal density was quantified using ImageJ software.

**Assessment of Inflammation**—Mice were anesthetized with isoflurane (2%), and baseline paw thickness was measured using a digital caliper (Mitutoyo, Aurora, IL). PAR2-AP (50 μg/paw) or 0.9% NaCl (50 μl) were administered by intraplantar injection. The paw thickness was measured from 30–180 min after injection. In some experiments, mice were treated with 17-ODYA (5 mg/kg, 150 μl, intraperitoneal) or vehicle (25% DMSO, 0.9% NaCl, 150 μl, intraperitoneal) 30 min before the intraplantar injections. The paw thickness was normalized to base line (0 min). Mice were killed 6 h after the injection. Paws were collected, snap-frozen in liquid nitrogen, and assessed for tissue myeloperoxidase (MPO) activity as described (55). MPO was solubilized with hexadecyltrimethylammonium bromide, and MPO activity was measured with a diansidine-H₂O₂ assay. Changes in absorbance at 450 nm over a 15-min period were determined using a microplate reader (Molecular Devices).
Data were expressed as MPO activity relative to total protein (units/mg) and normalized to controls.

**Statistical Analysis**—Results were expressed as the mean ± S.E. and were compared by Student’s or one-sample t test (one-tailed) or one-way analysis of variance and Newman-Keuls test, as indicated, using GraphPad Prism (v5.0). Differences were considered significant when p < 0.05.

**RESULTS**

**PAR<sub>2</sub> Couples to TRPV4, Which Mediates Influx of Extracellular Ca<sup>2+</sup> Ions**—We confirmed that Flp-In<sup>TM</sup> T-Rex<sup>TM</sup> HEK293 cells express endogenous PAR<sub>2</sub> by examining the effects of graded concentrations of PAR<sub>2</sub>-AP (SLIGRL-NH<sub>2</sub>) on [Ca<sup>2+</sup>]<sub>i</sub>. PAR<sub>2</sub>-AP caused a transient and concentration-dependent increase in [Ca<sup>2+</sup>]<sub>i</sub> with a pEC<sub>50</sub> (negative logarithm of the EC<sub>50</sub>) of 4.67 ± 0.15 and a maximal response to 100 μM (Fig. 1, A and B).

To examine the effect of TRPV4 expression on PAR<sub>2</sub>-evoked Ca<sup>2+</sup><sup>+</sup> signaling, we generated HEK cell lines expressing TRPV4 with or without an HA tag. TRPV4 was expressed under control of a tetracycline-inducible promoter, which enabled controlled expression of the channel. The expression of TRPV4 was examined by measuring changes in [Ca<sup>2+</sup>]<sub>i</sub> in response to the TRPV4 activator 4α-PDD and by immunofluorescence and confocal microscopy using an antibody to the HA.11 epitope. In HEK-TRPV4 cells not exposed to tetracycline, 4α-PDD had no effect on [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 1C), and immunoreactive TRPV4 was undetectable (D). Incubation of HEK-TRPV4 cells with graded concentrations of tetracycline (0.0001–0.1 μg/ml) induced graded expression of functional and immunoreactive TRPV4. In tetracycline-treated cells, 4α-PDD elicited a gradual increase in [Ca<sup>2+</sup>]<sub>i</sub> that was sustained for the period of observation (200 s) and was graded with the concentration of tetracycline (Fig. 1C). Responses to 4α-PDD were detected after incubation with 0.001 μg/ml tetracycline and were maximal after 0.1 μg/ml tetracycline. The basal [Ca<sup>2+</sup>]<sub>i</sub> was also elevated by the highest expression levels of TRPV4 (0.01, 0.1 μg/ml), although the cells were microscopically normal and remained responsive to TRPV4 and PAR<sub>2</sub> agonists. Immunoreactive TRPV4 was detected in some cells after incubation with 0.001 μg/ml tetracycline and present at the plasma membrane of all cells after incubation with 0.1 μg/ml tetracycline (Fig. 1D).

