

Epac Mediates a cAMP-to-PKC Signaling in Inflammatory Pain: An Isolectin B4(+) Neuron-Specific Mechanism

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The ϵ isoform of protein kinase C (PKC ϵ) has emerged as a critical second messenger in sensitization toward mechanical stimulation in models of neuropathic (diabetes, alcoholism, and cancer therapy) as well as acute and chronic inflammatory pain. Signaling pathways leading to activation of PKC ϵ remain unknown. Recent results indicate signaling from cAMP to PKC. A mechanism connecting cAMP and PKC, two ubiquitous, commonly considered separate pathways, remains elusive. We found that, in cultured DRG neurons, signaling from cAMP to PKC ϵ is not mediated by PKA but by the recently identified cAMP-activated guanine exchange factor Epac. Epac, in turn, was upstream of phospholipase C (PLC) and PLD, both of which were necessary for translocation and activation of PKC ϵ . This signaling pathway was specific to isolectin B4-positive [IB4(+)] nociceptors. Also, in a behavioral model, cAMP produced mechanical hyperalgesia (tenderness) through Epac, PLC/PLD, and PKC ϵ . By delineating this signaling pathway, we provide a mechanism for cAMP-to-PKC signaling, give proof of principle that the mitogen-activated protein kinase pathway-activating protein Epac also stimulates PKC, describe the first physiological function unique for the IB4(+) subpopulation of sensory neurons, and find proof of principle that G-protein-coupled receptors can activate PKC not only through the G-proteins α_q and $\beta\gamma$ but also through α_s .

Key words: nociception; inflammation; intracellular signaling; β -adrenergic receptor; cAMP; PKC; Epac; PLD; IB4

Introduction

The cardinal symptom of inflammation is increased sensitivity to mechanical stimuli (mechanical hyperalgesia or tenderness). The understanding of the underlying intracellular signaling pathways, as well as the mechanoreceptors involved, remains fragmentary. Nevertheless, one signaling component, the ϵ isoform of protein kinase C (PKC ϵ), has turned out to be important in nociceptor sensitization caused by inflammation (Khasar et al., 1999b; Numazaki et al., 2002; Sweitzer et al., 2004), peripheral neuropathies such as diabetes (Joseph and Levine, 2003a), chronic alcoholism (Dina et al., 2000), and cancer chemotherapy (Dina et al., 2001; Joseph and Levine, 2003b), as well as the transition from acute to chronic pain (Aley et al., 2000; Parada et al., 2003a,b). However, a signaling pathway leading to activation of PKC ϵ remains still to be elucidated.

Surprisingly, we recently found an indication for signaling from cAMP to PKC, suggesting that the signaling through adenylyl cyclase (AC)/cAMP does not involve PKA but branches upstream of PKA to activate PKC (Gold et al., 1998; Parada et al., 2005). However, a mechanism to account for signaling from cAMP to PKC, in nociception or other functional context, has yet to be established.

Recently, in a non-neuronal cell line, cAMP has been shown to

activate not only PKA but also the guanine exchange factor Epac (de Rooij et al., 1998; Kawasaki et al., 1998). Epac in turn activates a newly identified phospholipase, phospholipase C ϵ (PLC ϵ) (Schmidt et al., 2001), and could therefore potentially activate novel PKCs, such as PKC ϵ , through phospholipase-produced diacylglycerol (DAG) (Parekh et al., 2000). While the role of Epac in activation of mitogen-activated protein kinases (MAPKs) is a matter of intensive ongoing investigation (Enserink et al., 2002; Keiper et al., 2004), Epac is thus far not known to mediate activation of PKCs.

In the model of epinephrine-induced PKC ϵ -mediated hyperalgesia (Khasar et al., 1999b; Parada et al., 2003b), we tested the hypothesis that cAMP is upstream of PKC ϵ and that Epac, through phospholipases, mediates the cAMP–PKC cross talk, leading to translocation and activation of PKC ϵ and to the establishment of inflammatory pain.

Materials and Methods

Antibodies. Antibodies and lectins used in this study were as follows: PKC ϵ -specific monoclonal mouse antibody (BD Biosciences, Franklin Lakes, NJ), β_2 -adrenergic receptor (β_2 -AR)-specific polyclonal rabbit antiserum, polyclonal rabbit anti-Epac1 serum (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal mouse NeuN (neuronal nuclear antigen)-specific antibody (Chemicon, Temecula, CA), isolectin B4 (IB4)–Alexa Fluor 568 (Molecular Probes, Eugene, OR), donkey rabbit-specific FITC-conjugated, donkey mouse-specific rhodamine-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA), and PKC ϵ -specific rabbit serum [SN134; provided by Dr. Robert Messing, University of California, San Francisco (UCSF)].

Drugs. 1-Butanol, alprenolol, D-609, epinephrine, forskolin, (–)-isoproterenol, and 1-[6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidine-dione (U73122) were purchased from

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Sigma (St. Louis, MO); 2-butanol was purchased from Fluka (Sigma); bisindolylmaleimide I (BIM), cholera toxin, ϵ V1–2, 1-[6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione (U73122), 4-cyano-3-methylisoquinoline (CMIQ), and 8-(4-chlorophenylthio)-2'-O-methyl-cAMP (CPTOMe) were purchased from Calbiochem (La Jolla, CA); Nembutoal was purchased from Abbott Laboratories (Irving, TX); (+)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol-hydrochloride (ICI 118,551) was purchased from Tocris Cookson (Ellisville, MO); the PKA catalytic subunit was purchased from New England BioLabs (Beverly, MA).

Chemicals. Chemicals used in this study were as follows: trypsin from Worthington Biochemical (Lakewood, NJ); collagenase from Roche Diagnostics (Indianapolis, IN); NeurobasalA (without phenol red) and B27 from Invitrogen (Carlsbad, CA); glutamine, MEM, HBSS, and penicillin/streptavidin solution from UCSF Cell Culture Facility; bovine serum albumin (BSA), DMSO, glutamate, paraformaldehyde (PFA), and Triton X-100 from Sigma; normal goat serum and Vectashield from Vector Laboratories (Burlingame, CA).

