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F-actin links Epac-PKC signaling to purinergic P2X3 receptor sensitization in dorsal root ganglia following inflammation

Yanping Gu, MD[1], Congying Wang, PhD[1], GuangWen Li, DDS[1] and Li-Yen M Huang, PhD[1]

Abstract

Sensitization of purinergic P2X3 receptors (P2X3Rs) contributes to the production of exaggerated nociceptive responses following inflammatory injury. We showed previously that prostaglandin E2 (PGE2) potentiates P2X3R-mediated ATP currents in dorsal root ganglion neurons isolated from both control and complete Freund's adjuvant-induced inflamed rats. PGE2 potentiation of ATP currents depends only on PKA signaling in control neurons, but it depends on both PKA and PKC signaling in inflamed neurons. We further found that inflammation evokes an increase in exchange proteins directly activated by cAMP (Epacs) in dorsal root ganglions. This increase promotes the activation of PKC to produce a much enhanced PGE2 effect on ATP currents and to elicit Epac-dependent flinch nocifensive behavioral responses in complete Freund's adjuvant rats. The link between Epac-PKC signaling and P2X3R sensitization remains unexplored. Here, we show that the activation of Epacs promotes the expression of phosphorylated PKC and leads to an increase in the cytoskeleton, F-actin, expression at the cell perimeter. Depolymerization of F-actin blocks PGE2-enhanced ATP currents and inhibits P2X3R-mediated nocifensive responses after inflammation. Thus, F-actin is dynamically involved in the Epac-PKC-dependent P2X3R sensitization. Furthermore, Epacs induce a PKC-dependent increase in the membrane expression of P2X3Rs. This increase is abolished by F-actin depolymerization, suggesting that F-actin mediates Epac-PKC signaling of P2X3R membrane expression. Thus, after inflammation, an Epac-PKC dependent increase in F-actin in dorsal root ganglion neurons enhances the membrane expression of P2X3Rs to bring about sensitization of P2X3Rs and abnormal pain behaviors.

Keywords

F-actin, purinergic P2X3R, Epac, PKC, dorsal root ganglion, complete Freund's adjuvant, inflammation

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Introduction

P2X3 receptors mediate enhanced pain responses after injury

Purinergic P2X3 receptors (P2X3Rs), which are abundantly expressed in dorsal root ganglion (DRG) neurons,[1–6] are thought to be directly involved in the increase in neuronal activity in response to inflammation and nerve injury.[7] We showed that inflammation-induced upregulation of P2X3R expression[8] and/or high-frequency stimulation- and nerve injury-induced increase of membrane trafficking of P2X3Rs result in a large increase in P2X3R-mediated current responses.[9,10] Furthermore, P2X3R responses to prostaglandin E2 (PGE2), a lipid mediator synthesized and released from damaged tissues surrounding nerve terminals of sensory neurons, are altered after cell damage. We found that PGE2 enhances P2X3R responses by increasing only

[1]Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX, USA

Corresponding Author:
Li-Yen M Huang, Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX 77555, USA.
Email: LmHuang@utmb.edu

PKA activity under normal conditions[11] but greatly sensitizes P2X3R responses by increasing both PKA and PKC activities after inflammation.[12,13] In addition, the exchange proteins directly activated by cAMP (Epacs)[14–18] are critically important in activating PKC-dependent signaling of PGE2 to affect P2X3R-mediated currents after injury.[12] Hucho et al.[19] found that Epac is involved in carrageenan primed independent epinephrine (Epi)-induced hyperalgesia in cultured DRG neurons.

Actin is important in PKCε-mediated nociception

Actin is one of the major cytoskeleton proteins expressed in the nervous system.[20–23] In addition to providing mechanical support of cells, axonal growth, and dendritic branching,[21,23] actin also participates in many cell functions, including protein and receptor trafficking and vesicle movements.[24,25] For instance, F-actin was found to mediate the trafficking of AMPA receptors (AMPARs),[26] transient receptor potential subfamily V cation channels (TRPV),[27] and Ca^{2+} channels inside neurons.[28,29] As a result, actin is essential in cell signaling. Cytoskeletal influence on the plasticity of cell signaling after tissue injury is likely an important mechanism underlying acute to chronic pain conversion.

Cytoskeletal proteins appear to participate in PKCε signaling in nociception after inflammation. In normal rats, cytochalasin D (CD), a microtoxin that inhibits polymerization and prolongation of F-actin, affects Epi-induced mechanical hyperalgesia, which depends on PKA and PKCε activities, but has no effect on PGE2-induced hyperalgesia, which depends on PKA activity alone.[30] When tissue is primed by a prior treatment with carrageenan to elicit transient inflammation, PGE2-induced primed hyperalgesia becomes PKCε and ERK-dependent[31,32] and is completely abolished by CD.[30,33] Thus, cytoskeletal proteins are directly linked to PKCε-mediated nociception.

We found, in our previous study, that Epac-PKC signaling is essential for enhancing P2X3R-mediated currents and behavioral responses after complete Freund's adjuvant (CFA)-induced inflammation.[12] However, it has been shown that even though PKC increases Ca^{2+} influx and magnitude of P2X3R responses in HEK cells or Aplysia oocytes, it does not directly phosphorylate P2X3Rs.[34,35] We asked if actin has any role in PGE2 potentiation of P2X3R-mediated ATP currents (I_{ATP}s) and if actin links Epac-PKC signaling to P2X3Rs in sensory DRG neurons. We show here that disruption of actin polymerization blocks PGE2 enhancement of P2X3R responses only under inflammatory conditions. Furthermore, F-actin, which is not involved in PKA signaling, is an essential link between Epac-PKC signaling and plasma membrane expression of P2X3Rs in DRG neurons.