In HEK control cells (expressing the empty vector without a TRPV4 insert), PAR<sub>2</sub>-AP (50 μM) evoked a transient increase in [Ca<sup>2+</sup>]<sub>i</sub> that was maximal after 10–20 s and that declined to baseline after ~75 s of stimulation (Fig. 2A). In tetracycline-treated (0.1 μg/ml, 16 h) HEK-TRPV4 cells, PAR<sub>2</sub>-AP evoked a similar rapid increase in [Ca<sup>2+</sup>]<sub>i</sub> that was markedly sustained (Fig. 2A). The sustained response was quantified by determining the ratio of the [Ca<sup>2+</sup>]<sub>i</sub> at the maximal point of the transient phase (10–20 s) and during the sustained phase (50–60 s) (Fig. 2B). This analysis revealed that the magnitude of the sustained phase was proportional to the concentration of tetracycline and, thus, the level of TRPV4 expression (Fig. 2C). TRPV4 expression similarly enhanced the sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> to trypsin (300 units/ml), a physiologically relevant PAR<sub>2</sub> agonist (Fig. 2D).

To determine whether TRPV4 similarly affects signaling by other GPCRs, we examined the consequences of TRPV4 expression on responses to ATP, which mobilizes Ca<sup>2+</sup><sup>+</sup> in HEK293 cells by activating P2Y receptors (56). ATP (1 μM) evoked a rapid and transient increase in [Ca<sup>2+</sup>]<sub>i</sub> in HEK control cells (Fig. 2F). The magnitude and duration of the ATP-evoked Ca<sup>2+</sup><sup>+</sup> response were unaffected by TRPV4 expression (p = 0.6 and p = 0.7 to control cells, respectively). Thus, not all GPCRs can activate TRPV4.

To determine whether PAR<sub>2</sub> can activate other TRP channels, which would represent a more general mechanism of TRP regulation, we examined the effect of expression of TRPA1 on the responses to PAR<sub>2</sub>-AP. TRPA1 was selected because it is coexpressed with PAR<sub>2</sub> by nociceptive neurons and because
pretreatment with PAR2 agonists amplifies responses to TRPA1 agonists, which is indicative of channel sensitization. TRPA1 was expressed in HEK cells using a tetracycline-inducible promoter to allow for regulated expression. Tetracycline-induced expression of TRPA1 was confirmed by responsiveness to allyl-isothiocyanate or cinnamaldehyde (data not shown). In contrast to TRPV4, TRPA1 expression did not affect the amplitude or duration of the response to PAR2-AP relative to identically treated control cells (Fig. 2F). Thus, TRPA1 does not contribute to PAR2-evoked Ca\(^{2+}\) signaling.

Our results suggest that PAR2 can activate TRPV4, possibly by generating endogenous TRPV4 agonists or by activating signaling pathways that alter channel gating or localization. We refer to this activation as PAR2 “coupling” to TRPV4. The mechanism of this coupling is not related to the rapid mobilization of intracellular Ca\(^{2+}\) per se because ATP stimulation of P2Y receptors did not activate TRPV4 despite eliciting a substantial release of intracellular Ca\(^{2+}\) ions.

PAR2 Stimulates an Influx of Extracellular Ca\(^{2+}\) through TRPV4—To determine the source of the sustained TRPV4-dependent Ca\(^{2+}\) response and to further assess the involvement of TRPV4, we either removed extracellular Ca\(^{2+}\) ions or treated cells with the non-selective TRPV inhibitor Ruthenium Red.
Omission of extracellular Ca\(^{2+}\) abolished the sustained phase of the PAR2-AP response of tetracycline-induced HEK-TRPV4 cells (Fig. 4, A and B; \(p < 0.002\)). Ruthenium Red (10 \(\mu\)M) also abolished the sustained phase of the PAR2-AP response (Fig. 4, C and D). However, removal of extracellular Ca\(^{2+}\) or addition of Ruthenium Red had no effect on the transient phase of the response to PAR2-AP. These results suggest that PAR2 couples to TRPV4, which mediates an influx of extracellular Ca\(^{2+}\) ions that comprise the sustained phase of the PAR2 response.