Animals. Behavioral experiments were performed on male Sprague Dawley rats (200–300 g; Charles River Laboratories, Hollister, CA). Animals were housed in a controlled environment in the Animal Care Facility of the University of California, San Francisco, under a 12 h light/dark cycle. Food and water were available *ad libitum*. Care and use of animals conformed to National Institutes of Health guidelines. The UCSF Committee on Animal Research approved the experimental protocols. All efforts were made to minimize the number of animals used.

Dorsal root ganglia cultures. Cultures of dissociated dorsal root ganglia (DRG) were prepared from male Sprague Dawley rats (200–300 g; Charles River Laboratories), adapting a protocol used by us previously (Khasar et al., 1999b). Rats were anesthetized with an overdose of Nembutal (50 mg/animal, s.c.). L1–L6 DRGs were removed, desheathed, pooled, and incubated with collagenase [final concentration (f.c.), 0.125%; 1 h; 37°C], followed by a trypsin digest (f.c., 0.25%; 7 min, 37°C). Cells were separated by trituration with a fire-polished Pasteur pipette. Axon stumps and dead cells were removed by straining (40 μ m mesh), followed by centrifugation (3 min, 500 g). Cells were resuspended in 12 ml of NeurobasalA/B27 media and plated 0.5 ml/culture (0.5 DRG equivalents) onto polyornithine/laminin-precoated glass coverslips (12 mm diameter) and incubated overnight in 24 well plates at 37°C in 5% CO₂. Variability between batches of NeurobasalA media and its B27 supplement, resulting in varying translocation of PKC ϵ after stimulation, was counteracted by the addition of up to 1 μ M ethanol (according to the technical support of Invitrogen, <2.5% of the ethanol concentration already in the medium).

Stimulation. After a 15–20 h incubation, to allow cells to adhere to coverslips, the cells were stimulated. To ensure homogeneous dispersion of the stimulants, 250 μ l of the 500 μ l medium was removed, mixed thoroughly with the respective activator/inhibitor, and added back to the same culture. Inhibitors were added at a concentration of 10 \times IC₅₀ values, 15 min before stimulation. Activators were added for the indicated time in concentrations based on literature reports. For all agonists, time courses were established (some data not shown). Negative controls (unstimulated cells) were treated alike but without the addition of any pharmaceutical reagent. Although we used the physiological stimulus epinephrine for the *in vivo* experiments, we used the β -AR agonist isoproterenol *in vitro* for identification of the β -AR subtype. After treatment, the cells were washed once with PBS and fixed with PFA [4%, 10 min, room temperature (RT)].

Immunocytochemistry. PFA-fixed cells were permeabilized with 0.1% Triton X-100 (10 min, RT), followed by three washes with 0.1% BSA/PBS (5 min, RT). After blockage of unspecific binding sites (5% BSA/10% normal goat serum/PBS, 1 h, RT), the cultures were probed with the respective primary antibody in 1% BSA/PBS (overnight, 4°C), washed three times (1% BSA/PBS, 5 min, RT), and incubated with the secondary fluorophore-coupled antiserum (f.c., 1:200, 1 h, RT). After three final washes (PBS, 5 min, RT), the cultures were mounted with Vectashield onto microscope slides and sealed with nail polish.

Evaluation of PKC ϵ translocation. Cells were evaluated with a Nikon (Tokyo, Japan) Microphot FXA microscope, using a 50 \times oil-immersion

objective. Fifty randomly selected cells per culture were evaluated. Data are plotted as mean percentage of translocating cells per evaluated culture \pm SEM based on the number of evaluated cultures. All counting was done in blind manner by the same observer. All treatments have been repeated with DRG neurons from different rats, on at least 2 separate days. Confocal images were taken with a 100 \times oil-immersion objective using a Zeiss (Oberkochen, Germany) Axiovert 100 microscope attached to an MRC 1000 confocal microscope (Bio-Rad, Hercules, CA). Epifluorescent images were taken with a 63 \times oil-immersion objective using a Zeiss Axiovert 100 microscope.

Testing of mechanical nociceptive threshold. The nociceptive flexion reflex was quantified using the Randall Selitto paw pressure device (Analgesymeter; Stoelting, Wood Dale, IL), which applies a linearly increasing mechanical force to the dorsum of the rat's hindpaw. The nociceptive mechanical threshold was defined as the force in grams at which the rat withdrew its paw. The protocols for this procedure have been described previously (Taiwo and Levine, 1989; Dina et al., 2003). Baseline paw-withdrawal threshold was defined as the mean of six readings before test agents were injected. Each paw was treated as an independent measure, and each experiment was performed on a separate group of rats. Each group of rats was treated with only one agonist and/or antagonist injected peripherally by the intradermal route. Measurement of nociceptive threshold was taken 30 min after the administration of the hyperalgesic mediator. All behavioral testing was done between 10:00 A.M. and 4:00 P.M.. The blocking agents were injected as described previously (Khasar et al., 1995, 1999a). Because it is less membrane permeable, injections of the PKC ϵ inhibitor (ϵ V1–2) (Johnson et al., 1996) and the PKA catalytic subunit (Slice and Taylor, 1989) were always preceded by administration of 2.5 μ l of distilled water in the same syringe, separated by a small air bubble, to produce hypo-osmotic shock, thereby enhancing cell membrane permeability to the drug (Khasar et al., 1995, 1999a). The onset of mechanical hyperalgesia is already statistically significant after 2 min (Khasar et al., 1999a) mirroring the cellular results. The mechanical threshold was tested 30 min after injection of the respective stimulus, a time point of plateauing maximal response.

Statistical analysis. All statistical comparisons were made with one-way ANOVAs followed by Dunnett's multiple-comparison *post hoc* test, the *p* values of which are given.

