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Protein Kinase D Isoforms Are Expressed In Rat and Mouse Primary Sensory Neurons and Are activated by Agonists Of Protease-Activated Receptor 2

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Abstract

Serine proteases generated during injury and inflammation cleave protease-activated receptor 2 (PAR₂) on primary sensory neurons to induce neurogenic inflammation and hyperalgesia. Hyperalgesia requires sensitization of transient receptor potential vanilloid (TRPV) ion channels by mechanisms involving phospholipase C and protein kinase C (PKC). The protein kinase D (PKD) serine/threonine kinases are activated by diacylglycerol and PKCs, and can phosphorylate TRPV1. Thus, PKDs may participate in novel signal transduction pathways triggered by serine proteases during inflammation and pain. However, it is not known whether PAR₂ activates PKD, and the expression of PKD isoforms by nociceptive neurons is poorly characterized. Using HEK293 cells transfected with PKDs, we found that PAR₂ stimulation promoted plasma membrane translocation and phosphorylation of PKD1, PKD2 and PKD3, indicating activation. This effect was partially dependent on PKC ϵ . Using immunofluorescence and confocal microscopy, with antibodies against PKD1/PKD2, PKD3 and neuronal markers, we found that PKDs were expressed in rat and mouse dorsal root ganglia (DRG) neurons, including nociceptive neurons that expressed TRPV1, PAR₂ and neuropeptides. PAR₂ agonist induced phosphorylation of PKD in cultured DRG neurons, indicating PKD activation. Intraplantar injection of PAR₂ agonist also caused phosphorylation of PKD in neurons of lumbar DRG, confirming activation *in vivo*. Thus, PKD1, PKD2 and PKD3 are expressed in primary sensory neurons that mediate neurogenic inflammation and pain transmission, and PAR₂ agonists activate PKDs in HEK293 cells and DRG neurons in culture and in intact animals. PKD may be a novel component of a signal transduction pathway for protease-induced activation of nociceptive neurons and an important new target for anti-inflammatory and analgesic therapies.

Keywords

Serine protease; GPCR; kinase; dorsal root ganglia; phosphorylation

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Introduction

Injury and inflammation result in the release and activation of multiple serine proteases that can regulate cells by cleaving protease-activated receptors (PARs), a family of four G protein-coupled receptors (Ossovskaia and Bunnett, 2004; Ramachandran and Hollenberg, 2008). Proteases from the circulation (e.g., thrombin, factors VIIa, Xa), inflammatory cells (e.g., mast cell tryptase), and the epithelium (e.g., trypsin I, II and IV) cleave PARs within the extracellular amino-terminus to expose a tethered ligand, which binds to and activates the receptor. PAR₂ is prominently expressed by a subpopulation of primary spinal afferent neurons containing the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) (Steinhoff et al., 2000). PAR₂ agonists stimulate the release of SP and CGRP from these nerves to cause neurogenic inflammation of peripheral tissues and hyperalgesia to thermal and mechanical stimuli (Knecht et al., 2007; Nguyen et al., 2003; Steinhoff et al., 2000; Vergnolle et al., 2001; Vergnolle et al., 1999). PAR₂-induced hyperalgesia requires sensitization of transient receptor potential vanilloid (TRPV) ion channels TRPV1 and TRPV4, non-selective cation channels that permit nociceptors to detect thermal and mechanical stimuli. This sensitization occurs by mechanisms dependent on phospholipase C (PLC) and protein kinase C epsilon (PKCε) (Amadesi et al., 2006; Amadesi et al., 2004; Grant et al., 2007). Although protein kinase D (PKD) is also regulated by PLC and PKCε, it is not known whether PAR₂ activates PKD or whether PKD also mediates PAR₂-induced sensitization of TRPV1 or TRPV4.

The three isoforms of PKD, PKD1 (also known as PKCμ), PKD2 and PKD3, comprise a new family of serine/threonine kinases within the Ca²⁺/calmodulin-dependent protein kinase (CaMKs) group. PKDs play a unique role as direct downstream targets of diacylglycerol (DAG) and PKCs (Rozengurt et al., 2005). Structurally, PKDs are characterized by a regulatory domain that is homologous with the DAG/phorbol ester-sensitive PKCs, and a catalytic domain that is homologous with CaMKs (Rozengurt et al., 2005). PKDs are activated by phosphorylation of serine residues within the activation loop of the catalytic domain through a rapid PKC-dependent transduction pathway (Waldron and Rozengurt, 2003). However, a PKC-independent activation of PKDs has also been described (Jacamo et al., 2008; Rozengurt et al., 2005). Pharmacological agents, such as the DAG analog phorbol esters (Zugaza et al., 1996), and physiological stimuli that elevate intracellular DAG, such as agonists of G protein-coupled receptors (e.g., bradykinin and thrombin) and growth factors, activate PKD through a PKC-dependent mechanism (Poole et al., 2008; Tan et al., 2004; Tan et al., 2003; Zugaza et al., 1997). DAG induces translocation of PKD from the cytosol to the plasma membrane where PKC can phosphorylate and activate PKD. Active PKD dissociates from the plasma membrane and translocates back to the cytosol and, in the case of PKD3, to the nucleus (Rozengurt et al., 2005).

PKDs are expressed in multiple tissues and cell types, including in the nervous system (Song et al., 2007; Wang et al., 2004), and PKDs regulate diverse cellular processes, including signal transduction, membrane trafficking and protein transport (Rozengurt et al., 2005). Recent studies suggest that PKD also regulates inflammation and pain transmission. For example, PKC-dependent activation of PKD in spinal neurons has been observed during endotoxemia and inflammation (Song et al., 2007). PKD also directly interacts with TRPV1, a multimodal cation channel that mediates neurogenic inflammation and pain (Caterina et al., 1997), in cell lines and primary sensory neurons, suggesting that PKD regulates TRPV1 and thereby controls sensory perception and hyperalgesia (Wang et al., 2004). Thus, PKD may be a novel component of a signal transduction pathway, which is activated in primary sensory neurons during inflammation, that sensitizes ion channels that regulate neurogenic inflammation and hyperalgesia. However, the expression and the distribution of PKD isoforms in primary spinal sensory neurons is poorly characterized. It is not known if PKD

isoforms are expressed by nociceptive neurons that also express PAR₂, or whether inflammatory agents, including agonists of PAR₂, activate PKD in these neurons.

In this study, we initially determined if stimulation of PAR₂ induces sub-cellular redistribution and phosphorylation of PKD isoforms, consistent with their activation. To do so, we studied a model system of HEK293 cells, which naturally express PAR₂, overexpressing PKD isoforms tagged with green fluorescent protein (GFP). This system allowed us to specifically examine PAR₂-induced activation of PKD in an isoform-specific manner by examining membrane translocation and phosphorylation by immunostaining, confocal microscopy and Western blotting. We then sought to determine if similar activation occurs in primary sensory neurons. We first examined the expression and the distribution of PKD isoforms in neurons of dorsal root ganglia (DRG). We localized PKD isoforms by immunostaining and confocal microscopy, using markers of different populations of neurons to characterize those expressing PKD. To assess whether agonists of PAR₂ and other proinflammatory mediators activate PKD in these neurons, we incubated cultured DRG neurons with a PAR₂-selective agonist or a mixture of proinflammatory mediators, and used an antibody to phosphorylated (activated) PKD to assess activation. To determine whether this activation can also occur in the intact animal, we administered a PAR₂ agonist by intraplantar injection, and studied phosphorylation of PKD in neurons of the lumbar DRG. Our results show that PKD isoforms are expressed by primary spinal afferent neurons, including those that express PAR₂, TRPV1, SP and CGRP, which mediate protease-induced neurogenic inflammation and hyperalgesia. PAR₂ agonists activate all isoforms of PKD in model systems, cultured neurons and the intact animal. These results support the hypothesis that PKD isoforms mediate protease-induced neurogenic inflammation and pain.