Materials and methods

Animals

Animal experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch and performed according to the guidelines of the National Institutes of Health and the International Association for the Study of Pain. Male Sprague–Dawley rats four to eight weeks of age were used in patch recordings, immunocytochemical, and Western analyses. Seven- to nine-week-old rats were used in behavioral experiments. To induce inflammation, animals were anesthetized with isoflurane (5%), and CFA (50 µl) was injected into the plantar surface of the rat left hindpaw. CFA solution was prepared by mixing *Mycobacterium butyricum* (10 mg/ml) (Difco, Detroit, MI, USA) in a peanut oil–saline (1:1) emulsion.[8] The injected paw showed signs of localized inflammation, i.e., redness, swelling, and/or hyperalgesia a day later and the inflammatory condition remained stable for 14 days. Experiments were performed 3–14 days after the CFA treatment. Rats that developed polyarthritis were euthanized with CO_2 asphyxiation.

Behavioral experiments

Flinching of the rat left hindpaw in response to an intradermal paw injection of the P2X receptor agonist, α,β-meATP, was used to assess nociception elicited by activation of purinergic receptor.[10,36] The nocifensive behavior was analyzed according to a previously described method.[10,12] In response to α,β-meATP injection, rats not only lifted the injected paw more frequently but also kept the paw in the air for a longer period. Instead of using flinching frequency, i.e., number of paw lifts per minute, a parameter commonly used to assess flinching behaviors, paw withdrawal (PW) duration, i.e., the accumulative duration that the hindpaw was lifted in the air in a 1 min time bin, was used. Since PW duration depends on both paw lift frequency and duration, it gives a more accurate measure of nociception. All behavioral studies were performed under blind conditions.

Pharamacological agents

The Epac activator, 8-(4-chlorophenylthio)-2-*O*-methyl-cAMP (CPT), the PKC inhibitor, Bisindolylmaleimide I (Bis), and the PKA inhibitor, dihydrochloride (H89), were purchased from Calbiochem (La Jolla, CA). The P2XR agonists, ATP and α,β-meATP, the F-actin formation inhibitors, CD and Latrunculin A (LaA), and Phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO). PGE2 was purchased from Cayman Chemical (Ann Arbor, MI). *Mycobacterium*

butyrium (CFA) was purchased from Fisher scientific (Waltham, MA).

DRG cell culture

DRGs were removed from 150 to 200 g male Sprague-Dawley rats and dissected in an ice-cold, oxygenated dissecting solution consisting of (mM) 135 NaCl, 5KCl, 2 KH_2PO_4, 1.5 $CaCl_2$, 6 $MgCl_2$, 10 glucose, 10 HEPES, and pH 7.2 (osmolarity, 300–310 mosmol/l). The ganglia were incubated in a dissecting solution containing trypsin (1 mg/ml, Sigma) and collagenase D (1 mg/ml, Life Technologies, Grand Island, NY) at 37°C for 1 h. DRGs were then taken out of the enzyme solution, washed, and dissociated by trituration with fire-polished glass pipettes. Isolated cells were plated on glass coverslips and placed in culture dishes and grown with medium containing DMEM/F12 (50:50, Life Technologies) plus 2.5% fetal bovine serum and antibiotics. Experiments were performed on DRG cells cultured for 18–24 h.

Immunocytochemistry

For immunocytochemical staining, cultured DRG cells were treated with different chemicals for various periods. Immediately after treatment, DRG cells were fixed with 4% paraformaldehyde at room temperature for 20 min, washed with phosphate-buffered saline (PBS), and blocked with PBS containing 5% normal goat serum for 30 min. To determine the membrane expression of P2X3Rs in DRG cells, the primary antibody rabbit anti-P2X3R extracellular epitope, (ext-P2X3R), (1:100, Alomone Lab, Jerusalem), was used. Cells were incubated with a primary antibody at 4°C overnight. After washing the antibody out with PBS next morning, cells were incubated with secondary antibody at room temperature for 1 h. The secondary antibody used was Alexa Fluor 488 (green) goat anti-rabbit IgG (H + L) (1:500; A11034, Life Technologies). To test the specificity of the primary antibodies, we performed pre-absorption experiments on the rat DRG preparations, i.e., treating the anti-ext-P2X3R antibody with a peptide made against the epitope of the antibody. When we treated cells with the pre-absorbed primary antibody, no label was observed, suggesting that our primary ex-P2X3R antibody was specific.

To detect F-actin expression, cells were permeabilized with 0.2% triton and stained with the high-affinity F-actin probe, Alexa Fluor 594 (red) phalloidin (1:2000, Life Technologies). After sealing the coverslips onto glass slides with nail polish, cells were visualized with a laser scanning confocal microscope. ImageJ was used for analyses.