PLA\(_2\) and Cytochrome P450 Epoxygenase Contribute to PAR\(_2\)-induced Activation of TRPV4—PAR2 couples to PLA\(_2\), which generates arachidonic acid (57), a substrate for synthesis of endogenous TRPV4 activators including 5,6-EET (25). We used a pharmacological approach to determine the contribution of PLA\(_2\) and downstream enzymes to PAR\(_2\)-dependent activation of TRPV4. The irreversible PLA\(_2\) inhibitor MAFP (1, 10 \(\mu\)M) inhibited both the transient and sustained phases of the response to PAR2-AP (Fig. 5, A and F), but lower concentrations of MAFP had no effect (data not shown). Thus, we could not ascribe a selective effect to inhibition of PLA\(_2\) by this compound. MAFP also slightly inhibited TRPV4 activation by the synthetic agonist GSK1016790A (100 nM, Fig. 5A, \(p = 0.045\)). The cytochrome P450 epoxygenase inhibitor 17-ODYA (50 \(\mu\)M) inhibited the sustained phase of the response to PAR2-AP without reducing the transient phase of the response (Fig. 5, B and F; \(p < 0.0001\)). 17-ODYA had a minor inhibitory effect on the response to the TRPV4 agonist 4\(\alpha\)PDD (100 nM), indicating that TRPV4 activity was mostly retained (Fig. 5B). Inhibition of cyclooxy-
genase by indomethacin (50 μM) did not alter the responses to PAR2-AP (p = 0.27; Fig. 5, C and F). Thus, PAR2 coupling to TRPV4 appears to involve activation of cytochrome P450 epoxygenase, which can generate arachidonic acid-derived TRPV4 activators such as 5,6-EET.

Certain GPCRs, including PAR2, can sensitize TRP channels through activation of second messenger kinases, including PKC, which can phosphorylate TRPs and regulate channel gating (12, 13, 16–18). Therefore, we examined the effects of kinase inhibitors on PAR2-AP-evoked changes in [Ca^{2+}]i in HEK-TRPV4 cells (0.1 μg/ml tetracycline). A, the PLA2 inhibitor MAFP inhibited the transient and sustained phases of the Ca^{2+} response to PAR2-AP and also had a small inhibitory effect on the response to GSK1016790A. B, the cytochrome P450 epoxygenase inhibitor 17-ODYA inhibited the sustained phase of the Ca^{2+} response to PAR2-AP and also had a small inhibitory effect on the response to 4α-PDD. C and D, the cyclooxygenase 1/2 inhibitor indomethacin (C) and PKC inhibitor BIM-1 (D) did not reduce the sustained phase of the Ca^{2+} response to PAR2-AP. E, the Src family inhibitor Src1 did not affect the transient response to PAR2-AP but reduced the sustained phase in TRPV4 cells compared with vehicle. F, the summarized data demonstrate the effects of inhibitors on the sustained phase by comparing the sustained:transient ratio for inhibitors and matching vehicle treatments. "Control" sustained: transient data compare HEK control and TRPV4-HEK cells (0.1 μg/ml tetracycline) from Fig. 2A. * p < 0.05; ** p < 0.01; *** p < 0.001 relative to vehicle; Student’s t test; n = 3 experiments.

These data suggest an involvement of tyrosine kinases in the generation of the sustained phase of the PAR2 response, presumably through modulation of TRPV4 by phosphorylation of Tyr-110, which we have identified as a key residue required for the TRPV4-dependent response to PAR2 activation.

Tyrosine 110 Is Required for TRPV4 Activation—To identify the putative sites of tyrosine phosphorylation, we mutated tyrosine residues within the N and C termini of TRPV4 (Y110F, Y805F, and Y110/805F). Cell surface biotinylation and Western blotting for TRPV4 indicated that wild-type TRPV4 and all TRPV4 mutants were expressed at similar levels at the cell surface (Fig. 6A). PAR2-AP induced a rapid and transient increase in [Ca^{2+}]i that was similar in cells expressing wild-type and mutant TRPV4 channels (Fig. 6B). However, the sustained phase of the response was markedly attenuated in cells expressing TRPV4-Y110F (Fig. 6, B and C; p < 0.0001 compared with the wild type). The sustained response to PAR2-AP of cells expressing TRPV4-Y805F was only slightly reduced (p = 0.03 compared with the wild type), whereas the response of cells expressing the double mutant TRPV4-Y110/805F resembled that of cells expressing the single mutant TRPV4-Y110F (Fig. 6, B and C; p = 0.001 compared with the wild type). Responses to the TRPV4 agonist GSK1016790A were unaffected by TRPV4 mutations (data not shown).