Results

β_2 -Adrenergic receptor activation translocates PKC ϵ in DRG neurons

To investigate the second-messenger signaling pathway upstream of PKC ϵ , we evaluated translocation of PKC ϵ in dissociated dorsal root ganglion neurons, a central step in the activation of PKCs (Dorn and Mochly-Rosen, 2002) and an established surrogate measurement of PKC activation (Cesare et al., 1999). As observed by stimulation with bradykinin and phorbol ester (Cesare et al., 1999), also the β -AR agonist isoproterenol induced translocation of PKC ϵ to the plasma membrane of DRG neurons (Fig. 1A). Because of the intense cytoplasmic PKC ϵ signal, potential translocation to other intracellular targets was not evaluated.

A dose–response curve established that 1 μ M isoproterenol produced translocation in a maximal number of cells (cultures evaluated, $n = 8$; 21.3% \pm 2.8% PKC ϵ -translocating cells) (Fig. 1B). Translocation of PKC ϵ was transient, peaking at \sim 30 s, decaying by 90 s, and returning to baseline after 5 min (Fig. 1C). Induction of translocation by isoproterenol was mediated by β_2 -AR as it was inhibited by the β_2 -AR-specific antagonist ICI 118,551 ($n = 8$; 3.0% \pm 1.1% PKC ϵ -translocating cells) (Fig. 1C). ICI 118,551 is described as an inverse agonist not only blocking receptor activation but also reducing its baseline activity (Bond et al., 1995), reflected by the decrease in baseline PKC ϵ translocation.

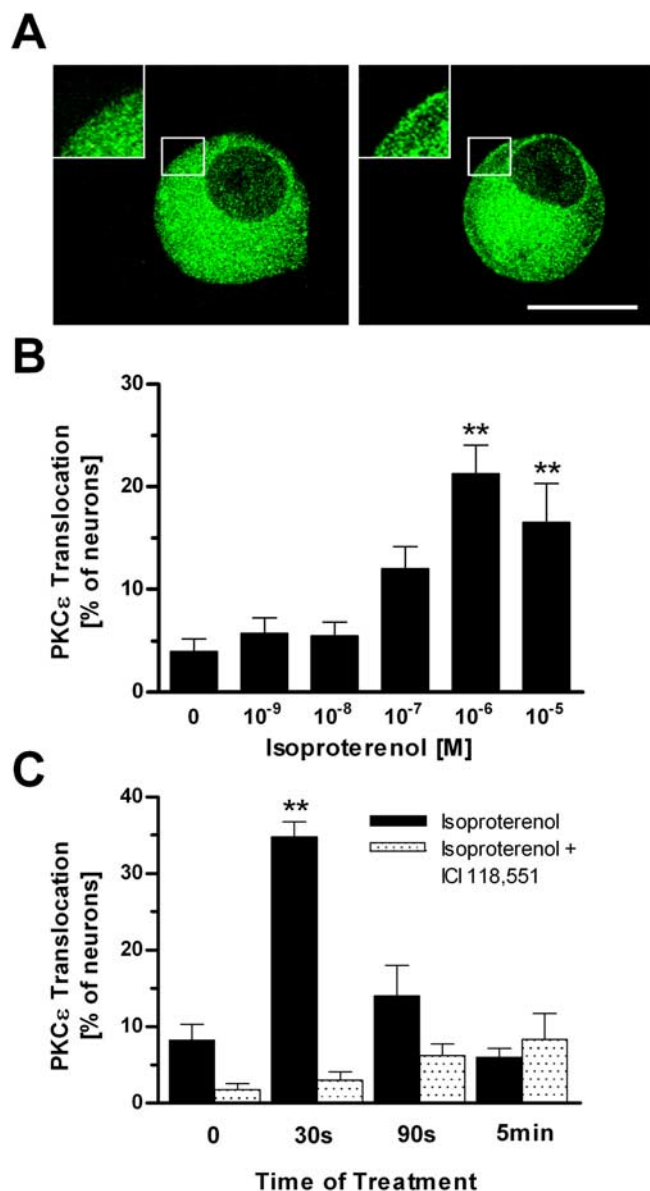


Figure 1. β_2 -AR agonist induced translocation of PKC ϵ in cultured DRG neurons. **A**, Confocal images of representative untreated (left) versus isoproterenol-treated ($1 \mu\text{M}$, 30 s; right) cultured DRG neurons. Cultures were fixed after treatment, incubated with the affinity-purified PKC ϵ -specific antiserum SN134 (1:1000), and detected with FITC-coupled donkey anti-rabbit IgG serum (1:200). Insets, Enlarged area, indicated by the white frame. After isoproterenol treatment, a portion of PKC ϵ can be seen to translocate to the plasma membrane. Scale bar, 20 μm . **B**, Percentage of neurons demonstrating, after 30 s, PKC ϵ translocation to the plasma membrane in response to indicated concentrations of isoproterenol. **C**, Percentage of neurons demonstrating PKC ϵ translocation with isoproterenol ($1 \mu\text{M}$) stimulation, for indicated time points. Filled bars represent cultures treated with $1 \mu\text{M}$ isoproterenol. Cultures represented by dotted bars were pretreated for 15 min with the β_2 -AR-specific inhibitor ICI 118,551 ($50 \mu\text{M}$) before stimulation with $1 \mu\text{M}$ isoproterenol. ** $p < 0.01$. Error bars represent SEM.

PKC ϵ translocation is mediated by α_s and AC but not by PKA

To establish that the β_2 -AR signals to PKC through cAMP, we applied the well described activator of α_s , cholera toxin, to the cell cultures, classically leading to a rise in intracellular cAMP. Translocation of PKC ϵ to the plasma membrane was observed, starting 30 s after treatment and peaking by 90 s ($n = 6$; $15.2 \pm 3.0\%$ and $24.0 \pm 1.9\%$, respectively, of neurons showing PKC ϵ translocation) (Fig. 2B).