Western blots and cell surface biotinylation

Rat DRG neurons were homogenized in radio-immunoprecipitation assay lysis buffer (Fisher, Waltham, MA). The homogenate was centrifuged at 10,000 g at 4°C for 12 min to pellet out cellular debris and nuclei. The concentration of proteins in the supernatant was determined using the BCA assay (Life Technologies). Protein samples were loaded into each well of a 7% SDS/PAGE gel and underwent 2 h electrophoresis. The proteins were then eletro-transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was subsequently incubated in a Tris Buffered Saline (TBS) blocking buffer containing 5% w/v fat-free dry milk for 1–2 h and immunoblotted with primary antibodies at 4°C, overnight. Antibodies used were rabbit anti-pPKCε (1:1000, Santa Cruz, Dallas, TX) and rabbit anti-P2X3R (1:2000, Alomone Labs). The neuronal marker, β-tubulin, probed with anti β-tubulin antibody (1:2000, Santa Cruz), was used as loading control. The PVDF membranes were then washed with T-TBS solution (1 X TBS and 0.1% Tween 20) and then incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. The immunoreactive proteins were detected by enhanced chemiluminescence (ECL kit, GE Health, Pittsburgh, PA) and visualized by exposing the PVDF membrane onto an x-ray film. Using ImageJ software, the intensities of protein bands were determined, quantified, and normalized with β-tubulin bands.

To detect membrane P2X3R protein, DRG cells were first incubated with Sulfo-NHS-LC-Biotin (1 mg/ml in PBS, Life Technologies) at 4°C for 30 min. Since biotin was impermeable to the cell membrane, only cell surface proteins were biotinylated. Cells were then washed with PBS and homogenized in radio-immunoprecipitation assay buffer. Biotin bound proteins were isolated by incubating the samples with streptavidan beads (Immuno Pure Immobilized Streptavidan, Life Technologies) at 4°C overnight. The beads were spun down after a brief centrifugation (2500 g), collected, and washed with PBS. The supernatant was the cytoplasm fraction. To isolate bound proteins, beads were put into the SDS-PAGE sample buffer and boiled. After pelleting the beads with centrifugation, the supernatant was collected and loaded onto a SDS-PAGE gel to probe for P2X3R expression. The expression of the membrane protein, Na/K-ATPase (1:1000, Santa Cruz), in the samples was also determined.

Electrophysiology

I_{ATP}s were recorded from acutely dissociated neurons (mean size: 28 μm in diameter; ~ 25 pF) isolated from L4-5 DRGs of normal rats or inflamed rats 3–14 days after CFA injection. Neurons were isolated according to the procedure described before[12] except papain

(20 unit/ml; Worthington, Lakewood, NJ) instead of trypsin was used. I_{ATP}s were recorded at room temperature with an external solution containing (mM) 130 NaCl, 5 KCl, 2 KH_2PO_4, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, and pH 7.3 (295–300 mosmol/l). I_{ATP} responses were obtained by applying 2 s ATP (10 μM, Sigma) pulses every 2 min through an electronic valve. Current recordings were made under voltage-clamp conditions using the whole-cell patch recording technique. Membrane potential was held at −60 mV. Unless indicated, patch-clamp electrodes had a resistance of 3–5 Mohm when filled with the pipette solution containing (mM) 145 potassium gluconate, 10 NaCl, 10 HEPES, 10 glucose, 5 BAPTA, and 1 $CaCl_2$, and pH was adjusted to 7.25 with KOH (290 mosmol/l). The current signals were filtered at 2–5 kHz and sampled at 100 µs per point.

Statistical analysis

All data are expressed as mean ± SEM. Differences between two means were analyzed with Student's paired or unpaired *t* test. Comparisons between multiple means were done with one-way analysis of variance followed by Newman–Keuls post hoc test. A $P < 0.05$ was considered significant.

Results

Disrupting actin assembly inhibits the potentiation of I_{ATP}s by PEG2 only in CFA inflamed neurons

ATP activated P2X3R-mediated currents were recorded in the DRG neurons dissociated from control rats and CFA rats (Figure 1). These fast-decaying currents could be blocked by the P2X3R antagonist, A317491.[12] PGE2 enhanced P2X3R-mediated currents and the enhancement effect of PGE2 was larger in CFA-inflamed neurons than in control cells. To determine the role of F-actin in the potentiation of P2X3R-mediated I_{ATP}s, we studied the effect of the F-actin formation inhibitor, CD (1µM), on the PGE2-elicited increase of I_{ATP}s in control and inflamed DRG neurons. CD had no effect on the current increase in control DRG neurons, but partially blocked the PGE2 enhancing effects on P2X3R-mediated I_{ATP}s in inflamed neurons (Figure 1).

