

Critical role for Epac1 in inflammatory pain controlled by GRK2-mediated phosphorylation of Epac1

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cAMP signaling plays a key role in regulating pain sensitivity. Here, we uncover a previously unidentified molecular mechanism in which direct phosphorylation of the exchange protein directly activated by cAMP 1 (EPAC1) by G protein kinase 2 (GRK2) suppresses Epac1-to-Rap1 signaling, thereby inhibiting persistent inflammatory pain. Epac1^{-/-} mice are protected against inflammatory hyperalgesia in the complete Freund's adjuvant (CFA) model. Moreover, the Epac-specific inhibitor ESI-09 inhibits established CFA-induced mechanical hyperalgesia without affecting normal mechanical sensitivity. At the mechanistic level, CFA increased activity of the Epac target Rap1 in dorsal root ganglia of WT, but not of Epac1^{-/-}, mice. Using sensory neuron-specific overexpression of GRK2 or its kinase-dead mutant *in vivo*, we demonstrate that GRK2 inhibits CFA-induced hyperalgesia in a kinase activity-dependent manner. *In vitro*, GRK2 inhibits Epac1-to-Rap1 signaling by phosphorylation of Epac1 at Ser-108 in the Disheveled/Egl-10/pleckstrin domain. This phosphorylation event inhibits agonist-induced translocation of Epac1 to the plasma membrane, thereby reducing Rap1 activation. Finally, we show that GRK2 inhibits Epac1-mediated sensitization of the mechanosensor Piezo2 and that Piezo2 contributes to inflammatory mechanical hyperalgesia. Collectively, these findings identify a key role of Epac1 in chronic inflammatory pain and a molecular mechanism for controlling Epac1 activity and chronic pain through phosphorylation of Epac1 at Ser-108. Importantly, using the Epac inhibitor ESI-09, we validate Epac1 as a potential therapeutic target for chronic pain.

GRK2 | Epac1 | chronic pain | Piezo2 | Epac1 translocation

Today >116 million adults in the United States are affected by chronic pain (1), which is accompanied by disability, depression, and reduced quality of life. Chronic pain is associated with multiple inflammatory conditions, such as rheumatoid arthritis, inflammatory bowel disease, osteoarthritis, and postsurgical inflammation (1). Inflammatory mediators (including PGE₂, substance P, bradykinin, ATP, and IL-1 β) act on primary sensory neurons (nociceptors) and alter the excitability of nociceptors via transcriptional and/or posttranslational mechanisms to cause pain hypersensitivity (hyperalgesia). cAMP signaling plays a key role in pain signaling, and mice deficient in adenylate cyclase activity are protected against inflammatory pain (2, 3). The contribution of the cAMP sensor protein kinase A (PKA) to inflammatory pain has been described extensively (3–5). Exchange protein directly activated by cAMP 1 (Epac1) is a more recently discovered effector of cAMP signaling. The first evidence for a role of Epac1 signaling in pain came from Levine and coworkers, who reported that intraplantar injection of the Epac agonist 8-(4-chlorophenylthio)-2'-O-methyl-cAMP (8-pCPT) leads to long-lasting mechanical hyperalgesia (6). More recent evidence shows that Epac1^{-/-} mice are protected against development of chronic neuropathic

pain (7), but it is not known whether Epac1 is also required for inflammatory pain.

Epac1 is a guanine nucleotide exchange factor (GEF) catalyzing the exchange of GDP for GTP for the Ras-like GTPases Rap1 and Rap2, resulting in their activation (8–10). Subcellular localization of Epac1 is dynamic and spatiotemporally regulated. In cells, agonist stimulation induces rapid translocation of cytosolic Epac1 to the plasma membrane (PM), where it binds phosphatidic acid (PA) via its Disheveled/Egl-10/pleckstrin (DEP) domain and activates a local Rap1 pool (11, 12). *In vitro* it has been shown that activated Rap signals to, e.g., Akt (13), PLC- ϵ (14), PKC, and MAPKs (15). Our recently published work showed that the serine–threonine kinase G protein-coupled receptor kinase 2 (GRK2) inhibits Epac1-mediated pain signaling (16, 17). Specifically, we showed that chronic inflammatory pain is associated with a decrease in GRK2 levels in pain-sensing neurons and that either increasing GRK2 protein levels or reducing Epac1 levels prevents chronic pain (17). However, the mechanism via which GRK2 controls Epac1-mediated pain signaling remained to be identified. Here we demonstrate that Epac1 is required for inflammation-induced mechanical hyperalgesia