Materials and Methods

Animals

C57Bl6 mice (male, 6–8 weeks) and Sprague Dawley rats (male, 200–250 g) were from Charles River Laboratories (CA). The Institutional Animal Care and Use Committee of the University of California, San Francisco approved the study. Animals were humanely killed using sodium pentobarbital (200 mg/kg i.p.) and bilateral thoracotomy.

Antibodies and fluorescent probes

Primary antibodies are shown table 1. Secondary antibodies were: goat anti-rabbit, -mouse or -guinea pig IgG conjugated to fluorescein isothiocyanate, rhodamine red or Texas Red (Jackson Immuno-Research, West Grove, PA, 1:200); rabbit anti-horseradish peroxidase (Jackson Immuno-Research, 1:500); goat anti-mouse or rabbit conjugated to Alexa Fluor 680 (Invitrogen, 1:10,000) or IRDye™ 800 (Rockland Immunochemicals, Gilbertsville, PA, 1:10,000). Streptavidin conjugated to Alexa-488 was from Molecular Probes Invitrogen (Carlsbad, CA, 1:2,000).

Primary Antibody Characterization

The antibody to PKD1/2 (anti-PKD1/2) has been previously characterized (Rey et al., 2001; Rey et al., 2003b). PKD1/2 immunoreactivity was eliminated by preincubation of the diluted antiserum (1:100) with the synthetic peptide used for the immunization (Santa Cruz Biotechnology, sc-639P), suggesting specificity (see supplemental Figure 1 A). Moreover, in rat DRG lysates analyzed by Western blotting, the anti-PKD1/2 antibody recognizes a band of ≥100 KDa, consistent with the size of rat PKD1 (~102 KDa). Also in this case, the positive immunoreaction was prevented by preincubation with the peptide used for immunization (Zhu et al., 2008). The PKD1/2 antibody also recognized a faint band of ≤100 KDa, similar to the size of rat PKD2 (~96.5 KDa). This finding can be probably explained

since the antibody is not selective for PKD1 but also reacts with PKD2. The antibody to PKD3 (anti-PKD3) has been previously characterized (Rey et al., 2001; Rey et al., 2003b). The peptide used for the immunization and production of PKD3 antibody was not available. However, there is only one residue that is shared between PKD3 and PKD1 within the sequence of the immunogen peptide. Moreover, when the immunogen peptide sequence is subjected to Blast analysis, neither PKD1 nor PKD2 are identified as homologous. We further investigated specificity of the PKD3 antibody using HEK293 cells transfected with PKD1-, PKD2- and PKD3-GFP. PKD3-like immunoreactivity (LI) was detected only in cells expressing PKD3-GFP, confirming specific detection of PKD3 and not PKD1 or PKD2 (Supplemental figure 1 B-O). Using a similar approach, the lack of cross-reaction between the PKD3 antibody and PKD1 and PKD2 was confirmed in a recent study that characterized the expression of PKD3 in the developing mouse (Ellwanger et al., 2008). In this study, Western blotting analysis indicated that the anti-PKD3 recognizes a band of a ~100 kDa in HEK293 cells, consistent with the size of PKD3. The phospho-PKD/PKCmu antiserum has been previously characterized (Jacamo et al., 2008; Waldron et al., 2001). The antibody recognizes the phosphorylated state of the equivalent serines within the activation loop of the three PKD isoforms (PKD1: Ser⁷⁴⁴-Ser⁷⁴⁸; PKD2: Ser⁷⁰⁶-Ser⁷¹⁰; PKD3: Ser⁷³¹-Ser⁷³⁵) (Waldron et al., 2001). The peptide used for the immunization and production of the antibody was not available. However, we detected immunoreactivity only in cells transfected with PKD1-, PKD2- and PKD3-GFP when stimulated with phorbol ester (not shown), suggesting specificity. Moreover, in rat DRG lysates analyzed by Western blotting, the anti-phospho-PKD/PKCmu antibody recognized a band of ~100 kDa, consistent with the size of rat PKDs (Besirli and Johnson, 2006). The CGRP antibody has been characterized (Wong et al., 1993), including in enteric, DRG and spinal cord neurons of the rat (Cottrell et al., 2005). The pattern of CGRP immunoreactivity that we detected confirms the results of previous studies of peptidergic sensory neurons in rats (Steinhoff et al., 2000). Moreover, the lack of positive immunoreactivity in histological sections of DRG from alpha-CGRP knockout mice confirms specific detection of CGRP (Zhang et al., 2001). The SP antibody has been used to characterize peptidergic sensory neurons, and the pattern of expression we detected confirms previous studies (Steinhoff et al., 2000). Moreover, the lack of positive immunoreactivity in primary sensory neurons from preprotachykinin-A knockout mice confirms specific detection of SP (Sanderson Nydahl et al., 2004). The antibody to PAR₂ has been previously characterized and staining in vascular tissues and in pancreatic stellate cells was prevented by preadsorption with the antigen used for immunization (Masamune et al., 2005) and there was no staining when the antibody was replaced with normal murine IgG2a (Molino et al., 1998). Moreover, we detected PAR₂-LI only in rat kidney epithelial cells (KNRK) transfected with rat or human PAR₂ but not in untransfected cells suggesting specific detection of PAR₂ (Bunnett, unpublished observations). The pattern of PAR₂-LI we detected in DRG is identical to previous reports (Grant et al., 2007), and is similar to the distribution found using a different PAR₂ antiserum and by *in situ* hybridization (Steinhoff et al., 2000). The antibody to TRPV1 has been previously characterized in rat DRG (Amadesi et al., 2004), and the pattern of TRPV1-LI we detected was similar to previous studies that used a different TRPV1 antiserum (Caterina et al., 1997). Immunoreactivity was abolished by preadsorption with the antigen blocking peptide (not shown) (Steinhoff et al., 2000). Moreover, the lack of TRPV1-LI in DRG from TRPV1 knockout mice confirms specific detection of TRPV1 (Baiou et al., 2007). The antibody to neurofilament 200 kDa (N52) recognizes the phosphorylated and dephosphorylated H-chain of neurofilament 200 kDa, one of the three intermediate filament proteins that serve as major supporting elements of the neuronal cytoskeleton. As neurofilaments are typically restricted to neurons, N52 is used as neuronal markers to identify cells of neuronal origin (Shaw et al., 1986).