Because α_s activates AC (Neves et al., 2002), we used the well

established activator of AC, forskolin, to test for its involvement in the signaling cascade leading to PKC ϵ translocation. With a maximal response time of 30 s, forskolin also induced the translocation of PKC ϵ to the plasma membrane ($n = 6$; $22.3 \pm 4.2\%$ PKC ϵ -translocating cells) (Fig. 2B).

Because cAMP activates PKA, we tested for the involvement of PKA in isoproterenol-induced PKC ϵ activation. Because the commonly used PKA inhibitor H89 also inhibits ligand binding to the β_2 -AR (Penn et al., 1999), and inactive cAMP analogs such as Rp-cAMP also risk inhibiting other cAMP effectors, we used the PKA-specific membrane-permeable inhibitor CMIQ, which blocks the ATP-binding site of PKA (Lu et al., 1996). Preincubation (15 min) with CMIQ completely abrogated hyperalgesia *in vivo* induced by injection of the catalytic subunit of PKA (Slice and Taylor, 1989) (Fig. 2B). Therefore, we preincubated the DRG cultures with CMIQ for 15 min with concentrations up to 1000-fold greater than its IC_{50} value, before stimulation with isoproterenol. Intriguingly, in the presence of CMIQ, PKC ϵ still translocated to the plasma membrane, as in control conditions ($n = 6$) (Fig. 2A). Thus, although α_s and AC are involved in the signal transduction from the β_2 -AR to PKC ϵ , PKA is not.

Epac mediates PKC ϵ translocation

Because cAMP is not signaling through PKA to induce PKC ϵ activity, we tested whether cAMP activation of Epac leads to translocation of PKC ϵ in dissociated DRG neurons. An Epac-specific activator, the cAMP analog CPTOMe, has been developed for the differentiation of PKA and Epac-mediated effects (Enserink et al., 2002; Rehmann et al., 2003). Stimulating neurons with this compound led to robust translocation of PKC ϵ to the plasma membrane, as seen with the activators of β_2 -AR, α_s , and AC, corroborating the observation that the inhibition of PKA does not change the extent of PKC ϵ translocation (Fig. 2B) and, for the first time, placing Epac upstream of PKC.

Because CPTOMe induced a maximal number of cells with translocated PKC ϵ at 90 s ($n = 6$; $20.3 \pm 2.6\%$ vs $23.5 \pm 3.7\%$ translocating neurons after CPTOMe stimulation for 30 s and 90 s, respectively), this time point was used in the subsequent investigation of downstream events of CPTOMe stimulation of Epac.

PKC ϵ translocation is PLC and PLD dependent

DAG, a product of members of the PLC family, can activate novel PKCs, such as PKC ϵ . We tested for the involvement of phosphatidylcholate (PC) and phosphatidylinositol (PI) hydrolyzing PLCs, as well as for the involvement of phospholipase D in Epac-induced PKC ϵ translocation.

To check for the involvement of PI-PLC, of which PLC ϵ is a member (Schmidt et al., 2001), we preincubated the cultures for 15 min with the PI-PLC inhibitor U73122 before stimulating with isoproterenol for 30 s. The translocation of PKC ϵ was completely blocked by this inhibitor. In contrast, the inactive analog of U73122, U73343, produced only a slight reduction in translocation ($n = 8$; $0.3 \pm 0.3\%$, and $15.3 \pm 1.9\%$ PKC ϵ -translocating cells in U73122- vs U73343-pretreated cultures; $19.0 \pm 3.0\%$ PKC ϵ -translocating cells in isoproterenol controls) (Fig. 3A).

The PI-PLC inhibitor U73122 and its negative control U73343 were solubilized in DMSO. By itself DMSO does not show any inhibitory influence over a wide range of concentrations, as exemplified here by the highest concentration used (dilution 1:200; $n = 8$; $21.3 \pm 2.9\%$ PKC ϵ -translocating cells) (Fig. 3A).

In addition, DAG can be produced indirectly by PLD, which produces phosphatidic acid (PA) that is metabolized to DAG (Rizzo and Romero, 2002). Also, PLD-produced PA has been

shown, in rat basophilic leukemia (RBL-2H3) cells, to act on PKC ϵ directly (Jose Lopez-Andreo et al., 2003). Therefore, cultures were pretreated with the competitive PLD inhibitor 1-butanol or its control, 2-butanol, for 15 min and then stimulated with 1 μ M isoproterenol for 30 s. The use of 1-butanol, but not 2-butanol, results in a decrease in the number of PKC ϵ -translocating cells, to baseline levels ($n = 8$; $5.5 \pm 1.1\%$ PKC ϵ -translocating cells in 1-butanol-pretreated vs $16.0 \pm 2.4\%$ in 2-butanol-pretreated cultures) (Fig. 3A).

The compound D-609 has been shown to block PC-hydrolyzing PLCs (PC-PLC) and leave the activity of PI-PLC and other PLCs unchanged (Schutze et al., 1992). Preincubation of DRG neuron cultures with D-609 for 15 min before stimulating with isoproterenol for 30 s produced no change in isoproterenol-induced translocation of PKC ϵ [$n = 8$; $20.8 \pm 2.9\%$ (D-609 pretreated) PKC ϵ -translocating cells in contrast to $19.0 \pm 3.0\%$ in isoproterenol controls] (Fig. 3A), suggesting that PC-PLCs are not involved in the activation of PKC ϵ . Thus, both PI-PLC and PLD, but not PC-PLC, are necessary for the isoproterenol-induced translocation of PKC ϵ .

To exclude that the activity of PLC and PLD leads to the activation of a PKC subtype different from PKC ϵ , which then in turn contributes to the activation of PKC ϵ , we used the PKC inhibitor BIM, which inhibits PKC activity by blocking the ATP-binding site, but which does not inhibit translocation of PKCs in the course of activation (Toullec et al., 1991). Again, the cultures were pretreated for 15 min with the inhibitor before stimulation with isoproterenol. There was no reduction in cells, showing PKC ϵ translocation ($n = 8$; PKC ϵ translocation in $19.8 \pm 1.3\%$ in BIM-treated vs $19.0 \pm 3.0\%$ in untreated isoproterenol-stimulated cells) (Fig. 3A). Therefore, PI-PLC and PLD are acting not via a different PKC subtype but on PKC ϵ directly.