Significance

Chronic pain represents a massive therapeutic challenge that requires understanding of underlying mechanisms to develop novel treatments. We show that genetic deletion of the Rap1 guanine-nucleotide exchange factor Epac1 protects against chronic inflammatory mechanical hyperalgesia. Moreover, an orally active Epac inhibitor reverses existing inflammatory pain without affecting normal pain sensitivity. Epac1-mediated pain is controlled by G protein kinase 2 (GRK2) through inhibition of Epac1-to-Rap1 signaling and inhibition of sensitization of mechanocurrents mediated by Piezo2. Molecularly, GRK2 controls Epac1-to-Rap1 signaling by phosphorylating Epac1 at Ser-108 to inhibit plasma membrane accumulation of Epac1. Our findings provide further insights into the molecular pathways regulating Epac1 activity, demonstrate its key role in chronic pain, and identify Epac1 as a potential target for treatment of chronic pain.

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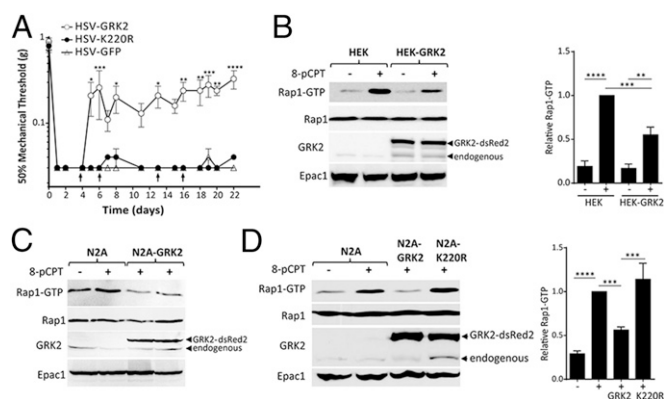


Fig. 2. GRK2-mediated regulation of Epac1-to-Rap1 signaling and chronic pain. (A) Effect of overexpression of kinase dead GRK2 (K220R) on mechanical hyperalgesia ($n = 8$ per group). Mice were treated intraplantarly with HSV-GRK2 or -GFP (empty vector) or HSV-K220R on days 4, 6, 13, and 16 after intraplantar CFA injection. Changes in 50% paw withdrawal threshold were monitored over time. Data represent mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Data were analyzed by using two-way ANOVA followed by Tukey's post hoc analyses. (B) Rap1 activation in HEK293 (HEK) or HEK293 cells overexpressing GRK2 (HEK-GRK2) cells. Cells transfected with HA-Epac1 were treated with 8-pCPT or vehicle for 20 min followed by a Rap1-GTP pull-down assay. Bar graph depicts means \pm SEM of at least three independent experiments. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ (analyzed using t test). (C) Pull-down of Rap1-GTP from N2A cells or N2A overexpressing GRK2 (N2A-GRK2). The two right lanes represent two different GRK2-overexpressing clones. (D) Rap1 activation in N2A-GRK2 or -K220R cells transfected with HA-Epac1. Cells were stimulated as in B. Data are representative of at least three independent experiments. Bar graphs represent band density for Rap1-GTP levels normalized to total Rap1, with Rap1-GTP levels in 8-pCPT-treated control N2A cells set as 1. **** $P < 0.0001$; **** $P < 0.0001$.

by GRK2 in vitro identified Ser-108 in the DEP domain of human Epac1 as a putative GRK2 phosphorylation site (Fig. 3C and Fig. S7). Phosphorylation of Ser-108 in Epac1, a conserved residue across species (Fig. 3C), was also detected in N2A cells overexpressing both GRK2 and Epac1, underlining its physiological relevance. Mutation of Ser-108 to alanine inhibited the ability of GRK2 to phosphorylate Epac1 in vitro by $\sim 50\%$, indicating that this serine residue is the major GRK2 phosphorylation site in Epac1 (Fig. 3D).

Mutation of Ser-108 to alanine, which precludes phosphorylation of the residue by GRK2, prevented GRK2-mediated inhibition of Rap1 activation (Fig. 3E). Conversely, 8-pCPT-induced Rap1 activation was reduced in N2A cells expressing a phospho-mimic for Ser108, S108E (Fig. 3E). Collectively, the data suggest that in cells overexpressing GRK2, phosphorylation of Epac1 at Ser-108 underlies the inhibition of 8-pCPT-induced Rap1 activation.