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FIGURE 5. PLA2, cytochrome P450 epoxygenase, and Src kinase-dependent mechanisms mediate PAR2 activation of TRPV4. The effects of inhibitors on PAR2-AP-evoked changes in [Ca^{2+}]i in HEK-TRPV4 cells (0.1 μg/ml tetracycline). A, the PLA2 inhibitor MAFP inhibited the transient and sustained phases of the Ca^{2+} response to PAR2-AP and also had a small inhibitory effect on the response to GSK1016790A. B, the cytochrome P450 epoxygenase inhibitor 17-ODYA inhibited the sustained phase of the Ca^{2+} response to PAR2-AP and also had a small inhibitory effect on the response to 4α-PDD. C and D, the cyclooxygenase 1/2 inhibitor indomethacin (C) and PKC inhibitor BIM-1 (D) did not reduce the sustained phase of the Ca^{2+} response to PAR2-AP. E, the Src family inhibitor Src1 did not affect the transient response to PAR2-AP but reduced the sustained phase in TRPV4 cells compared with vehicle. F, the summarized data demonstrate the effects of inhibitors on the sustained phase by comparing the sustained:transient ratio for inhibitors and matching vehicle treatments. "Control" sustained: transient data compare HEK control and TRPV4-HEK cells (0.1 μg/ml tetracycline) from Fig. 2A. * p < 0.05; ** p < 0.01; *** p < 0.001 relative to vehicle; Student’s t test; n = 3 experiments.
TRPV4 mutant (expressed as percentage relative to the wild-type control, 0.03 (mean response of 50 neurons) to sequential stimulation with trypsin (25 s, PAR2 agonist) (60 s post-PAR2-AP treatment (quartiles of GSK1016790A responsiveness. Analysis of the area under the curve, measured up to 125 s post-PAR2-AP, indicated that neurons in the upper and lower quartiles of capsaicin responsiveness (100 nM) exhibited larger transient and sustained responses to PAR2-AP compared with neurons in the lower quartile (maximal transient responses at 30 s; 0.017; p = 0.05; Student’s t test; n = 9 experiments).

TRP Channels Regulate PAR2-dependent Ca2+ Signaling in DRG Neurons—To determine whether PAR2 couples to TRPV4 in cells that naturally express these proteins, we examined responses of DRG neurons from rats to agonists of PAR2 and TRP channels. DRG were first challenged with trypsin (300 units/ml), which was selected as a physiological agonist of PAR2 that gave more robust responses than PAR2-AP (data not shown). DRG cultures were then challenged sequentially with the TRPV4 agonist GSK1016790A (10 nM) and the TRPV1 agonist capsaicin (100 nM). Cells were finally exposed to K+ (50 mM), which depolarizes neurons. Only those cells that responded to K+ and that were neurons were analyzed. Trypsin stimulated a rapid increase in [Ca2+]i, in neurons, with a peak within 5–15 s that gradually returned to prestimulation levels after 120 s (Fig. 7A). Approximately half of all PAR2-responsive neurons also responded to GSK1016790A (52.78 ± 12.69%, 649 neurons, n = 5 experiments) with a prompt and sustained elevation in [Ca2+]i, although the magnitude of those responses was variable. Capsaicin elicited further responses in many of the neurons, which were generally sustained. Not every neuron that responded to GSK1016790A also responded to capsaicin and vice versa. These results indicate that PAR2 and TRPV4 are coexpressed by > 50% of DRG neurons.

The omission of extracellular Ca2+ ions reduced the basal [Ca2+]i, and blunted the peak and sustained phase of the Ca2+ response to trypsin (Fig. 7B). The TRPV4 antagonist HC067047 (10 μM) did not affect the basal [Ca2+]i, but markedly attenuated the peak and sustained Ca2+ response to trypsin (Fig. 7B).