PLC and PLD are downstream of Epac

To investigate whether PI-PLC and PLD are downstream of the α_s /AC/cAMP/Epac signaling pathway delineated with the previous experiments and not downstream of a possible parallel-signaling cascade [e.g., transactivation of a receptor tyrosine kinase leading to lipase activation (Luttrell et al., 1997; Lee and Chao, 2001)], we evaluated for the influence of U73122 and U73343 or 1-butanol and 2-butanol on PKC ϵ translocation after direct activation of Epac with CPTOMe. Again, cultures were pretreated with the respective inhibitor or its inactive control compound for 15 min before adding the Epac activator CPTOMe and stimulating for 90 s. As shown in Figure 3B, the activity of both phospholipases is also necessary for the translocation of PKC ϵ to occur in response to direct activation of Epac ($n = 6$;

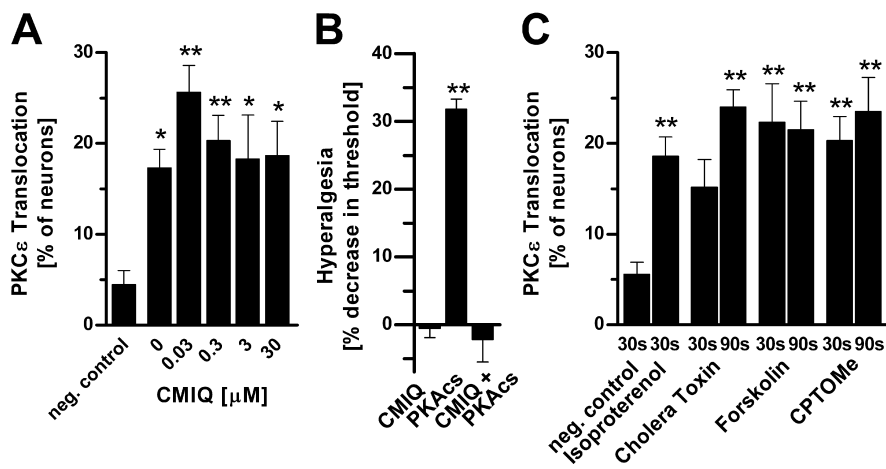


Figure 2. G-protein α_s , adenylyl cyclase, and Epac, but not PKA, are involved in PKC ϵ translocation. **A**, DRG cultures were pretreated for 15 min with indicated concentrations [1, 10, 100, and 1000 \times the CMIQ-IC $_{50}$ (0.03–30 μ M)] of the PKA-specific inhibitor CMIQ (Lu et al., 1996) before stimulation with 1 μ M isoproterenol for 30 s. Cultures not treated with either CMIQ or isoproterenol served as negative controls. **B**, Injection of the PKA inhibitor CMIQ (2.5 μ g/2.5 μ l) intradermally in rat paws did not change the mechanical paw-withdrawal threshold. Injection of active PKA [PKA catalytic subunit (PKAcs); 25 U/2.5 μ l] induced robust mechanical hyperalgesia. Preinjection of CMIQ 15 min before the injection of PKAcs completely blocked the PKA-induced hyperalgesia *in vivo*. **C**, Cultures were stimulated with activators of G-protein α_s (cholera toxin; 1 μ g/ml), adenylyl cyclase (forskolin, 5 μ M), and Epac (CPTOMe, 10 μ M), for the indicated time. Unstimulated cells served as a negative control, and isoproterenol (1 μ M, 30 s)-treated cells served as a positive control. * $p < 0.05$; ** $p < 0.01$. Error bars represent SEM.

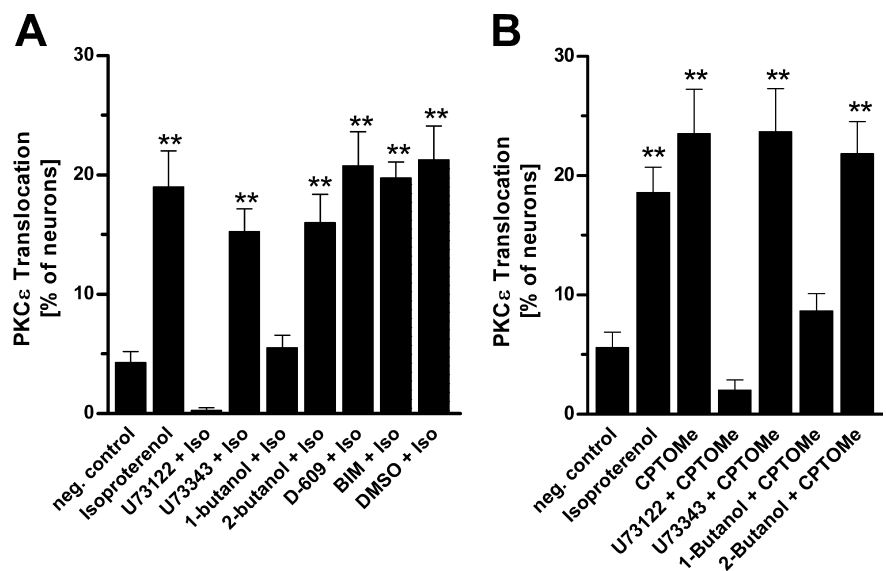


Figure 3. β_2 -AR-induced Epac-mediated translocation of PKC ϵ requires the activity of both PI-PLC and PLD. **A**, Percentage of neurons demonstrating PKC ϵ translocation to the plasma membrane after stimulation with isoproterenol (Iso; 1 μ M, 30 s). Cultures were pretreated for 15 min with the indicated inhibitors: PC-PLC inhibitor (D-609; 30 μ M), PI-PLC inhibitor (U73122; 10 μ M), inactive homolog of U73122 (U73343; 10 μ M), PLD inhibitor (1-butanol; 50 mM), inactive homolog of 1-butanol (2-butanol; 50 mM); PKC kinase inhibitor (BIM; 100 nM). Concentrations used are ~ 10 times the reported IC $_{50}$ values. **B**, Percentage of neurons demonstrating PKC ϵ translocation to the plasma membrane after stimulation with the Epac-specific activator CPTOMe (10 μ M, 90 s). Inhibitors were used as in **A**. ** $p < 0.01$. Error bars represent SEM.