Reagents

PAR₂-activating peptide (AP) (SLIGRL-NH₂) and the inactive PAR₂-reverse peptide (RP) (LRGILS-NH₂) were from SynPep Corp. (Dublin, CA). Phorbol-12, 13-dibutyrate (PDBu) and phorbol-12-myristate-13-acetate (PMA) were from Calbiochem. Bradykinin and SP were from Bachem (Torrance, CA). 5-Hydroxytryptamine and histamine were from Sigma (St. Louis, MO). The membrane-permeable PKC ϵ translocation inhibitor peptide, conjugated to an 11-amino-acid protein-transduction domain from the human immunodeficiency virus TAT protein (YGRKKRRQRRRC-disulfide bond-CEAVSLKPT-COOH, TAT-PKC ϵ I) (Schwarze et al., 1999), and the inactive scrambled sequence (TAT-PKC ϵ I-sc) were from SynPep. Biotin-conjugated isolectin IB4 from *Bandeiraea simplicifolia* was from Sigma (L3759). It was incubated with histological sections of DRG (1:100) to detect nociceptive non-peptidergic, small diameter, unmyelinated neurons (Silverman and Kruger, 1990). IB4 binds to alpha-D-galactose expressed on sensory ganglion cells and their central terminals in the spinal cord.

HEK293 cell lines

HEK-FLP cells (Invitrogen) were maintained in DMEM, 10% FBS and Zeocin (100 μ g/ml). Cells were transiently transfected with 2–3 μ g of PKD1, PKD2 and PKD3 (GFP tagged), using Lipofectamine 2000TM (Invitrogen). At 48 h after transfection, cells were incubated in low-serum medium (0.5% FBS) for 16–18 h (Poole et al., 2008). Cells were treated at different time points with test substances, for then processed for immunofluorescence or Western blot analysis.

DRG neurons

Cultures of DRG neurons from thoracic and lumbar spinal regions were prepared as described (Amadesi et al., 2004). Neurons were incubated in low-serum medium (0.2% FBS) for 16–18 h. Cells were treated with PAR₂-AP or PAR₂-RP (100 μ M, 1, 5, 15 min), PDBu (1 μ M, 5 min), or with an inflammatory mixture of bradykinin (1 μ M), SP (1 μ M), 5-hydroxytryptamine (10 μ M) and histamine (10 μ M) (1–5 min), and then processed for immunofluorescence.

Animal treatments

Mice under light anesthesia (2.5% isoflurane) received an intraplantar injection of saline, PAR₂-AP or PAR₂-RP (10 μ g in 10 μ l saline per paw). After 30 min, animals were transcardially perfused with 4% paraformaldehyde (PFA) in 100 mM PBS, pH 7.4 and lumbar DRG (L4 – L6) were collected and processed for immunofluorescence.

Immunofluorescence

Histological sections—Animals were transcardially perfused with 4% PFA in 100 mM PBS, pH 7.4. DRGs were removed and fixed in 4% PFA for 4 h at room temperature, then washed, incubated in 25% sucrose in PBS overnight at 4°C, and embedded in OCT. Sections of mouse and rat DRG (10–16 μ m) were cut and mounted on slides coated with poly-L-lysine. Histological sections were washed and incubated in 10 mM PBS, pH 7.4, containing 5% normal goat or donkey serum and 0.3% Triton X-100 for 1 h at room temperature. Specimens were incubated with primary antibodies for 16 h at 4°C, then washed and incubated with secondary antibodies: goat anti-rabbit, -mouse or -guinea pig IgG conjugated to fluorescein isothiocyanate, rhodamine red, Texas Red (2 h, room temperature), or streptavidin conjugated to Alexa-488 (2 h, room temperature). TRPV1 signals were amplified using a Fluorescein Tyramide Signal Amplification System (Perkin Elmer, Boston, MA). To determine the specificity of the PKD1/2 antibody, we pre-absorbed the antibody with the peptide used for immunization. To determine the specificity of the PKD3

antibody, as the immunizing peptide is not available, we localized PKD3 in HEK293 cells transfected with PKD1-, PKD2- or PKD3-GFP and activated with PDBu. Histological sections were mounted in Prolong Gold (Invitrogen).

Cultured Cells—HEK293 cells transiently transfected with PKD1-, PKD2- or PKD3-GFP and cultured rat DRG were fixed with 4% PFA for 20 min at 4°C. Cells were washed and incubated with 10 mM PBS, pH 7.4 containing 1% normal goat serum and 0.1% saponin for 30 min at room temperature. HEK293 cells were incubated with rabbit anti-pSer^{744/748} (16 h, 4°C), washed, and incubated with goat anti-rabbit IgG conjugated to rhodamine red-X (1 h, room temperature). DRG were treated with the Fluorescein Tyramide Signal Amplification System (Perkin Elmer) according to the manufacturer's instructions. DRG were incubated with the rabbit anti-pSer^{744/748} (16 h, 4°C), washed, and incubated with rabbit anti-horseradish peroxidase (1 h, room temperature). Cultured cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA).

Confocal microscopy and image analysis

Specimens were observed with a Zeiss Axiovert and an LSM 510 META laser scanning confocal microscope with Zeiss EC Plan-Neofluar 40x/1.3 or Zeiss Plan-Apo 100x/1.4 oil objectives. Images of 512 × 512 pixels were collected. For colocalization, single optical sections at the same focal plane were taken separately and the corresponding channels were merged. Images were collected, colored to represent the appropriate fluorophores, and processed to adjust contrast and brightness using Adobe Photoshop CS2 9.0 (Adobe Systems, Mountain View, CA). The pixel intensity of images was quantified using the Zeiss LSM Image Browser, Version 4.2.0.121. Neurons were considered positive for the expression of the proteins of interest if the immunoreactive signal was above the average background fluorescence signal used as minimum threshold. The activation of PKD in HEK293 cells was expressed as the ratio between the pixel intensity of the pSer^{744/748} and GFP signals. The activation of PKD in cultured DRG neurons was expressed as the ratio between the intensity of the pSer^{744/748} signal per area analyzed (pixel intensity/μm). Ratios were then normalized to the appropriate control. The activation of PKD in histological sections of mouse DRG was expressed as average pixel intensity of the pSer^{744/748} signal. Neuronal sub-populations were distinguished on the basis of cell diameter (large neurons, >30 μm; medium/small neurons, ≤30 μm). Values were compared to controls within the same neuronal sub-population.

SDS-PAGE and Western blotting

Cells were washed and lysed in 50 mM Tris-HCl pH 7.4, 50 mM NaF, 1 mM Na₃VO₄, 1% SDS and protease inhibitors, boiled, and centrifuged. Lysates (40 μg) were separated by SDS-PAGE (8% acrylamide), transferred to polyvinylidene difluoride membrane (Immobilon FL, Millipore Corp.), and blocked in blocking buffer (LI-COR, Lincoln, NE), and incubated with rabbit anti-pSer^{744/748} (1:1,000) and mouse anti-GFP (1:10,000) (overnight, 4°C). Membranes were washed and then incubated with secondary antibodies coupled to either Alexa Fluor 680 (1:10,000) or IRDyeTM 800 (1:10,000) (1 h, room temperature). Immunoreactive proteins were detected using an Odyssey Infrared Imaging System (LI-COR). For the densitometric analysis, the pSer^{744/748} signal and the GFP-signal (used as loading control) were calculated, then expressed as a ratio and compared to non-treated cells.