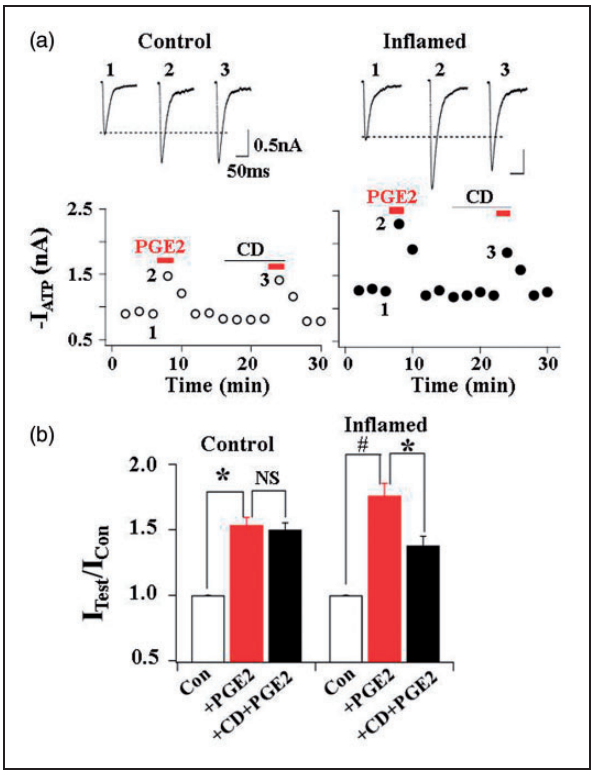

Figure 1. Effects of the F-actin assembly inhibitor, CD, on the PGE2 potentiation of I_{ATP}s in DRG neurons. (a) Changes in I_{ATP}s in response to PGE2 or CD + PGE2 applications. I_{ATP}s elicited by ATP pulse (10 µM, 2 s) applied every 2 min were recorded from DRG neurons isolated from control (left) and inflamed rats (right). The number above each trace corresponds to the current recordings indicated in the graph below. Application of PGE2 (1 µM) with ATP potentiated I_{ATP}s. In a control neuron, CD (1 µM, 10 min) pretreatment did not affect the enhancing effect of PGE2. In a CFA-inflamed neuron, PGE2-induced potentiation was partially inhibited by CD. (b) Summary of CD effects. In control neurons, PGE2/Con = 1.54 and (CD + PGE2)/PGE2 = 0.97. In CFA neurons, PGE2/Con = 1.76 and (CD + PGE2)/PGE2 = 0.78. $N = 4$–6 cells. $^{\#}P < 0.01$, $^{*}P < 0.05$. NS: not significant.

PKC participates in the F-actin effects on the PGE2-induced potentiation of I_{ATP}s in inflamed neurons

To define the role of protein kinases in F-actin dependent modulation of I_{ATP}s after inflammation, the effects of the PKA inhibitor, H89, and of the PKC inhibitor, Bis, on P2X3R-mediated I_{ATP}s were studied in dissociated DRG neurons isolated from inflamed rats. After inflammation, PGE2 elicited a large increase in I_{ATP}s. H89 (1 µM) blocked the increase partially. The F-actin formation inhibitor, CD, was effective in inhibiting the rest of the PGE2-enhanced currents (Figure 2(a)). In contrast, when the PKC pathway was blocked by Bis (1 µM), CD could no longer alter the PGE2-enhanced currents (Figure 2(b)). Thus, F-actin only affects I_{ATP}s in the presence of PKC signaling. Since Epac-dependent PKC-mediated signaling occurs only after inflammation,[12] the observation suggests that F-actin has a critical role in Epac-dependent PKC signaling of I_{ATP}s in inflamed neurons.

Activation of Epac is required for the F-actin-dependent modulation of P2X3R-mediated I_{ATP}s

To determine the role of Epac in the F-actin-dependent modulation, we used CPT, a specific Epac agonist,[14] to

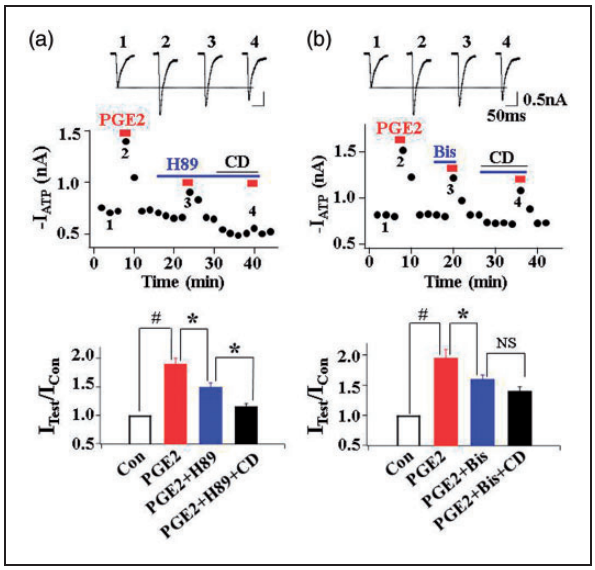

Figure 2. F-actin no longer mediates PGE2-induced potentiation of I_{ATP}s in the presence of Bis. (a) In CFA-induced inflamed neurons, following the block of PKA activity by H89, CD remained effective in blocking PGE2-induced potentiation of I_{ATP}s, i.e., (PGE2 + H89 + CD)/(PGE2 + H89) = 0.77. (b) In CFA-induced inflamed neurons, following the block of PKC activity by Bis, CD could no longer block PGE2-induced potentiation of I_{ATP}s ((PGE2 + Bis + CD)/(PGE2 + Bis) = 0.94. Thus, F-actin effect depends on the PKC pathway. $N = 4$–6 cells. $^{\#}P < 0.01$, $^{*}P < 0.05$. NS: not significant.

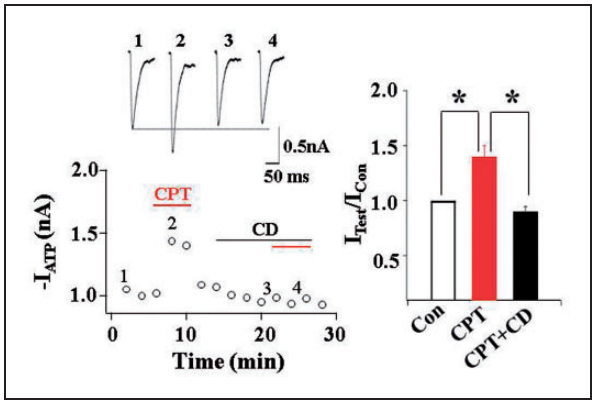

Figure 3. CD blocks CPT enhancement of I_{ATP}s in DRG neurons isolated from control rats. Activation of Epac by CPT enhanced I_{ATP}s in control DRG neurons ($I_{CPT}/I_{Con} = 1.40$). Disruption of F-actin polymerization by CD abolished the Epac-induced enhancement of I_{ATP}s ($I_{CD+CPT}/I_{Con} = 0.95$). $N = 5$, $^{*}P < 0.05$.

activate Epac in DRG neurons dissociated from control rats and determined the effect of the F-actin formation inhibitor, CD, on P2X3R-mediated currents in these neurons. CD blocked CPT-induced enhancement of I_{ATP} in control DRG neurons (Figure 3). Thus, F-actin effectively modulates I_{ATP}s when Epac is activated by CPT. This result, together with the observation that PKC inhibitor blocks F-actin effects on I_{ATP}s in inflamed neurons (Figure 2(b)) led us to conclude that activation of Epac-PKC signaling pathway is required for F-actin modulation of I_{ATP}s.