PKC ϵ translocation in $2.0 \pm 0.9\%$ of U73122, $23.7 \pm 3.6\%$ of U73343, $8.7 \pm 1.5\%$ of 1-butanol, $21.8 \pm 2.7\%$ of 2-butanol-pretreated and CPTOMe-stimulated cells, and $23.5 \pm 3.7\%$ in CPTOMe stimulated cells) (Fig. 3B).

β_2 -Adrenergic receptor-induced translocation of PKC ϵ occurs in IB4(+) neurons

Although almost all neurons expressed both PKC ϵ and β_2 -AR [$94.9\% \pm 1.8\%$ ($n = 409$) and $99.8\% \pm 0.2\%$ ($n = 407$) of

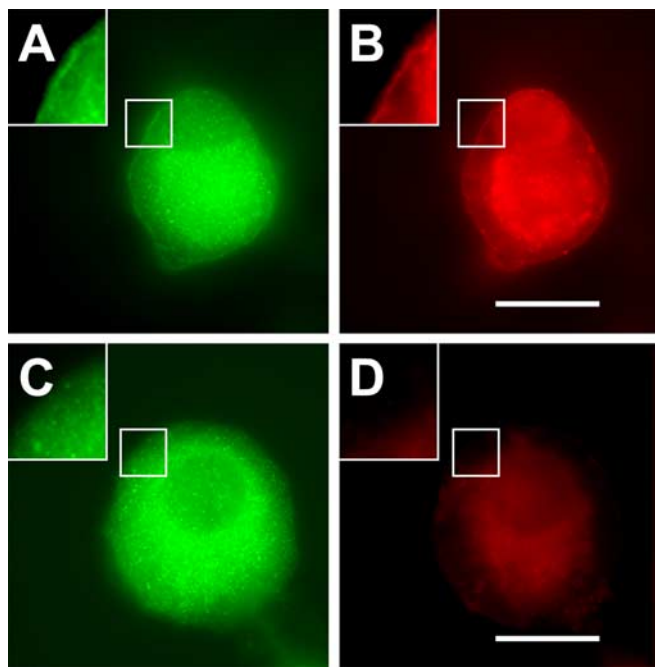


Figure 4. *A–D*, PKC ϵ translocation and IB4 double-staining epifluorescence images of double-stained cells tested for PKC ϵ (*A* and *C*; 1:1000 diluted) and IB4 (*B* and *D*; 1:100 diluted). *A, C*, Although the cell in the top row is translocating PKC ϵ to the plasma membrane (*A*), it also shows clear plasma membrane staining of the IB4 epitope (*C*). In contrast, the cell in *C* does not translocate PKC ϵ and is not positive for the IB4 epitope (*D*). Insets, Enlarged area indicated by the white frame. Scale bar, 20 μ m.

neurons, respectively], translocation of PKC ϵ was detected in only 20–35% of neurons. Because the culture system used comprises a mixture of sensory neurons subserving different function, we sought to identify a subpopulation of neurons in which translocation occurs using double staining with the marker for nonpeptidergic nociceptive neurons, IB4. The vast majority of neurons translocating PKC ϵ after β_2 -AR stimulation showed strong IB4 plasma-membrane fluorescence signal (Fig. 4) (88.6 \pm 2.4% of PKC ϵ -translocating cells show IB4 staining (evaluated cultures, n = 4; percentage of IB4 positive per PKC ϵ -translocating cells, 88.9, 92.9, 81.8, and 90.9%; total number of PKC ϵ -translocating cells, 45).

Epac activates PKC ϵ to induce hyperalgesia

To determine whether the signaling pathway upstream of PKC ϵ in cultured nociceptive neurons also applied to PKC ϵ signaling in nociceptor sensitization/hyperalgesia *in vivo*, behavioral experiments were performed. The same model as that used to establish the role of PKC ϵ in β -AR agonist-induced hyperalgesia was applied (Khasar et al., 1999b). We found that, *in vivo*, direct activation of Epac with CPTOMe robustly induces mechanical hyperalgesia [reduction of paw-withdrawal threshold by 37.7 \pm 1.9% after epinephrine (n = 6), 34.6 \pm 1.6% after CPTOMe (n = 12), and increase of threshold by 0.4 \pm 1.8 after saline injection (n = 6)] (Fig. 5A).

Subsequently, we tested whether Epac mediates hyperalgesia through the activation of PKC ϵ . We therefore stimulated the onset of hyperalgesia with CPTOMe after the injection of the specific PKC ϵ inhibitor ϵ V1–2. As shown in Figure 5A, ϵ V1–2 inhibited hyperalgesia induced by CPTOMe [reduction of paw-withdrawal threshold by 34.6 \pm 1.6% after CPTOMe (n = 12), increase by 1.6 \pm 2.7% after ϵ V1–2 (n = 6), and increase by 3.9 \pm

2.1% after CPTOMe into ϵ V1–2-pretreated paws (n = 6)]. Therefore, the *in vivo* activation of Epac also leads to the activation of PKC ϵ , which in turn leads to mechanical hyperalgesia.