RT-PCR

Total RNA from rat DRG was isolated using Trizol (Invitrogen). RNA (5 μg) was reverse transcribed using standard protocols with random hexamers and TaqMan reverse

transcription reagents (Applied Biosystems, Foster City, CA). Subsequent PCR reactions used primers specific for rat PKD1 (forward: 5'-ggagactggacagcaaaagc-3' and reverse 5'-gtccagaaccagaacctca-3'), PKD2 (forward: 5'-gatccgtttctcaggtgaa-3' and reverse: 5'-tggcgtctgatacaaaactg-3') and PKD3 (forward 5'-aatgtgctgcttgcactg-3' and reverse 5'-acgttctcaatgcgagttt-3'). Control reactions omitted reverse transcriptase. PCR products were separated by electrophoresis (2% agarose gel), and visualized using ethidium bromide. Product identity was confirmed by sequencing.

Statistical analysis

Results were normalized to the appropriate control and expressed as mean $\pm$ SEM. Groups were compared using ANOVA and Bonferroni tests or Student's *t* test analysis. Differences were considered significant when *p* was ≤ 0.05 .

Results

PAR₂ agonist induces redistribution and activation of PKD1, PKD2 and PKD3 in HEK293 cells

To investigate whether stimulation of PAR₂ activates PKD1, PKD2 and PKD3, we studied trafficking and phosphorylation of GFP-tagged kinases expressed in HEK293 cells. By immunostaining and confocal microscopy, we studied redistribution of kinases and the levels of phosphorylated kinases as a measure of activation. To monitor the activation of the PKD isoforms we used an antibody raised against a peptide phosphorylated on serines equivalent to Ser⁷⁴⁴ and Ser⁷⁴⁸ of PKD but that predominantly detects the phosphorylated state of Ser⁷⁴⁴, as originally shown (Waldron et al., 2001) and verified in a recent study (Jacamo et al., 2008). This antibody (pSer^{744/748}) detects the phosphorylated state of the equivalent serines within the activation loop of the three PKD isoforms (PKD1: Ser-744; PKD2: Ser-706; and PKD3: Ser-731). In non-treated cells (0 min), PKD1- (Figure 1, A1-C1) PKD2- (Figure 1, A2-C2) and PKD3-GFP (Figure 1, A3-C3) were localized in the cytosol and a pSer^{744/748}-LI was not detected. PAR₂-AP (100 μ M, 1 min) induced translocation of each PKD isoform from the cytosol to the plasma membrane and increased the pSer^{744/748}-LI for all the three PKDs (PKD1, figure 1, D1-F1; PKD2, figure 1, D2-F2; PKD3, figure 1, D3-F3). After 5 min, PKDs started to return to the cytosol, but remained phosphorylated, suggesting a sustained activation (PKD1, figure 1, G1-I1; PKD2, figure 1, G2-I2; PKD3, figure 1, G3-I3). In a similar manner, PMA, a non-selective PKC activator used as a positive control (1 μ M, 5 min), induced a robust translocation of PKD1-, PKD2- and PKD3-GFP from the cytosol to the plasma membrane and a large increase of the pSer^{744/748} signal for all the three PKDs (PKD1, figure 1, A4-C4; PKD2, figure 1, D4-F4; PKD3, figure 1, G4-I4).

To investigate the role of PKC ϵ in PAR₂-mediated activation of PKDs, we treated cells with the PKC ϵ inhibitor (TAT-PKC ϵ I) or with the inactive scrambled sequence used as control (TAT-PKC ϵ I-sc) (10 μ M, 15 min). In cells pretreated with TAT-PKC ϵ I-sc, PAR₂-AP (5 min) increased the levels of phosphorylated PKDs-GFP (fold-increases compared to non-treated cells: PKD1, 1.03; PKD2, 1.47; PKD3, 0.48; figure 2A-C). After 15 min, phosphorylation of PKDs was still sustained (fold-increases compared to control: PKD1, 0.46; PKD2, 0.76; PKD3, 0.47; figure 2A-C). Pretreatment with TAT-PKC ϵ I reduced PKDs phosphorylation induced by PAR₂-AP applied for 5 min (phosphorylation reduction % compared to control: PKD1, 13.7; PKD2, 16.3; PKD3, 14.7; figure 2A-C) and PKD3 phosphorylation induced by PAR₂-AP applied for 15 min (phosphorylation reduction %: 15.0, figure 2C) compared to cells treated with TAT-PKC ϵ I-sc for the same time.

Activation of PKDs was confirmed by Western blot analysis using the pSer^{744/748} antibody and densitometry. PAR₂-AP (1–30 min) increased the level of phosphorylated PKD1-GFP (1.5-, 4.2-, 4.1- and 7.8-fold increases at 1, 5, 15, 30 min. Figure 3A, B), PKD2-GFP (6.2-, 8.8-, 7.2- and 3.5-fold increases at 1, 5, 15, 30 min. Figure 3C, D), and PKD3-GFP (2-, 2.5-, 2.8- and 2.4-fold increases at 1, 5, 15, 30 min. Figure 3E, F) when compared to non-treated cells. As expected, the PKD activator PMA also increased the level of phosphorylated PKDs-GFP, whereas PAR₂-RP was completely inactive (Figure 3A–F). Thus, stimulation of PAR₂ induces translocation and a sustained activation of PKD1-, PKD2- and PKD3-GFP in HEK293 cells. PAR₂-mediated PKD phosphorylation depends on PKC ϵ activation.

PKD1, PKD2 and PKD3 are expressed by primary sensory nociceptive neurons

PKD1 is expressed in rat DRG (Wang et al., 2004). However, the distribution of the PKD isoforms within the different neuronal types has not been characterized. By RT-PCR, we found that rat DRG neurons expressed mRNA of the sizes predicted for PKD1 (448 bp) and PKD2 (479 bp) and PKD3 (488 bp) (Fig. 4). By immunofluorescence and confocal microscopy, we localized PKD-LI in histological section of rat DRG neurons. Using an antibody that recognizes both PKD1 and PKD2 (PKD1/2, figure 5) we detected PKD1/2-LI in 64% of small, medium and large diameters neurons (255/401 neurons). In some neurons, PKD1/2-LI co-localized with CGRP (Figure 5, A–C) and TRPV1 (Figure 5, D–F), which are markers for peptidergic small-diameter neurons considered to be nociceptors, and also with PAR₂ (Figure 5, G–I) that is known to be expressed by a subset of nociceptive neurons (Steinhoff et al., 2000). PKD1/2-LI also co-localized with IB4, a marker for nociceptive non-peptidergic, small diameter, unmyelinated neurons (IB4, figure 5, J–L), and with N52, a marker for large diameter neurons with myelinated fibers (Figure 5, M–O). Table 2 summarizes the expression pattern for PKD1/2-LI and neuronal markers. Using an antibody that recognizes PKD3, we detected PKD3-LI with a distribution pattern similar to that for PKD1/2-LI (Figure 6). PKD3-LI was detected in 77% of small, medium and large diameter neurons (210/273 neurons), in peptidergic nociceptive neurons (CGRP and TRPV1 positive neurons. Figure 6, A–C and D–F), in non-peptidergic unmyelinated neurons (IB4 positive. Figure 6 J–L), and in myelinated neurons (N52 positive. Figure 6, M–O). In some neurons, PKD3-LI co-localized with PAR₂ (Figure 6, G–I). Table 2 summarizes the expression pattern for PKD3-LI and neuronal markers. Incubating the PKD1/2 antibody with the peptide used for the immunization or omitting primary antibodies prevented staining, suggesting antibody specificity (Supplemental figure 1A). Since the peptide used for the immunization and production of PKD3 antibody was not available, we further confirmed specificity of the PKD3 antibody using HEK293 cells transfected with PKD1-, PKD2- and PKD3-GFP (Supplemental figure 1, B–O). Cells were treated with PDBu (1 μ M, 5 min), a non-selective PKC/PKD activator, to induce kinase activation (Rey et al., 2004). In non-treated cells, PKDs-GFP were localized in cytosolic vesicles, but when treated with PDBu, PKDs-GFP moved to the plasma membrane. The anti-PKD3 antibody showed positive immunoreactivity only in cells transfected with PKD3-GFP (Supplemental figure 1, J–O), where it was colocalized with the GFP-signal, suggesting specific interaction of the antibody only with PKD3. There were no detectable signals in cells expressing PKD1- or PKD2-GFP.