Epac-PKC signaling promotes the expression and re-arrangement of F-actin in DRG neurons

It is well documented that PKC plays important roles in the rearrangement and expression of F-actin.[37–42] To determine if a link exists between Epac-PKC signaling and F-actin expression in DRG neurons, we stained F-actin with rhodamine-labeled pholloidin and studied the effect of CPT and (CPT + Bis) on the F-actin expression in small and medium (<35 μm in diameter) cultured DRG neurons. The F-actin expression level in satellite glial cells, which encircled each neuron, was in general much higher than that in neurons thus confounding the presence of F-actin at the neuronal perimeter (data not shown). Therefore, isolated cultured DRG neurons devoid of attached satellite glial cells were used in our study. Weak F-actin staining was seen in control cells (Figure 4(a)). Following CPT treatment, F-actin expression in neurons was greatly enhanced. High-intensity punctuate phalloidin labels were seen in the cytoplasm and around the perimeter of cells. Treating cells with Bis significantly reduced the enhanced F-actin expression at the neuronal perimeter. F-actin staining in the cytoplasm became diffused while the average intensity of the label remained elevated. These results suggest that activation of Epac-PKC signaling promotes F-actin expression and reorganization especially at the perimeter of DRG neurons.

We then determined the effect of direct PKC activation on F-actin expression in DRG neurons. Treating neurons with the PKC activator, PMA, drastically increased F-actin expression at the cell perimeter. The increase was blocked almost completely by Bis (Figure 4(b)), suggesting that F-actin expression at the cell perimeter especially depends on the activity of PKC. CPT had a complex effect on F-actin (Figure 4(a)). It affected F-actin expression not only through the activation of PKC but also through the activities of other proteins in DRG neurons.

F-actin is downstream of Epac-PKCε signaling

An actin-binding motif has been identified in the PKCε molecule.[43] It was proposed that activation of PKCε exposes the binding motif and allows actin to bind, thus stabilizing the active conformation of the kinase.[43] We explored the possibility that F-actin affects PKCε activation in cultured DRG neurons. The expression of phosphorylated PKCε (pPKCε) increased significantly following 15 min pre-incubation of DRGs with CPT.

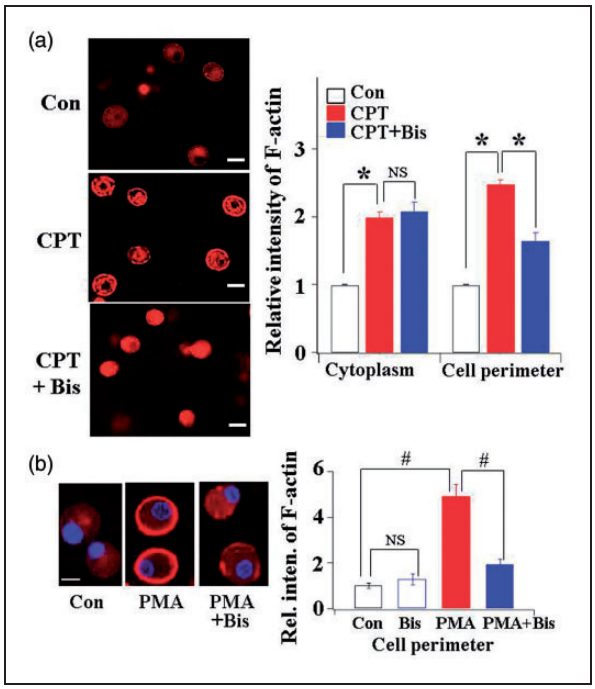

Figure 4. Effects of CPT and PMA on F-actin expression. (a) Epac-PKC signaling increases F-actin expression. F-actin expression levels were determined by the average intensity of rhodamine-labeled phalloidin in the cytoplasm and around the perimeter of cultured DRG neurons. Following 15 min incubation of CPT (1 μM), the relative intensity was 2.00 fold higher in the cytoplasm and 2.50 fold higher at the neuronal perimeter than those in the control. Bis (1 μM) did not change the F-actin label intensity in the cytoplasm ((Bis + CPT)/Con = 2.10), but significantly reduced the F-actin label at the cell perimeter ((Bis + CPT)/Con = 1.66). $N = 50$ cells, two experiments. $^*P < 0.05$. Bars = 25 μm. (b) Direct PKC activation by PMA enhances F-actin expression in the perimeter of culture DRG neurons. Following 15 min PMA (1 μM) treatment, the relative intensity of F-actin in the cell perimeter was dramatically increased (PMA/Con = 4.93). Bis (1 μM) significantly blocked PMA-induced F-actin increase ((Bis + PMA)/Con = 1.93). Bis (1 μM), by itself, had no significant effect on F-actin formation (Bis/Con = 1.28). Data obtained from 118 cells, three experiments. $^*P < 0.05$. NS: not significant. Bars = 15 μm.