Finally, to establish the role of PI–PLC and PLD as mediators of β_2 -AR agonist and Epac-induced hyperalgesia, we preinjected the paws with the respective inhibitors and their inactive controls, U73122/U73343 (PI–PLC) and 1-butanol/2-butanol (PLD), before the stimulation with either epinephrine or CPTOMe. Without stimulation, neither inhibitor influenced the baseline sensitivity of the rats (n = 6; U73122, 4.4% \pm 1.3%; U73343, 2.8% \pm 1.9%; 1-butanol, –0.0% \pm 1.8%; 2-butanol, –8% \pm 1.7%) (Fig. 5B). However, as seen *in vitro*, U73122 as well as 1-butanol inhibited the onset of CPTOMe-induced hyperalgesia [CPTOMe, 31.7% \pm 0.8% (n = 12); U73122/CPTOMe, –2.5% \pm 2.0% (n = 6); 1-butanol/CPTOMe, –0.5% \pm 1.8% (n = 6)], whereas the respective negative controls, U73343 and 2-butanol, did not [U73343/CPTOMe, 30.9% \pm 1.9% (n = 6); 2-butanol/CPTOMe, 33.4% \pm 1.7% (n = 6)]. Thus, *in vivo*, both PI–PLC and PLD could be established as essential mediators of mechanical hyperalgesia.

Discussion

Epac mediates cross talk $G\alpha_s$ /cAMP to PKC ϵ

Extensive research on G-protein-coupled receptor (GPCR) signaling has identified different pathways leading to the activation of PKC. The G-proteins α_q and $\beta\gamma$ (Gudermann et al., 1997) or transactivation of growth factor receptors (Luttrell et al., 1997; Lee and Chao, 2001), but not the G-protein α_s , have been shown to lead to the activation of phospholipases and thereby the activation of PKCs. Recently, studies of nociceptor sensitization, in which AC was activated with forskolin, suggested that the G-protein α_s might activate PKC (Gold et al., 1998; Parada et al., 2005). Using the well established activator of α_s , cholera toxin, of AC, forskolin, and the cAMP analog, CPTOMe, we provide proof of principle that, indeed, the G-protein α_s also mediates GPCR signaling toward activation of PKC ϵ .

A mechanism connecting the cAMP and PKC signaling pathways has not been elucidated. We found that the PKA inhibitor CM1Q, which blocks the ATP-binding site of PKA (Lu et al., 1996), did not abolish β_2 -AR-induced translocation of PKC ϵ in DRG neurons, indicating that the α_s /cAMP second-messenger signaling pathway branches upstream of PKA before activating PKC ϵ . We tested the hypothesis that the recently identified downstream mediator of cAMP, Epac (de Rooij et al., 1998; Kawasaki et al., 1998), could mediate this cross talk. Although Epac is known to activate MAPKs, it is not known to activate PKCs. Using the Epac-specific cAMP analog CPTOMe, we show that the activation of Epac induces PKC ϵ translocation and therefore mediates the cAMP/PKC cross talk in DRG neurons.

Epac activates PKC ϵ via PI–PLC and PLD

DAG generated by phospholipases can activate novel PKCs, such as PKC ϵ . The β_2 -AR has been shown in HEK293 (human embryonic kidney 293) cells to lead to activation of PLC ϵ (Schmidt et al., 2001), a PLC isoform for which there are no specific activators or inhibitors. Nevertheless, using the inhibitor U73122, we were able to narrow down the phospholipase involved in PKC ϵ activation to be of the same class as PLC ϵ , namely a PI–PLC, and to demonstrate its essential role in β_2 -AR/Epac-induced activation of PKC ϵ .

Although PLCs often provide the first surge in DAG production, PLD can also contribute to a rise in DAG by production of PA, which then can be converted into DAG (Nishizuka, 1992;

Clapham and Neer, 1993). Surprisingly, however, we found that not only is PLD involved, but it is necessary for the translocation of PKC ϵ . Of note in this regard, DAG as well as PA have been shown recently in RBL-2H3 cells to bind PKC ϵ at two different sites and to contribute to both the attachment and activation of PKC ϵ to the plasma membrane (Jose Lopez-Andreo et al., 2003). We now show that PLD is an essential component of PKC ϵ translocation in nociceptors.

Epac mediates PKC ϵ -dependent mechanical hyperalgesia

We investigated whether activation of Epac also leads, through PI–PLC and PLD *in vivo*, to PKC ϵ -dependent mechanical hyperalgesia. PKC ϵ -dependent hyperalgesia has been shown to require the activity of PKC ϵ in nociceptive neurons, leading to modulation of the tetrodotoxin-resistant (TTX-R) sodium channel, which is one effector component in peripheral pain (Khasar et al., 1999b; Parada et al., 2003b). Injection of modulators of PKC ϵ into the hindpaw of rats has been proven to be a suitable way to modulate the activity of PKC ϵ in nociceptive neurons and thereby to modulate PKC ϵ -dependent hyperalgesia (Khasar et al., 1999b; Parada et al., 2003b). We found that injections of the Epac activator CPTOMe induces mechanical hyperalgesia to an extent similar to the β_2 -AR acting epinephrine. Epac activation also leads *in vivo* to activation of PKC ϵ , because the use of the PKC ϵ -specific inhibitor, ϵ V1–2, completely attenuated Epac activator-induced mechanical hyperalgesia, confirming the *in vitro* delineated signaling pathway.

The central role of both phospholipases, PI–PLC and PLD, in Epac/PKC ϵ -induced hyperalgesia was confirmed *in vivo*. Inhibition of either PI–PLC with U73122 or inhibition of PLD with the inhibitor 1-butanol completely abolished Epac-mediated mechanical hyperalgesia, whereas the respective inhibitor controls, U73343 and 2-butanol, showed no effect. PI–PLC and PLD activity have been thereby introduced as new and essential components to β_2 -AR-induced/PKC ϵ -mediated mechanical hyperalgesia.