The variability in responsiveness to GSK1016790A probably reflects variable levels of TRPV4 expression in different neurons. Therefore, we sought to determine whether the magnitude of the PAR2 responses correlated with the magnitude of the responses to GSK1016790A. To do so, we compared the

![FIGURE 6. PAR2-dependent activation of TRPV4 requires key tyrosine residues. A, TRPV4 WT and the TRPV4 Y110F, Y805F, and Y110F/Y805F mutants were expressed at similar levels at the cell surface of HEK cells and detected by cell surface biotinylation and TRPV4 Western blotting. B and C, the sustained phase of the Ca2+ response to PAR2-AP was significantly reduced by mutation of the phosphorylation sites Tyr-110 and Tyr-805. A marked reduction in [Ca2+]i for Y110F and the double mutant (Y110F and Y805F) was evident at 60 s post-PAR2-AP treatment (p < 0.001) compared with the Y805F single TRPV4 mutant (expressed as percentage relative to the wild-type control, p = 0.03 (C)). * p < 0.05; **, p < 0.001 relative to the wild type; one sampled t test; n = 9 experiments.](image1)

![FIGURE 7. PAR2 activates TRPV4 in DRG neurons. A, Ca2+ responses of 50 randomly selected rat DRG neurons (gray traces, individual neurons; black trace, mean response of 50 neurons) to sequential stimulation with trypsin (25 s, PAR2 agonist) (i), GSK1016790A (150 s, TRPV4 agonist) (ii), capsaicin (270 s, TRPV1 agonist) (iii), and KCl (50 mM, 380 s, positive selection for neurons) (iv). B, omission of extracellular Ca2+ ions reduced the baseline [Ca2+]i and lowered the peak and sustained phase of the Ca2+ response to PAR2-AP. The TRPV4 antagonist HC067047 significantly reduced the magnitude of the transient phase (0.440 ± 0.012 to 0.391 ± 0.017; p = 0.047) but not the sustained phase (0.379 ± 0.020 to 0.357 ± 0.012; p = 0.355) of the PAR2-AP response. N ≥ 3 experiments. C and D, the magnitude of the response to GSK1016790A was used to assess TRPV4 expression and activity. Neurons were segregated into upper and lower quartiles of GSK1016790A responsiveness. Analysis of the area under the curve, measured up to 125 s post-PAR2-AP, indicated that neurons in the upper quartile exhibited larger transient and sustained responses to PAR2-AP compared with neurons in the lower quartile (maximal transient responses at 30 s post-PAR2-AP: upper quartile, 0.137 ± 0.036; lower quartile, 0.067 ± 0.014; p = 0.024; Student’s t test). Neurons were similarly segregated into upper and lower quartiles of capsaicin responsiveness (p = 0.346). *, p < 0.05; Student’s t test; n = 5 experiments.](image2)
Ca\textsuperscript{2+} response to trypsin, which was quantified as the area under the curve measured up to 125 s after challenge with trypsin, for those neurons in the upper and lower quartiles of the GSK1016790A responses (Fig. 7C). The PAR\textsubscript{2} responses of the neurons in the upper quartile of GSK1016790A responsiveness were ~2-fold greater than the responses of the lower quartile (Fig. 7D, p = 0.002). This finding is consistent with a role for TRPV4 in mediating the Ca\textsuperscript{2+} responses of the neurons to PAR\textsubscript{2} activation. In contrast, when neuronal populations were similarly subdivided on the basis of their responsiveness to TRPV1 activation with capsaicin, there was no difference between the responsiveness of the upper and lower quartiles (Fig. 7D, p = 0.06). Our results indicate that TRPV4 largely mediates the PAR\textsubscript{2}-induced Ca\textsuperscript{2+} signals in DRG neurons.

TRPV4 Mediates PAR\textsubscript{2}-evoked Inflammation—PAR\textsubscript{2} agonists evoke neurogenic inflammation that depends on the local release of neuropeptides from primary spinal afferent neurons (3, 5). To investigate the functional relevance of PAR\textsubscript{2} coupling to TRPV4 in the intact animal, we assessed the effects of PAR\textsubscript{2}-AP on peripheral inflammation in trpv4\textsuperscript{+/+} and trpv4\textsuperscript{−/−} mice. Intraplantar injection of PAR\textsubscript{2}-AP into trpv4\textsuperscript{+/+} mice resulted in an increase in paw thickness that was maximal within 30 min and maintained for 180 min, indicative of tissue edema and consistent with previous reports (Fig. 8A). The magnitude of PAR\textsubscript{2}-induced paw edema at early time points was reduced in trpv4\textsuperscript{−/−} mice compared with trpv4\textsuperscript{+/+} mice (p = 0.006 at 30 min, p = 0.052 at 60 min, n = 10 mice). Paw thickness was similar in both groups at 120 and 180 min after PAR\textsubscript{2}-AP (Fig. 8A, p < 0.05 relative to NaCl-treated mice). No difference in paw thickness was detected between trpv4\textsuperscript{+/+} and trpv4\textsuperscript{−/−} mice after intraplantar injection of 0.9% NaCl.