Phosphorylation of Epac1 at Ser-108 Inhibits Rap1 Activation by Inhibiting PM Association. Based on NMR studies of Epac1 and X-ray crystallographic studies of Epac2, it has been suggested that binding of cAMP or 8-pCPT induces an open conformation of Epac1, allowing the CDC25-HD domain to exert GEF activity toward Rap1 (8, 21–23). An intramolecular Epac1 FRET probe (24) stimulated with 8-pCPT induced a similar decrease in fluorescence energy transfer in cells expressing WT-Epac1 as in cells expressing the phospho-mimic mutant of Epac1 (Fig. 4A). These data indicate that phosphorylation of Ser-108 does not impair the 8-pCPT-induced conformational dynamics of Epac1 or its capacity to activate Rap1 in vitro.

An in vitro fluorescence-based GEF assay with purified Rap1 showed that WT, phospho-mimic, and phospho-deficient Epac1 have a similar capability to catalyze the GDP to GTP exchange

on Rap1 (Fig. 4B). Moreover, addition of GRK2 and ATP as a phosphate donor did not affect the agonist-induced GEF activity of WT GST-Epac1 (Fig. 4C).

In cells, 8-pCPT stimulation induces translocation of cytosolic Epac1 to the PM, where it activates a local Rap1 pool. The results in Fig. 5A demonstrate that 8-pCPT-induced translocation of the phospho-mimic mutant of Epac1 (Epac1-S108E) was significantly reduced compared with WT Epac1. Conversely, siRNA-mediated knockdown of GRK2 increased the 8-pCPT-induced PM translocation of WT Epac1 (Fig. 5B; Fig. S8). Moreover, tethering the phospho-mimic mutant of Epac1 to the PM by adding a CAAX-motif (ICUE1-PM-S108E) restored 8-pCPT-induced Rap1 activation (Fig. 5C), supporting the model that phosphorylation of Epac1 by GRK2 at Ser-108 inhibits Epac1-to-Rap1 signaling by preventing its binding to the PM.

GRK2-Mediated Regulation of Epac1 Signaling to Piezo2. In vitro evidence has shown that Epac1 signaling sensitizes Piezo2-mediated mechanocurrents. To first examine the role of Piezo2 signaling in CFA-induced mechanical hyperalgesia, we used an antisense oligodeoxynucleotide (ODN) strategy (7). Intrathecal administration of Piezo2 antisense ODN (asODN) starting on day 3 after injection of CFA to target L4–L5 DRG inhibited mechanical hyperalgesia, but not thermal hyperalgesia (Fig. 6A and B). A previous study reported that deletion of Piezo2 from advillin-positive neurons did not affect mechanical hyperalgesia, as measured 24 h after intraplantar injection of CFA. In line with these data, Piezo2 asODN treatment starting 3 d before CFA injection did not affect mechanical hyperalgesia, as measured at 24 h after CFA (Fig. S9).

Next, we investigated the effect of GRK2 on Piezo2-mediated mechanically evoked currents in HEK cells overexpressing Piezo2 and Epac1 (HEK-PE) in response to the Epac1 agonist 8-pCPT. In line with a previous study (7), activation of Epac1 by 8-pCPT shifted the stimulus/response curve to the left, indicating sensitization to mechanical stimulation. The data in Fig. 6C show that coexpression of GRK2 (HEK-PE-GRK2) abrogated this sensitizing effect of