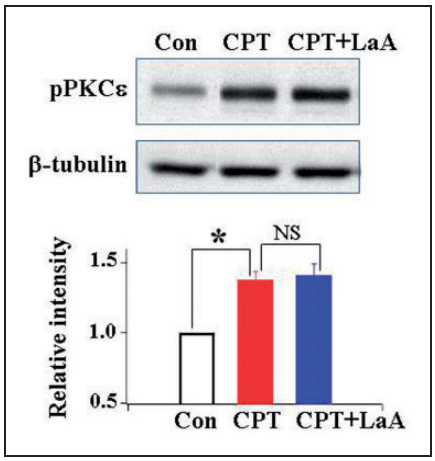

Figure 5. Epac-induced increase in pPKCε is insensitive to the disruption of F-actin polymerization. CPT (10 μM) enhanced the expression of pPKCε (CPT/Con = 1.38, n = 3) in cultured rat DRG neurons. LaA (6 μM, 30 min) preincubation did not affect the enhancing effect of CPT ((CPT + LaA)/Con = 1.41). $N = 3$, $^*P < 0.05$; NS: not significant. The expressions of β-tubulin were used as sample loading controls.

The F-actin destabilizing agent, LaA, which was applied 30 min prior to the application of CPT, did not alter CPT-induced increase in the expression pPKCε (Figure 5). Thus, F-actin had no effect on pPKCε expression. The effect of F-actin on P2X3R-mediated responses is likely downstream of Epac-PKCε signaling.

F-actin mediates PGE2-induced hyperalgesia in CFA rats

We then studied the functional consequence of F-actin involvement. In a previous study, we showed that paw-injected PGE2 enhances α,β-meATP-induced paw withdrawal duration through both PKA and PKC signaling pathways under inflammatory injury conditions.[12] Since activation of Epac promotes the expression of F-actin (Figure 4(a)), it is of interest to determine if F-actin participates in the PGE2 modulation of P2X3R-mediated behavioral responses. We studied the effect of paw-injected CD on α,β-meATP-induced flinch responses in saline-injected control rats and in CFA rats (Figure 6). The F-actin formation inhibitor, CD, by itself did not affect basal flinch responses in saline control rats. PGE2 enhanced the flinch responses in these control rats and CD had no effect on the PGE2-enhancing effects. In CFA rats, basal α,β-meATP-induced responses were higher than those in control rats. CD again had no effect on the basal flinch responses. PGE2 enhancing effect on α,β-meATP responses in CFA rats was much more pronounced. Unlike control rats, CD effectively blocked the enhanced flinch responses induced by PGE2. These observations suggest that F-actin is involved in PGE2-induced flinch responses only in CFA rats.

Activation of Epac increases the membrane expression of P2X3Rs in DRG neurons

To determine the mechanism underlying the F-actin actions on P2X3R-dependent responses, we examined the effects of Epac activation on the levels of P2X3R expression in the lysates prepared from total, membrane, and cytoplasmic proteins isolated from cultured DRGs. In response to CPT (1 μM, 10 min) stimulation, there was a 19% increase in total P2X3Rs, a 2-fold increase in the expression of membrane P2X3Rs, and a 25% decrease in

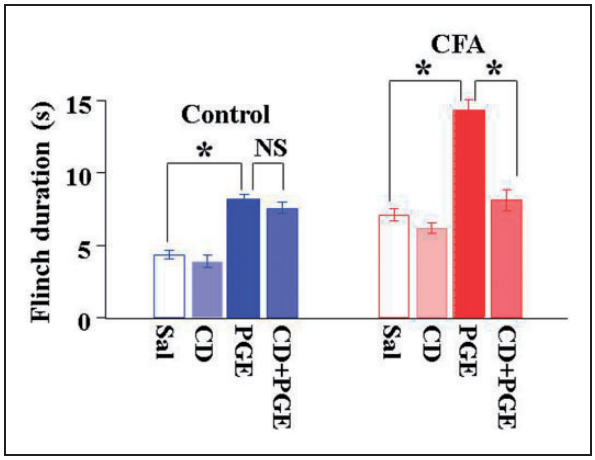

Figure 6. F-actin mediates PGE2-induced hyperalgesia only after inflammation. In control rats, PGE2 enhanced α,β-meATP-induced flinch responses (PGE2/saline = 1.89). The enhancing effect was not affected by CD treatment ((CD + PGE2)/saline = 1.78). In CFA rats, PGE2-induced hyperalgesia (PGE2/saline = 2.01) was significantly blocked when F-action polymerization was disrupted by CD ((CD + PGE2)/saline = 1.15). $N = 3$. $^{*}P < 0.05$, NS: not significant.