PAR\textsubscript{2}-AP also increased MPO activity in the paws of trpv4\textsuperscript{+/+} and trpv4\textsuperscript{−/−} mice (Fig. 8B, p = 0.0028 and p = 0.0088, respectively, compared with NaCl; n = 8–10). However, MPO activity was similar in trpv4\textsuperscript{+/+} and trpv4\textsuperscript{−/−} mice (p = 0.8340).

Pretreatment with 17-ODYA (5 mg/kg, intraperitoneal, 30 min before PAR\textsubscript{2}-AP) reduced the paw edema response to PAR\textsubscript{2}-AP at an early time point (Fig. 8C, p = 0.0305 at 30 min compared with vehicle, n = 5 mice). Paw thickness was similar in both groups at other time points after PAR\textsubscript{2}-AP (Fig. 8C, p < 0.05 relative to NaCl-treated mice). 17-ODYA did not affect the PAR\textsubscript{2}-induced MPO activity (Fig. 8D).

These results indicate that the release of arachidonic acid metabolites and the activation of TRPV4 contributes to the initial phases of PAR\textsubscript{2}-evoked edema. However, TRPV4 is not involved in PAR\textsubscript{2}-mediated recruitment of inflammatory cells.

**DISCUSSION**

Our results reveal an unexpected functional coupling between PAR\textsubscript{2} and TRP channels, which involves the generation of an arachidonic acid-derived TRPV4 activator and a tyrosine kinase signaling pathway (Fig. 9). We identified this coupling in a model HEK cell line and in primary nociceptive neurons. The coupling gives rise to long-lasting TRPV4-regulated Ca\textsuperscript{2+} signals and demonstrates a direct involvement of TRPV4 in the cellular response to PAR\textsubscript{2} activation, which contributes to the proinflammatory effects of this receptor. We propose that this coupling represents a mechanism of TRP channel regulation that is distinct from the process of sensitization. This convergence of GPCR and TRP signaling may provide a mechanism through which the specificity and magnitude of cellular responses is conferred and controlled. This proposal...
These observations suggest that the mechanism of PAR2 coupling may influence the duration of PAR2-dependent inflammation development (13, 16–18). In our proposed model, the major mechanism through which both neurogenic pain and inflammation develop with PAR2 in nociceptive spinal-afferent neurons, and the magnitude and duration of PAR2 signaling are affected by TRPV4 expression. The PAR2 response was greatly reduced in DRG neurons treated with the TRPV4 antagonist HC067047 or after omission of extracellular Ca\(^{2+}\). However, a substantial component of the increase in [Ca\(^{2+}\)]\(_i\) also derives from intracellular Ca\(^{2+}\) stores.

In this study, we have demonstrated a role for PAR\(_2\)-TRPV4 coupling in the regulation of PAR\(_2\)-mediated paw edema but not in granulocyte infiltration. These findings are consistent with a role for TRPV4 in PAR\(_2\)-dependent signaling in DRG neurons, as the development of paw edema is neurogenic in origin, whereas granulocyte recruitment occurs independently of sensory innervation (3).

**PAR\(_2\)-TRPV4 Coupling in Other Systems**—Our observations may have important functional implications for other cell types in which PAR\(_2\) and TRPV4 are coexpressed, such as bladder urothelium, colonic epithelium, and bronchial epithelial cells (18, 57–60). Whether physiological and pathophysiological responses to PAR\(_2\) activation are augmented by TRPV4 in these tissues has yet to be determined. PAR\(_2\) activation in these cell types leads to increased barrier permeability and is associated with inflammation (61). Similarly, TRPV4 activation results in inflammation and altered cellular permeability of colonic epithelial cells (58). Thus, as with DRG neurons, TRPV4 channels may also regulate PAR\(_2\)-dependent signaling in these cells.