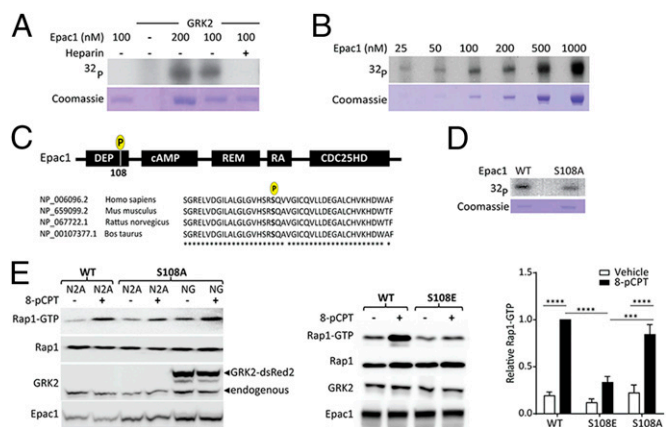


Fig. 3. GRK2 phosphorylates Epac1 at Ser-108 residue. (A) In vitro phosphorylation of Epac1 by GRK2. Visualization of ^{32}P incorporation by autoradiography analysis of GST-Epac1 incubated with purified recombinant GRK2 (50 nM) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (^{32}P) in the absence or presence of 0.01 U/ μL heparin. (B) Representative autoradiogram of ^{32}P incorporation in 25 to 1,000 nM GST-Epac1 incubated with 25 nM GRK2. (C) Alignment of Epac1 sequence surrounding Ser-108 across multiple species. (D) Autoradiogram of ^{32}P incorporation in WT-Epac1 and Epac1-S108A incubated with purified recombinant GRK2 (50 nM). (E) Rap1 activation in N2A and N2A-GRK2 (NG) cells transfected with WT-Epac1 (WT), Epac1-S108A (S108A), or Epac1-S108E (S108E) treated with 8-pCPT. Shown are data representative of three independent experiments. Bar graph represents band density for Rap1-GTP levels normalized to total Rap1, with Rap1-GTP levels in 8-pCPT-treated cells expressing WT Epac1 set as 1. **** $P < 0.0001$; **** $P < 0.0001$.

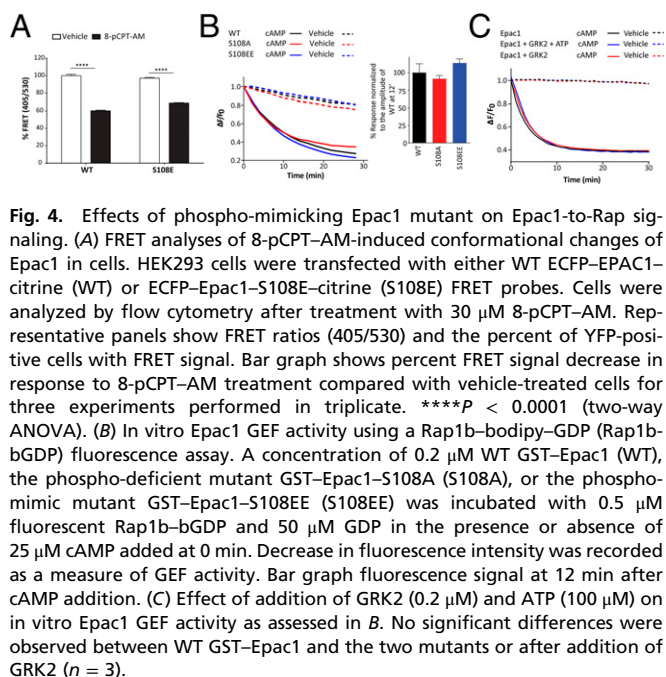


Fig. 4. Effects of phospho-mimicking Epac1 mutant on Epac1-to-Rap signaling. (A) FRET analyses of 8-pCPT-AM-induced conformational changes of Epac1 in cells. HEK293 cells were transfected with either WT ECFP-EPAC1-citrine (WT) or ECFP-Epac1-S108E-citrine (S108E) FRET probes. Cells were analyzed by flow cytometry after treatment with 30 μ M 8-pCPT-AM. Representative panels show FRET ratios (405/530) and the percent of YFP-positive cells with FRET signal. Bar graph shows percent FRET signal decrease in response to 8-pCPT-AM treatment compared with vehicle-treated cells for three experiments performed in triplicate. **** $P < 0.0001$ (two-way ANOVA). (B) In vitro Epac1 GEF activity using a Rap1b-bodipy-GDP (Rap1b-bGDP) fluorescence assay. A concentration of 0.2 μ M WT GST-Epac1 (WT), the phospho-deficient mutant GST-Epac1-S108A (S108A), or the phospho-mimic mutant GST-Epac1-S108EE (S108EE) was incubated with 0.5 μ M fluorescent Rap1b-bGDP and 50 μ M GDP in the presence or absence of 25 μ M cAMP added at 0 min. Decrease in fluorescence intensity was recorded as a measure of GEF activity. Bar graph fluorescence signal at 12 min after cAMP addition. (C) Effect of addition of GRK2 (0.2 μ M) and ATP (100 μ M) on in vitro Epac1 GEF activity as assessed in B. No significant differences were observed between WT GST-Epac1 and the two mutants or after addition of GRK2 ($n = 3$).

Epac1 stimulation on Piezo2-mediated mechanocurrents. The maximal mechanically evoked inward current was recorded before whole cell configuration was lost. At a distension of 8 μ m, 8-pCPT increased this maximal current, and overexpression of GRK2 blocked this effect of the Epac agonist (Fig. 6D). Under baseline conditions, the stimulus/response curve was similar in HEK and HEK-GRK2 cells expressing Piezo2 and Epac1.