Epac activates PKC ϵ in IB4(+) nociceptors

The IB4 epitope is found on GDNF (glial cell line-derived neurotrophic factor)-dependent, small-diameter nociceptive neurons and marks ~30% of DRG neurons (Molliver and Snider, 1997; Hunt and Mantyh, 2001). These neurons make up ~70% of neurons innervating the epidermis (Lu et al., 2001), project to lamina III of the spinal cord, and are suggested to be involved in neuropathic pain (Molliver et al., 1997; Bennett et al., 1998; Boucher et al., 2000). Although IB4 has been used extensively for descriptive investigations of changes in protein expression in models of pain, little is known about the functional and mechanistic importance of these neurons. We observed translocation of PKC ϵ in only ~20–35% of cultured DRG neurons. Although we found that β_2 -AR, Epac (data not shown), and PKC ϵ are expressed in nearly all DRG neurons, translocation of PKC ϵ was highly correlated with the expression of the IB4 epitope. The molecular basis for

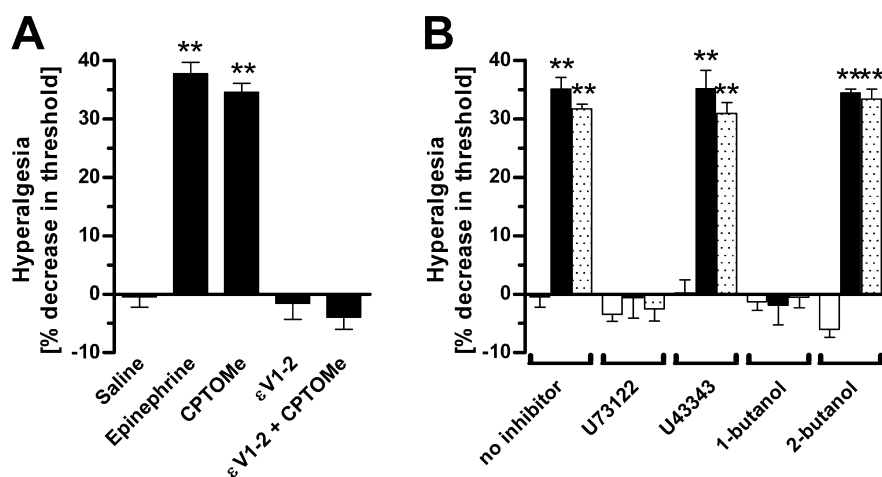


Figure 5. *In vivo* Epac mediates epinephrine-induced hyperalgesia via PI–PLC, PLD, and PKC ϵ . **A**, Injection of epinephrine (0.1 μ g in 2.5 μ l) and CPTOMe (6.3 μ g in 2.5 μ l) produce hyperalgesia of similar magnitude, whereas saline vehicle injection has no effect. CPTOMe-induced sensitization can be completely blocked by the preinjection of the specific PKC ϵ inhibitory peptide ϵ V1–2 (1 μ g in 2.5 μ l) 30 min before stimulation with CPTOMe, demonstrating that, *in vivo*, Epac also induces mechanical hyperalgesia through activation of PKC ϵ . **B**, Epinephrine-induced (filled bars) and CPTOMe-induced (dotted bars) mechanical hyperalgesia can be completely blocked by preinjection of the PI–PLC inhibitor U73122 (2.5 μ l, 1 μ g/ μ l), 30 min before epinephrine/CPTOMe stimulation. Likewise, resembling our *in vitro* data, the injection of the PLD inhibitor 1-butanol (2.5 μ l, 10.9 mM), but not its inactive control, 2-butanol, completely inhibits the sensitization through epinephrine/CPTOMe injection. The inhibitors show little or no effect on saline control injections (open bars). Both phospholipases are therefore also necessary for the mediation of β_2 -AR-stimulated/Epac/PKC ϵ -mediated mechanical hyperalgesia *in vivo*. ** p < 0.01. Error bars represent SEM.

this specificity remains elusive and could be based on differential expression of additional signaling components not identified thus far or on differential regulation of signaling components. Our observation corroborates IB4(+) neurons as nociceptors and suggests a functional difference between IB4-positive and IB4-negative/peptidergic nociceptors. Because the TTX-R sodium current is modulated by PKC ϵ as well as other mechanisms (Khasar et al., 1999b), it will be interesting to also investigate whether these additional mechanisms are present in IB4(+) neurons. Although other molecules involved in nociception, such as TRPV1 (transient receptor potential vanilloid receptor-1), are expressed overlapping with different subpopulations of DRG neurons (Guo et al., 1999; Zwick et al., 2002), we describe with this observation the first cellular mechanism restricted to the IB4(+) subtype of nociceptive neurons.

Epac and other signaling pathways mediating hyperalgesia

In conclusion, by delineating a signaling pathway leading to the activation of PKC ϵ *in vitro* and *in vivo* (for overview, see supplemental data, available at www.jneurosci.org as supplemental material), we present a detailed analysis of an intracellular signaling pathway in sensory neurons underlying pain. We thereby elucidate a novel mechanism of signal transduction, a cross talk between the two ubiquitous signaling pathways cAMP and PKC, which are both used in a wide variety of mechanisms, such as neuronal plasticity. We provide evidence that Epac is a key element in this cross talk. Importantly, this signaling pathway in DRG neurons is restricted to the IB4(+) nociceptors, establishing a first mechanism specific to this subpopulation of nociceptors. Delineating the cascade leading to activation of PKC ϵ , we introduce two new potential targets for treatment of pain, Epac and PLD. In addition, we provide proof of principle that not only the G-proteins α_q and $\beta\gamma$ but also α_s can activate PKC.

Our results provide a powerful entry point to also start investigating the intracellular signaling pathways in hyperalgesia in-

duced by other inflammatory mediators. Intriguingly, the literature on Epac and its downstream target, Rap1, also suggests even further cross talk to other pathways important in nociceptor function, such as PKA and MAPK (Khasar et al., 1999b; Aley et al., 2001; Stork and Schmitt, 2002), integrins (Bos et al., 2003; Rangarajan et al., 2003; Dina et al., 2004), and growth factor receptors (Boucher et al., 2000).

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