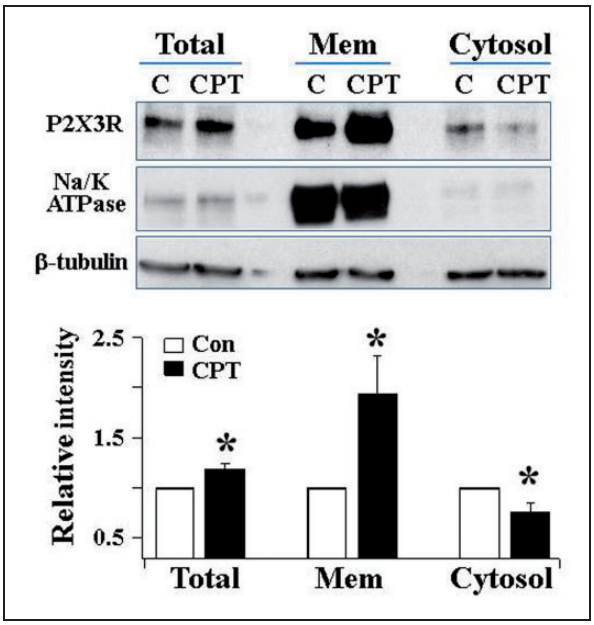

Figure 7. CPT increases the membrane expression of P2X3Rs in cultured DRGs. The P2X3R levels in total protein (Total), membrane protein (Mem), and cytoplasmic protein samples (Cytosol) before (Con) and after CPT (1 μM, 10 min) treatment were determined. Total, membrane, and cytosol protein samples were loaded onto gels. The expression of the ubiquitous membrane protein Na+/K+ ATPase was probed to indicate the membrane expressed protein levels in our samples. The expressions of β-tubulin used as sample loading controls were also indicated. CPT significantly increased P2X3Rs expressed in the cell membrane (total protein: CPT/Con = 1.19; membrane protein: CPT/Con = 1.93 and cytoplasmic protein: CPT/Con = 0.75). $N = 3$. $^{*}P < 0.05$.

cytoplasmic P2X3Rs (Figure 7). Thus, one of the major effects of Epac activation is to promote membrane expression of P2X3Rs.

F-actin mediates the Epac-enhanced membrane expression of P2X3Rs in DRG neurons

To determine the role of F-actin in the Epac-enhanced membrane expression of P2X3Rs, a specific P2X3R antibody directed against an extracellular epitope of the receptor (ext-P2X3R) was used to probe the membrane expression of P2X3Rs in cultured DRG neurons. As observed previously in DRGs,[10,44] P2X3Rs were expressed only in neurons. Activation of Epac with CPT greatly increased the average intensity of ext-P2X3R labeling and the number of ext-P2X3R-labeled cells (Figure 8). Pre-incubation of the F-actin disruptor, LaA (1 μM, 60 min), abolished the CPT-induced increase in the ext-P2X3R expression. Similar results were also observed with CD (data not shown). Thus, activation of Epac-PKC signaling enhances the membrane expression of P2X3R in DRG neurons (Figures 7 and 8) and the effect is F-actin mediated.

Discussion

F-actin plays an essential role in PGE2-induced increase of I_{ATP}s and P2X3R-mediated nocifensive behaviors after inflammation

We showed previously that PGE2 activates PKA and increases P2X3R-mediated I_{ATP}s under normal conditions.[11] The potentiating effect of PGE2 after CFA-induced inflammation is greatly enhanced as a result of activation of Epac-PKCε signaling in addition to the activation of PKA.[12] We found in this study that blocking the polymerization of actin does not affect the PKA-mediated action of PGE2 on P2X3R-mediated currents and nocifensive behaviors under control conditions. In contrast, F-actin depolymerization inhibits the Epac-PKC-mediated enhancing effects of PGE2 after CFA-induced inflammation (Figures 1, 2, and 6) or that of CPT treatment under the control condition (Figure 3). These results led us to conclude that F-actin is directly involved in Epac-PKC signaling and P2X3R sensitization.

F-actin is a downstream effector of Epac-PKC signaling to enhance membrane expression of P2X3Rs

Despite the possibility that F-actin may bind to PKCε directly to alter the kinase activity,[43] we showed that disruption of F-actin polymerization by LaA does not

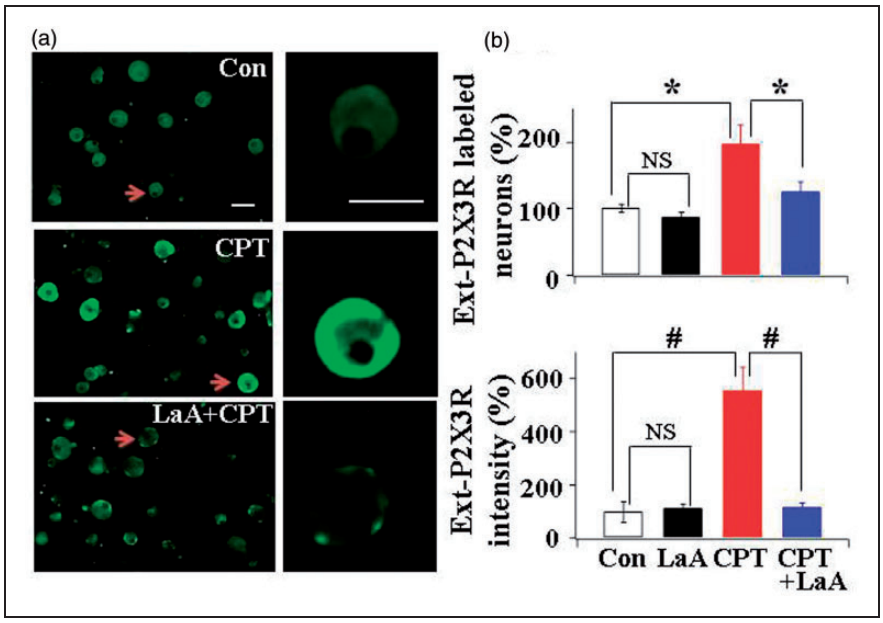

Figure 8. Epac control of the membrane expression of P2X3Rs in DRG neurons is F-actin mediated. (a) The membrane expression of P2X3Rs was examined in cultured DRG neurons using anti-ext-P2X3R antibody. The enlarged views of neurons (indicated by red arrows) are shown on the right side of each panel. Bars = 25 μm. (b) In the presence of CPT (1 μM), the percentage of cells expressing ext-P2X3R increased by 1.80 fold and the intensity of ext-P2X3R labels increased by 5.57 fold. The CPT-induced increase in membrane expressed P2X3Rs was significantly reduced by pre-incubating cells with the F-actin disrupter, LaA (1 μM, 60 min). A total of 1192 cells obtained from three experiments were used for the analyses. $^{*}P < 0.05$, $^{\#}P < 0.01$. NS: not significant.