Discussion

We demonstrate here that Epac1 is required for inflammatory mechanical hyperalgesia and describe a previously unidentified mechanism for controlling Epac1 signaling that inhibits inflammatory pain in vivo. Specifically, we demonstrate that Rap1 is activated in the DRG of mice with CFA-induced inflammatory pain, but not in Epac1^{-/-} mice that are protected from mechanical hyperalgesia. At the molecular level, we demonstrate that phosphorylation of Ser-108 in Epac1 by the kinase GRK2 inhibits Epac1-to-Rap1 signaling via preventing agonist-induced accumulation of Epac1 at the PM. We propose that this molecular mechanism underlies the protective effect of GRK2 on chronic inflammatory pain.

The competitive Epac inhibitor ESI-09 (25, 26) inhibits mechanical hyperalgesia in the CFA model of inflammatory pain, and preliminary data indicate that the same is true for the spared nerve injury model of chronic neuropathic pain. Conversely, intraplantar administration of the Epac agonist 8-pCPT induces mechanical hyperalgesia (6, 7, 16, 27). In vitro, ESI-09 inhibits both Epac1 and Epac2 (27, 28), and there is evidence that both Epac1 (7) and Epac2 (29) contribute to sensitization of pain-sensing neurons. We demonstrate here that Epac1^{-/-} mice are protected from mechanical hyperalgesia in the CFA model of inflammatory pain and that these mice are also protected against neuropathic pain (7). Therefore, we propose that Epac1 is the main target for the pain-relieving effect of ESI-09. Notably, administration of ESI-09 does not affect baseline pain sensitivity. ESI-09 is orally active, and its beneficial effect is maintained for at least 24 h after a single dose. These properties make ESI-09 an attractive candidate for clinical translation.

Epac1 acts as a GEF for Rap1 (9, 10), and, in line with a key role of Epac1 in pain signaling, Rap1-GTP levels were increased

in DRG of WT mice with CFA-induced mechanical hyperalgesia, whereas we did not detect any change in Rap1-GTP in DRG of Epac1^{-/-} mice. Thus, although multiple signaling pathways can activate Rap1, Epac1 is required for Rap1 activation in the DRG in the context of inflammatory pain.

Earlier studies by us and others have shown that Epac1 protein levels in the DRG are increased in the CFA model of chronic inflammatory pain, whereas GRK2 levels are decreased (16, 17, 30–32). In addition, we presented evidence that Epac1-dependent pain signaling is inhibited by GRK2, but the mechanism remained to be determined (17). Here, we identify GRK2, to our knowledge, as the first kinase known to directly phosphorylate Epac1, identify the site of phosphorylation, and demonstrate that this phosphorylation event inhibits Epac1-to-Rap1 signaling. We also demonstrate that, in vivo, GRK2 kinase activity is required for inhibition of Epac1-dependent inflammatory mechanical hyperalgesia. These