Thus, PKDs are expressed in rat DRG neurons, localized in a subset of peptidergic nociceptive neurons, and, in some of these neurons, are co-expressed with PAR₂.

We similarly examined the localization of PKD1/2 and PKD3 in mouse DRG. We detected PKD1/2-LI in 59% of small, medium and large diameters neurons (72/123 neurons) (Figure 7, A–F) and PKD3-LI in 77% of small, medium and large diameters neurons (59/77 neurons) (Figure 7, G–L). Moreover, PKDs were co-localized in a subset of neurons with CGRP (PKD1/2, CGRP: figure 7, A–C; PKD3, CGRP: figure 7, G–I) and IB4 (PKD1/2, IB4: figure 7, D–F; PKD3, IB4: figure 7, J–L). Table 2 summarizes the expression pattern

for PKD1/2-LI, PKD3 and neuronal markers in histological section of mouse DRG. Thus, PKD1/2 and PKD3 are expressed in mouse peptidergic nociceptive neurons.

Inflammatory mediators and a selective agonist of PAR₂ activate PKDs in cultured primary sensory neurons

We next investigated whether inflammatory mediators activate PKDs in rat DRG in culture. To monitor the activation of the PKD isoforms we used the pSer^{744/748} antibody. In non-treated cells (treated with vehicle, 0.2% DMSO) the pSer^{744/748}-LI was detected at low levels, usually in cytosolic vesicles (Figure 8 A, J). PDBu (1 μ M, 5 min, used as a positive control, induced a 1.5-fold increase of the pSer^{744/748}-LI in the whole cell when compared to cells treated with vehicle (pixel intensity/ μ m: from 1.00 ± 0.08 to 2.55 ± 0.34), and also induced a redistribution of PKD from the cytosol to the plasma membrane (Figure 8 B, J). A mixture of inflammatory mediators applied for 1–5 min, also induced a ≥ 0.85 -fold increase of the pSer^{744/748}-LI in the whole cell (pixel intensity/ μ m: from 1.00 ± 0.08 to 1.85 ± 0.26 at 1 min, and 2.80 ± 0.52 at 5 min) (Figure 8 C, D and J). Similarly, PAR₂-AP, the selective PAR₂ agonist, induced a small but significant increase of the pSer^{744/748}-LI in the whole cell when applied for 5 min (pixel/ μ m: from 1.00 ± 0.07 to 1.42 ± 0.18 at 5 min) (Figure 8 F, G and J) when compared to cells treated with the inactive control peptide PAR₂-RP (Figure 8 E, J); PKD phosphorylation returned to basal levels 15 min after the treatment (Figure 8 H, J). There was almost no detectable signal when the primary antibody was omitted (Figure 8 I, J). After pre-treatment with inflammatory mixture and PAR₂-AP, pSer^{744/748}-LI was increased at the plasmamembrane in only a few cells, which may reflect different levels of expression of PKDs, PAR₂ and other receptors. For this reason, together with the finding that PKD1/2-LI and PKD3-LI were also detected at the plasma membrane of a few non-treated cells (Supplemental Figure 2), we were unable to examine whether PAR₂ agonists or inflammatory mediators caused membrane translocation of PKD1/2-LI and PKD3-LI in cultured neurons. However, our results suggest that inflammatory mediators and the agonist of PAR₂ can phosphorylate and activate PKD in primary sensory neurons in culture.

PAR₂ agonist activate PKDs in vivo

To investigate whether PAR₂-stimulation activates PKD *in vivo* in the intact animal, we injected saline, PAR₂-RP or PAR₂-AP into the mouse paw (10 μ g paw, 30 min). We monitored activation of PKD using the pSer^{744/748} antibody in histological sections of lumbar DRG (L4-6), that receive nervous projections from the paws (Takahashi et al., 1994). Intraplantar injection of PAR₂-AP induced a ≥ 0.14 -fold and ≥ 0.15 -fold increase of the pSer^{744/748}-LI in the total cell of medium/small diameter neurons (diameter ≤ 30 μ m) but not in large diameter neurons (diameter > 30 μ m), when compared to saline or to PAR₂-RP (average pixel: PAR₂-AP: 142.57 ± 1.74 , 161 neurons; saline: 124.51 ± 1.64 , 79 neurons; PAR₂-RP: 123.37 ± 2.28 , 118 neurons) (Figure 9 A–D). Thus, stimulation of PAR₂ can activate PKDs in primary sensory neurons, in the intact animal.

Discussion

This study shows, for the first time, that stimulation of PAR₂ promotes sub-cellular redistribution and PKC ϵ -dependent phosphorylation/activation of PKD1, PKD2 and PKD3 expressed in a heterologous system, HEK293 cells. Our results also show that PKDs are expressed in rat and mouse DRG neurons. Some of these neurons are peptidergic, nociceptive neurons that express PAR₂ and the PKD1 target, TRPV1. An agonist of PAR₂ and proinflammatory mediators activate PKDs in these neurons in culture and in the intact animals. Thus, PKD isoforms are present in nociceptive neurons and are activated by agents, notably PAR₂ agonist, that cause neurogenic inflammation and hyperalgesia.

PAR₂ agonist activates PKD1, PKD2 and PKD3 by a PKC ϵ -dependent mechanism

To determine if stimulation of PAR₂ induces activation of PKD, we used HEK293 cells transfected with GFP-tagged PKDs. HEK293 cells are a model system that has been extensively used to characterize molecular mechanisms of signaling, including neuron-specific pathways (Campbell et al., 2005; Shaw et al., 2002). Although originally derived from human embryonic kidney epithelial cells, HEK293 cells resemble neurons in that they express neuronal proteins and display neuronal characteristics (Campbell et al., 2005; Shaw et al., 2002). Moreover, HEK293 cells are an established model system used to characterize PAR₂ signaling and regulation of TRPV channels (Amadesi et al., 2004; Dai et al., 2007; Grant et al., 2007), including kinase activation (Amadesi et al., 2006).