affect CPT-induced increase in pPKCε (Figure 5). This result, together with the observation that F-actin does not affect PKCα activation in nerve cells,[45] suggests that F-actin is not involved in PKCα or PKCε activation. On the other hand, there is good evidence that PKC promotes F-actin polymerization[41,46] and reorganization.[38–40,42] We showed that CPT and PMA promote F-actin expression at the cell perimeter (Figure 4), suggesting that F-actin is downstream effector of Epac-PKC signaling. CPT was found to induce a large increase in the membrane expression of P2X3Rs in DRGs (Figure 7). The increase was abolished by the F-actin disruptor, LaA (Figure 8). These results have led us to conclude that F-actin is a critical link between Epac-PKC signaling and enhancement of membrane expression of P2X3Rs.

PKCε has been well established as the major PKC isoform mediating injury-induced hyperalgesic responses.[33,47] The role of PKCα in receptor sensitization and hyperalgesia is less clear. We recently showed that inflammatory tissue injury or CPT application enhances the activity of Epacs, which leads to a large increase in the expression of both pPKCα and pPKCε in DRGs to give rise to P2X3R-mediated hyperalgesia.[48] PKC inhibitor, Bis, blocks CPT- and PMA-induced increase in F-actin formation (Figure 4). This is consistent with the view that PKCα and PKCε promote F-actin formation in DRG neurons.

Mechanisms underlying the F-actin induced increase in P2X3R membrane expression have yet to be determined

We show here that F-actin mediates the Epac-PKC-dependent increase in the expression of membrane P2X3Rs, which results in an increase in I_{ATP} (Figures 1 to 3) and exaggerated nocifensive responses (Figure 6). The detailed mechanism by which F-actin regulates P2X3R membrane expression has yet to be identified. Some clues may be found from studies of the interactions between actin and TRPV channels in nonneuronal cells and neurons and from the F-actin action exerting on the trafficking AMPARs at synapses.

In F11 cells, a fusion cell line of rat DRG neurons and mouse neuroblastoma cells, the C-terminus of TRPV4 was found to interact directly with actin and microtubules.[49] The TRPV4-actin/microtubule complex also interacts with PKCε and CaMK. Microtubule dynamics can regulate Ca^{2+} influx through TRPV4 to modulate its activity. Reciprocally, TRPV4 modulates actin and microtubule stability to regulate growth cone movement.[49] Phosphorylation of Ser824 on TRPV4 expressed in CHO cells was found to be required for the interaction between actin and TRPV4 to promote channel activities.[50] The site of interaction between F-actin and P2X3Rs and the phosphorylation status of P2X3R for the interaction have yet to be identified.

In synaptic terminals, actin, which is a major cytoskeleton in presynaptic terminals and postsynaptic dendritic spines, regulates glutamate vesicle pools.[26,51] Polymerization of actin was found to convert silence boutons to active ones at glutamatergic terminals.[52,53] In addition to determining spine morphology, actin also has a role in the postsynaptic distribution of AMPARs and is required for functional long-term potentiation.[51] There is an elaborate F-actin control of AMPAR trafficking in dendritic spines. A number of actin-binding proteins were found to play critical roles in actin regulation of AMPAR activity.[26] Through binding to actin, these proteins regulate actin polymerization and depolymerization. By physical association with AMPARs, these actin-binding proteins also facilitate actin control of exocytosis, endocytosis, and endosomal recycling of AMPARs.[26] It is of interest to determine if F-actin interacts directly with P2X3Rs and/or indirectly through actin-binding proteins associated with the receptors.

Furthermore, Epac was found to promote interactions between actin and receptors. In microvascular smooth muscle, Epac was found to activate Ras-related small GTPase Rap1 and Rho-associated kinase to phosphorylate F-actin-binding protein, filamin-2. This results in an increase in F-actin expression and facilitates translocation of α2C-adrenergic receptors to the plasma membrane, thus causing vasoconstriction in small blood vessels.[54,55] Interaction of α2C-adrenergic receptors with A-kinase anchoring proteins increases the level of adrenergic receptors on the cell surface.[56] We showed that activation of Epac by CPT increases F-actin expression in the cell membrane and cytoplasm of DRG neurons and the PKC inhibitor, Bis, cannot completely block the increase (Figure 4(a)). The observations suggest that Epac-PKC is not the only pathway involved in Epac signaling. Other kinases such as Rap1- and Rho-associated kinases may also be involved. It is not known if any A-kinase anchoring proteins participate in the interactions among Epac-PKC, F-actin, and P2X3Rs.

Our data suggest that chronic inflammation induces an Epac-PKC dependent increase in F-actin expression at the cell perimeter to promote membrane expression of P2X3Rs in DRG neurons. Since sensitization of purinergic receptors is a major contributor to abnormal DRG neuronal activity after injury,[8,10,12,57] plasticity of Epac-PKC-F-actin control is likely the key mechanism used to switch between delimited acute pain and sustained chronic pain states after tissue injury, a phenomenon frequently observed in chronic pain patients.

Author contributions

YG, CW, GL, and LMH designed research. YG, CW, and GL performed experiments. YG and LMH wrote the paper. YG and CW contributed equally to this work.

Declaration of Conflicting Interests

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