**Signaling Pathways Involved in TRP Activation**—PARs signal through phospholipase C and PLA\(_2\) (57, 62, 63). Arachidonic acid derivatives are endogenous activators of TRPV1, TRPV4, and TRPA1 (24, 25, 43, 64). PAR\(_2\) coupling with TRPV4 involves production of arachidonic acid derivatives, as demonstrated using MAPF, an irreversible inhibitor of PLA\(_2\) (65). This observation is consistent with evidence that PAR\(_2\) activation leads to increased arachidonic acid release and prostaglandin production in enterocytes (57) and with other studies examining GPCR interaction with TRPV4 channels (47).

Whether this is a common mechanism in cells that coexpress GPCRs and TRPV4 channels remains to be determined. PLA\(_2\)-derived TRP channel activators contribute to both the transient and sustained phases of the PAR\(_2\) response because MAPF inhibited these observations suggest that the mechanism of PAR2 coupling may influence the duration of PAR2-dependent inflammation development (13, 16–18). In our proposed model, the major mechanism through which both neurogenic pain and inflammation develop with PAR2 in nociceptive spinal-afferent neurons, and the magnitude and duration of PAR2 signaling are affected by TRPV4 expression. The PAR2 response was greatly reduced in DRG neurons treated with the TRPV4 antagonist HC067047 or after omission of extracellular Ca\(^{2+}\). However, a substantial component of the increase in [Ca\(^{2+}\)]\(_i\) also derives from intracellular Ca\(^{2+}\) stores.

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PAR2 and TRPV4 Coupling

both components of the response. The inability of indomethacin to block TRPV4 activation excludes involvement of cyclooxygenase-derived prostaglandin in signaling to TRPV4. Lipoxigenase-derived arachidonic acid derivatives, such as hydroxyeicosatetraenoic acids, are endogenous TRPV1 agonists and are not reported to activate TRPV4 (28, 43, 66, 67). In our experiments, lipoxygenase inhibition reduced cell viability and was not examined further (data not shown). We did not observe coupling between PAR2 and TRPA1. This result is at variance with a recent study in which 5,6-EET-dependent neuronal activation of TRPA1 was reported (68). The reason for this discrepancy is presently unknown but may relate to the differences in the studied receptors and the experimental systems.

TRPV4 is modulated by a range of serine/threonine and tyrosine kinases and contains target residues, including those for tyrosine kinases (50, 69) and PKC (40). In addition, PKC-activated phorbol esters directly activate TRPV4 (41). We found that PKC-specific inhibition (BIM-1) had no effect on PAR2 signaling, which excludes a role for PKC in coupling with TRPV4.

Two observations implicate tyrosine phosphorylation of TRPV4 in PAR2-TRPV4 coupling. First, the Src family kinase inhibitor, Src1, attenuated TRPV4-dependent PAR2 signaling. Second, the magnitude of coupling was markedly attenuated by mutation of Tyr-110, a target residue for Src family kinases (50). A limitation of our work is that we have not identified the particular tyrosine kinase involved in PAR2-TRPV4 coupling. Although Src1 inhibited coupling, preliminary studies indicated that PP2 was less effective (data not shown). Thus, it is possible that other kinases are involved because nanomolar concentrations of PP2 block certain Src kinases (70). Another caveat of our study is that we did not directly demonstrate phosphorylation of TRPV4-Y110 in response to PAR2 activation. Further studies using pharmacological and genetic approaches for manipulating kinase activity are required to identify the kinases that mediate tyrosine phosphorylation. Proteomic analyses are necessary to study PAR2-induced tyrosine phosphorylation of TRPV4. Once the specific kinase is identified, it will be possible to examine its contribution to pro tease-evoked inflammation and pain in mice.

Summary—In summary, we have demonstrated coupling between PAR2 and TRPV4. This activation of TRP channels is mediated by production of endogenous activators and is dependent on key tyrosine residues of TRPV4. Our study has identified critical components of the intracellular signaling pathways underlying the activation of TRP channels. These may represent novel targets for therapeutics aimed at reducing augmented signaling under pathophysiological conditions while leaving normal responses intact. This selective inhibition of aberrant signaling is a more attractive target compared with global inhibition of TRP channels, which have important physiological roles in thermoregulation, osmoregulation, and nociception.

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