We found that PAR₂ stimulation promoted translocation to the plasma membrane and phosphorylation of all the three PKD isoforms expressed in HEK293 cells, indicative of activation. To our knowledge, this is the first report of a role of PKD as second messenger in the molecular pathway of PAR₂-mediated responses. However, the contribution of different PKD isoforms to PAR₂-mediated effects remains unknown. PKD1 directly interacts with TRPV1 in cell lines and in primary sensory neurons, thereby regulating TRPV1 functions, such as sensory perception and hyperalgesia (Wang et al., 2004). Thus, it is possible that PKD contributes to PAR₂-induced sensitization of TRPV channels. We recently showed that PAR₂ sensitizes TRPV1 and TRPV4 to induce hypersensitivity to thermal and mechanical stimuli, and that this sensitization requires activation of PKC, which phosphorylates TRP channels, enhancing activity (Amadesi et al., 2006; Grant et al., 2007). Further, PAR₂-mediated sensitization of TRPV1 and TRPV4 is prevented by a non-selective PKC and PKD inhibitor, Gö6978 (Amadesi et al., 2006; Grant et al., 2007), and sensitization of TRPV1 is prevented by a selective PKC ϵ antagonist (Amadesi et al., 2006; Grant et al., 2007). PKD is a known target of PKC, including PKC ϵ (Brandlin et al., 2002; Haworth et al., 2007; Poole et al., 2008; Rey et al., 2004; Romero et al., 2006; Waldron and Rozengurt, 2003), PKC- α , - δ and - η (Berna et al., 2007; Song et al., 2007; Tan et al., 2003; Tinsley et al., 2004; Waldron et al., 1999). In previous studies, we and others have shown that PAR₂ stimulation activates PKC ϵ in transfected cells, in primary spinal sensory neurons (Amadesi et al., 2006) and in enteric submucosal neurons (Poole et al., 2007). Here we showed that stimulation of PAR₂ induced activation of PKD1, PKD2 and PKD3 and this effect was reduced in cells treated with a PKC ϵ translocation inhibitor peptide. Together, these findings suggest that a PKC ϵ -dependent activation of PKD may be a novel signal transduction pathway that mediates protease-induced responses. The finding that the PKC ϵ inhibitor reduces PKD activation in particular at the early time point (5 min after PAR₂ stimulation) is in agreement with a recent report of a rapid PKC-dependent followed by a PKC-independent PKD activation pathway as a novel mechanism of prolonged PKD activation induced by agonists of GPCR such as bombesin (Jacamo et al., 2008). The small reduction of PAR₂-mediated activation of PKD obtained by the inhibition of PKC ϵ suggests that the activation of other upstream PKC isoforms is maybe involved. PAR₂ stimulation can activate other PKC isozymes such as PKC α (Ahamed and Ruf, 2004). However, it is also possible that, due to the heterologous system used in our study, it is difficult to prevent the activation of an over expressed kinase (PKD) while inhibiting an endogenously expressed kinase (PKC ϵ).

PKD isoforms are expressed by peptidergic nociceptive neurons

Having established that stimulation of PAR₂ induced activation of the three PKD isoforms over-expressed in HEK293 cells, we sought to investigate if similar mechanisms occur in primary sensory neurons that play an essential role on protease-induced neurogenic inflammation and transmission of pain. We first determined if PKD are expressed in rat DRG. The RT-PCR products and the positive immunoreactivity to previously characterized

PKD1/PKD2 and PKD3 antibodies (Rey et al., 2001; Rey et al., 2003b), suggest that PKDs are expressed in rat DRG neurons. The expression of PKD1/2 in DRG neurons has been reported using Western blotting (Wang et al., 2004). However our study, using RT-PCR and immunostaining to different antibodies, further characterizes the expression of the PKD isoforms in DRG neurons. In addition, we also used markers to define the pattern of PKD expression within different neuronal cell types. To our knowledge this is the first study to confirm expression of the three PKD isoforms and characterize PKD distribution in rat DRG neurons. We found that PKDs are expressed in IB4 positive cells, and co-expressed with TRPV1 and CGRP, all markers for small-diameter nociceptive and peptidergic neurons. These findings suggest that PKDs can potentially regulate neuronal functions including neurogenic inflammation and nociception.

PAR₂ agonist and pro inflammatory mediators activate PKD in DRG neurons

Our study shows that PKDs are co-expressed with PAR₂, supporting the possibility that PKDs may regulate PAR₂-mediated neurogenic effects including hyperalgesia and inflammation. Proteases activate PAR₂, which is expressed in small-diameter nociceptive neurons, inducing the release of SP and CGRP to promote neurogenic inflammation and hyperalgesia to mechanical and thermal stimuli (Amadesi et al., 2006; Amadesi et al., 2004; Grant et al., 2007; Knecht et al., 2007; Nguyen et al., 2003; Steinhoff et al., 2000; Vergnolle et al., 2001; Vergnolle et al., 1999). Stimulation of PAR₂ activates PLC, leading to production of inositol 1,4,5-triphosphate and DAG, which increases $[Ca^{2+}]_i$ and activates protein kinases, respectively (Mule et al., 2002; Swystun et al., 2005). Agonists of GPCRs, including bradykinin and thrombin, activate PKD1, PKD2 and PKD3 in many different cell types (Rey et al., 2004; Rey et al., 2003a; Tan et al., 2003; Yuan et al., 2005; Zugaza et al., 1997). We found that bradykinin, other inflammatory compounds and a selective PAR₂ agonist induced phosphorylation and activation of PKDs in cultured rat DRG. The less robust phosphorylation and activation of PKDs induced by the different agonists in DRG compared to HEK293 cells is expected as HEK293 overexpressed PKDs, and probably express higher level of endogenous PAR₂, as suggested by intracellular calcium imaging assay and immunostaining (Amadesi, unpublished observations).

Neuronal expression of PKD is not unique to rat. We found that PKDs are also expressed in mouse small-diameter nociceptive and peptidergic DRG neurons mouse. The proportion of PKD1/2-LI and PKD3-LI positive neurons is similar in rat and mice. Nevertheless, in mice, the neuronal expression pattern between PKDs appeared different, suggesting a higher expression of PKD1/2 in peptidergic nociceptive neurons compared to PKD3. However, this result remains to be confirmed using different approaches other than immunostaining.

More importantly, we showed that intraplantar injection of PAR₂-AP in mice, increased phosphorylation of PKDs in lumbar DRG. Thus, agonists of PAR₂, including proteases released during tissue injury and inflammation, can activate PKDs in the intact animal. In support of our findings, Song et al. (2007) observed that LPS treatment activates PKD in primary spinal neurons in culture, and that peripheral inflammation induced by carrageenan activates PKD in the spinal dorsal horn in rats. Together these findings suggest that PKD may be an important mediator in the signal transduction pathway that underlies responses produced in primary sensory neurons by different inflammatory mediators including serine proteases that activate PAR₂.

In conclusion, we characterized the expression and the distribution of PKD isoforms in DRG neurons and identified PKDs as a novel molecular pathway of PAR₂-mediated responses. Our findings suggest that stimulation of PAR₂, which is co-expressed with PKDs in a sub-population of medium-small diameter nociceptive sensory neurons, can promote phosphorylation and activation of PKDs. Thus, PKDs may participate in a novel signal

transduction pathway that underlies protease-mediated effects. PKD stimulation maybe involved in PAR₂-induces phosphorylation and sensitization of TRP channels, such as TRPV1 and TRPV4. Our study adds to our understanding of the molecular mechanisms regulating responses induced by proteases that activate PAR₂, including inflammation and hyperalgesia. Proteases that activate PAR₂ are released and generated during pathological conditions, such as irritable bowel syndrome (Cenac et al., 2007). Proteases can directly stimulate sensory neurons and generate hypersensitivity symptoms through the activation of PAR₂ (Cenac et al., 2007) and stimulation of PAR₂ produces visceral hyperalgesia that is mediated by TRPV4 (Cenac et al., 2008). Therefore, our study contributes to our understanding of the molecular mechanisms of PAR₂-mediated effects and of the role of proteases in human disease. However, the function of PKDs in PAR₂-mediated effects needs to be elucidated. Further studies are required to understand the role of the PKD pathway in PAR₂-mediated neurogenic inflammation, hyperalgesia and sensitization of TRP channels. The lack of selective PKD inhibitors and the possible overlapping roles of different PKD isoforms present challenges to understanding the roles of PKD isoforms in the nervous system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation used in the figures and tables

PKD	Protein kinase D
GFP	Green Fluorescent Protein
PAR₂-AP	PAR ₂ -activating peptide
PAR₂-RP	PAR ₂ -reverse peptide
aa	Amino acid
PMA	Phorbol-12-myristate-13-acetate
pSer^{744/748}	phospho-PKD/PKCmu antibody
NT	non-treated cells
PKCεI	Protein Kinase Cε inhibitor peptide
ScPKCεI	Scrambled sequence PKCε inhibitor peptide
bp	Base pair
PKD1/2	PKD1 and PKD2 antibody
CGRP	Calcitonin Gene-Related Peptide
TRPV1	Transient Receptor Potential Vanilloid 1
PAR₂	Protease-Activated Receptor 2
IB4	Isolectin B4

N52	Anti-neurofilament protein
Veh	Vehicle, DMSO 1:1000
PDBu	Phorbol-12,13-dibutyrate
Inf. Mix	Inflammatory mixture
SP	Substance P
Ab	Antibody
BP	Blocking Peptide

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Figure 1. Stimulation of PAR₂ induced translocation and phosphorylation of PKD-GFP in HEK293 cells determined by immunostaining

HEK293 cells expressing PKD1- (A1-I1, A4-C4), PKD2- (A2-I2, D4-F4) or PKD3-GFP (A3-I3, G4-I4) were incubated with PAR₂-AP (100 μM) for 0, 1 or 5 min or with PMA (100 nM) for 5 min. PKDs were localized by immunostaining using GFP (green) and activation was monitored with a pSer^{744/748} antibody for phosphorylated kinases (magenta). In non-treated cells (0 min), PKD1- (A1-C1), PKD2- (A2-C2) and PKD3-GFP (A3-C3) were localized in the cytosol (white arrows), and PKD3-GFP was also localized in the nucleus (asterisks). pSer^{744/748}-LI was undetectable. After incubation with PAR₂-AP for 1 min, GFP and pSer^{744/748} were detected at the plasma membrane (PKD1-GFP: D1-F1; PKD2-GFP: D2-F2; PKD3-GFP: D3-F3; arrow heads). At 5 min, GFP and the corresponding pSer^{744/748}-LI returned to the cytosol (PKD1-GFP: G1-I1; PKD2-GFP: G2-I2; PKD3-GFP: G3-I3; white arrows). Note that some PKD3-GFP and the corresponding pSer^{744/748}-LI were still detected at the plasma membrane. PMA induced translocation of PKD1-GFP (A4-C4), PKD2-GFP (D4-F4), and PKD3-GFP (G4-I4) to the plasma membrane and the appearance of pSer^{744/748}-LI (arrow heads). n ≥ 3 experiments. Scale bar = 20 μm.

Figure 2. PAR₂-induced activation of PKD-GFP is mediated by PKCε

HEK293 cells expressing PKD1- (A), PKD2- (B) or PKD3-GFP (C) were incubated with PAR₂-AP (100 μM) for 5 and 15 min in the presence of PKCε inhibitor peptide (PKCεI) or the inactive scrambled sequence (ScPKCεI) (10 μM, 15 min). Kinase activation was monitored by measuring the level of pSer^{744/748}-LI normalized to GFP signal. Treatment with PAR₂-AP increased the levels of pSer^{744/748}-LI compared to non-treated cells (NT), suggesting activation of PKDs. Pre-treatment with PKCεI reduced the increase of phosphorylated PKD1- and PKD2-GFP induced when PAR₂-AP was applied for 5 min, and of phosphorylated PKD3-GFP induced when PAR₂-AP was applied for 5 or 15 min. n=3 experiments. n=80-145 cells analyzed per group. **p* < 0.05 versus ScPKCεI-treated.

Figure 3. Stimulation of PAR₂ induced phosphorylation of PKD-GFP in HEK293 cells determined by Western blotting

HEK293 cells expressing PKD1- (A, B), PKD2- (C, D) or PKD3-GFP (E, F) were incubated with PAR₂-AP (100 μM) for 0–30 min, PMA (100 nM) or PAR₂-RP (100 μM) for 5 min. Kinase activation was monitored by Western blotting (A, C, E) and densitometry (B, D, F) measuring the level of pSer^{744/748} normalized to GFP signal. In non-treated cells (NT), a p-S^{744/748}-LI was undetectable. Treatment with PAR₂-AP and PMA (used as positive control) but not with PAR₂-RP (used as negative control) increased levels of pSer^{744/748}, suggesting activation. *n* ≥ 3 experiments. **p* < 0.05 versus NT.

Figure 4. RT-PCR analysis of rat DRG

Rat DRG express mRNA for PKD1, PKD2 and PKD3. Lanes on gels are as follows: lane 1, rat PKD1 control (no reverse transcriptase); lane 2, rat PKD1 product size 448 bp; lane 3, rat PKD2 control; lane 4, rat PKD2 product size 479 bp; lane 5, rat PKD3 control; lane 6, rat PKD3 product size 445 bp; lane 7, blank; lane 8, ladder.

Figure 5. Characterization of the distribution of PKD1 and PKD2 in histological sections of rat DRG

PKD1/2-LI was detected in histological sections of rat DRG using an antibody that recognizes both PKD1 and PKD2 and co-localized with neuronal markers. PKD1/2-LI (A,D,G,J,M; green) was detected at the plasma membrane (yellow arrow heads) and in the cytosol (asterisks) of large, medium and small diameter neurons. In some neurons, PKD1/2-LI was co-localized with neuronal markers (magenta): CGRP (B,C), TRPV1 (E,F), PAR₂ (H,I), IB4 (K,L) and N52 (N,O) (white arrows). *n* = 3 experiments. Scale bar = 25 μ m.

Figure 6. Characterization of the distribution of PKD3 in histological sections of rat DRG

PKD3-LI was detected in histological sections of rat DRG and co-localized with neuronal markers. PKD3-LI (A,D,G,J,M; green) was detected at the plasma membrane (yellow arrow heads) and in the cytosol (asterisks) of large, medium and small diameter neurons. In some neurons PKD3-LI was co-localized with neuronal markers (magenta): CGRP (B,C), TRPV1 (E,F), PAR₂ (H,I), IB4 (K,L) and N52 (N,O) (white arrows). n = 3 experiments. Scale bar = 25 μm.

Figure 7. Characterization of the distribution of PKD/2 and PKD3 in histological sections of mouse DRG

PKD1/2 -LI (A–F) and PKD3-LI (G–L) were detected in histological section of mouse DRG and co-localized with the neuronal markers CGRP and IB4. PKD1/2-LI (A,D; green) and PKD3-LI (G–J; green) were detected at the plasma membrane (yellow arrow heads) and in the cytosol (asterisks) of large, medium and small diameter neurons. In some neurons PKD1/2 was co-localized with neuronal markers (magenta): CGRP (B,C) and IB4 (E,F). PKD3 was co-localized with CGRP (H,I) and IB4 (K,L) (white arrows head). $n = 3$ experiments. Scale bar = 25 μm .

Figure 8. Stimulation of PAR₂ induced activation of PKD in rat DRG in culture

Rat DRG in culture were stimulated with different test compounds and PKD activation was monitored by immunostaining (A–I) and quantified by densitometry (J) using pSer^{744/748} antibody for phosphorylated kinases. Treatments: A, vehicle (veh); B, PDBu, 100 nM, 5 min; C, inflammatory mixture (Inf. Mix: bradykinin (1 μM), SP (1 μM), 5-hydroxytryptamine (10 μM) and histamine (10 μM)) for 1 min; D, inflammatory mixture, 5 min; E, PAR₂-RP, 100 μM, 5 min; F, PAR₂-AP, 100 μM, 1 min; G, PAR₂-AP, 100 μM, 5 min; H, PAR₂-AP, 100 μM, 15 min; I, no treatment and no primary antibody. Note that PDBu and the inflammatory mixture increased levels of pSer^{744/748}-LI compared to cells treated with vehicle, suggesting PKD activation. PAR₂-AP increased levels of pSer^{744/748}-LI compared to cells treated with PAR₂-RP (J). White asterisk indicates increased pSer^{744/748}-LI. No pSer^{744/748}-LI was detected when primary antibody was omitted. For the quantification, pSer^{744/748}-LI intensity (pixels) was normalized to cell area (μm). Scale bar = 25 μm. *, ** $p < 0.05$ compared to vehicle (*) or to PAR₂-RP (**). $n = 3$ experiments. $n \geq 3$ experiments. Numbers above bars = number of cells analyzed per group.

Figure 9. Intraplantar injection of PAR₂-AP activates PKD in lumbar DRG

Test compounds were injected into the mouse paw. After 30 min, lumbar DRG (L4-L6) were collected and PKD activation monitored by immunostaining (A–C) and quantified by densitometry (D) using pSer^{744/748} antibody for phosphorylated PKD in large (diameter > 30 μ m) and medium/small size (diameter $\leq$ 30 μ m) neurons of histological sections. PAR₂-AP (10 μ g/10 μ l/paw) increased levels of pSer^{744/748}-LI (C,D) in lumbar medium/small size neurons but not in large neurons, compared to mice treated with saline (A,D) or PAR₂-RP (B,D). Scale bar = 25 μ m. * p < 0.05 compared to saline or PAR₂-RP. $n \geq 3$ experiments. Numbers above bars = number of neurons analyzed per group.

Table 1**Primary antibodies**

The immunogen, source and dilution of primary antibodies used for immunofluorescence (IF) and Western blotting (WB) are shown. aa, amino acids.

ANTIGEN	IMMUNOGEN	MANUFACTURER, SPECIES, TYPE, CATALOG NUMBER	DILUTION USED
PKD1/2	Synthetic peptide, aa 899 PEAEEREMKALSERSVS 918, from C-terminus of mouse PKD	Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit polyclonal, SC-639	1:250 IF
PKD3	Synthetic peptide, aa 877 FIMAPNPDDMEEDP 890 from C-terminus of human PKD3	Bethyl Laboratories, (Montgomery, TX, USA), USA), rabbit polyclonal, A300-319	1:500 IF
Phospho-PKD/PKCmu (Ser744/748)	Synthetic phospho-peptide, CARIIGEKsFRRsVVGVT (the lower case letters indicate phosphorylated residues), aa surrounding Ser744/748 of mouse PKD	Cell Signaling Technology, Inc. (Danvers, MA, USA), rabbit polyclonal, 2054	1:100, IF, DRG 1:200, IF, HEK-293 1:1,000, WB
CGRP	Rat alpha-CGRP, whole peptide	CURE, Antibody Core, UCLA (Los Angeles, CA, USA), mouse monoclonal, #4901	1:500 IF
CGRP	Human CGRP (#6009, Peninsula), whole peptide	Research Diagnostics, Inc., (Concord MA, USA), guinea pig polyclonal, RDI-PRO16010	1:500 IF
SP	Rat SP, whole peptide	Millipore (Chemicon, Temecula, CA, USA), Guinea pig polyclonal, AB5892	1:1,000 IF
PAR ₂	Synthetic peptide, aa 37 SKGRSLIGKVDGTSHVT G 50, of human PAR2	Santa Cruz Biotechnology (Santa Cruz, CA, USA), mouse monoclonal, SC-13504	1:250 IF
TRPV1	Synthetic peptide, aa 817 YTGSLKPEDAEVFKDSM VPGEK 838 from C-terminus of rat TRPV1	Millipore (Chemicon, Temecula, CA, USA), guinea pig polyclonal, AB5566	1:7,500 IF
Neurofilament 200 kDa, clone N52	C-terminus of dephosphorylated porcine neurofilament H protein.	Millipore (Chemicon, Temecula, CA, USA), mouse monoclonal, MAB5266	1:500 IF
GFP	Green fluorescent protein of <i>Aequorea victoria</i> origin, aa acids 1-238.	Santa Cruz Biotechnology (Santa Cruz, CA, USA), mouse monoclonal, SC-9996	1:10,000 IF

Table 2
Pattern of PKD1/2 and PKD3 expression in histological sections of rat and mouse DRG

The total number of observed neurons, the number of neurons expressing PKD1/2 and PKD/3, and the number of those neurons that coexpressed CGRP, TRPV1, PAR₂, IB4 and N52 are shown.

Rat DRG.					
	Neurons #	Neurons %		Neurons #	Neurons %
PKD1/2	255/401	64%	CGRP	13/41	32%
			TRPV1	15/32	47%
			PAR ₂	43/85	51%
			IB4	27/41	66%
			N52	24/56	43%

	Neurons #	Neurons %		Neurons #	Neurons %
PKD3	176/232	76%	CGRP	7/21	33%
			TRPV1	19/58	33%
			PAR ₂	14/31	45%
			IB4	10/31	32%
			N52	39/69	57%

Mouse DRG.					
	Neurons #	Neurons %		Neurons #	Neurons %
PKD1/2	72/123	59%	CGRP	17/34	50%
			IB4	18/38	47%

	Neurons #	Neurons %		Neurons #	Neurons %
PKD3	59/77	77%	CGRP	8/29	28%
			IB4	6/30	20%