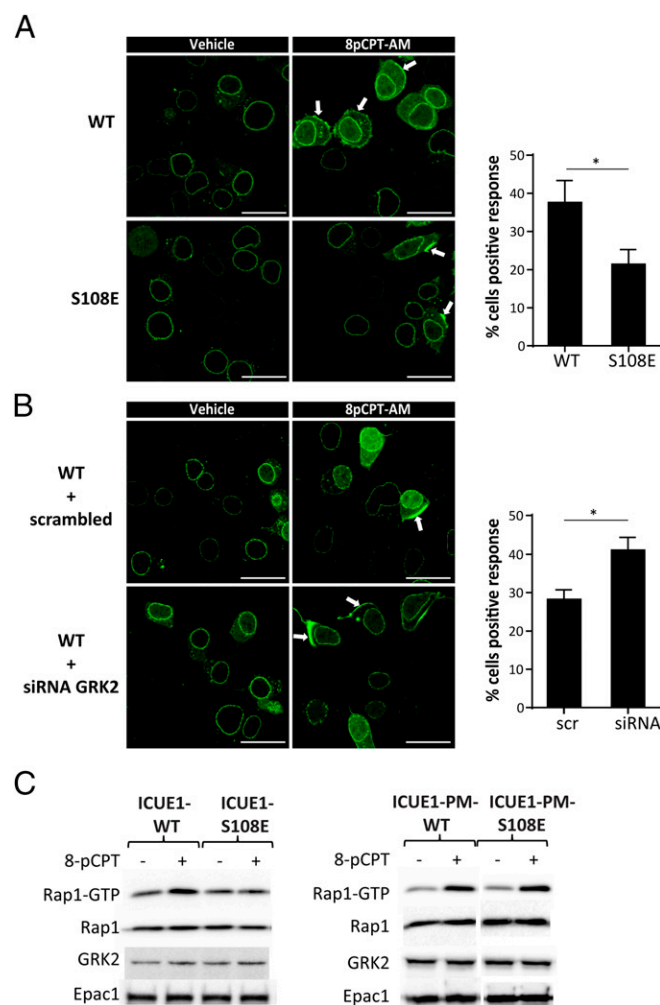


Fig. 5. Impaired 8-pCPT-induced PM translocation of Epac1-S108E. (A) N2A cells transfected with WT YFP-Epac1 (WT) or the phospho-mimic mutant YFP-Epac1-S108E, and stimulated with 8-pCPT-AM for 10 min. (Scale bars, 25 μ m.) * $P < 0.05$. (B) N2A cells transfected with WT YFP-Epac1 and siRNA GRK2 or scrambled siRNA (scr) and stimulated with 8-pCPT-AM as in A. Shown are representative images. Bar graph represents mean percentage of cells with Epac1 accumulation in the PM in response to 8-pCPT as determined in three independent experiments each including >50 cells per condition. (Scale bars, 25 μ m.) * $P < 0.05$. (C) Rap1 activation in N2A cells transfected with WT Epac1 (ICUE1-WT) or Epac1-S108E (ICUE1-S108E) (Left) or with PM-tagged WT Epac1 (ICUE1-PM-WT) or PM-tagged Epac1-S108E (ICUE1-PM-S108E) (Right), and stimulated with 8-pCPT-AM for 10 min.

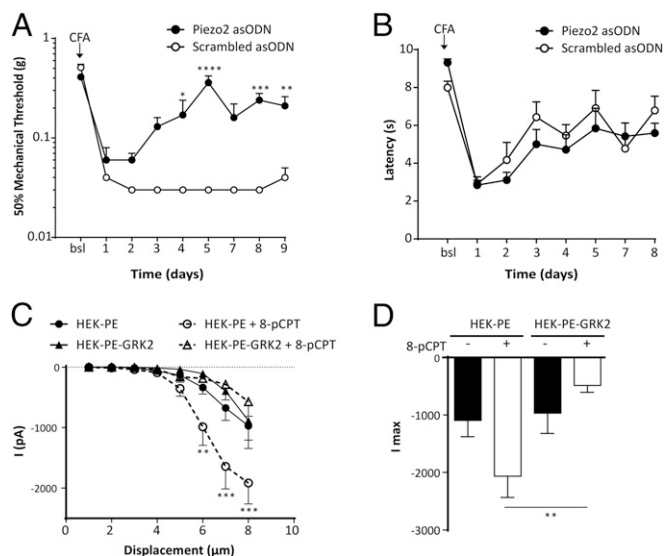


Fig. 6. Role of Piezo2 in CFA-induced mechanical hyperalgesia. (A) Effect of asODN against Piezo2 on CFA-induced mechanical hyperalgesia. Treatment, $P < 0.0001$; interaction, $P < 0.0001$ (two-way ANOVA). $*P < 0.1$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$ (Bonferroni posttest). (B) Effect of an asODN against Piezo2 on CFA-induced thermal hyperalgesia. For A and B, Piezo2 or scrambled asODNs were administered intrathecally on days 1, 2, 3, 5, 6, 7, and 8 after CFA injection. (C) Mechanically evoked currents in HEK cells transfected with Piezo2 and YFP-Epac1 overexpressing GRK2 (HEK-PE-GRK2) ($n = 22$ –26 cells) or control HEK-PE cells ($n = 20$ –22 cells). The 8-pCPT or bath solution was added, and cells were voltage-clamped at -60 mV in a whole-cell configuration. Mechanically evoked currents were elicited by increasing displacement of the cell membrane in $1\text{-}\mu\text{m}$ increments. $**P < 0.01$; $***P < 0.001$. (D) Peak current elicited by the largest mechanical stimulus before whole-cell configuration was lost. $**P < 0.01$.

findings collectively support a model in which GRK2-mediated phosphorylation of Epac1 prevents Rap1 activation and thereby reduces inflammatory mechanical hyperalgesia.

Epac1-to-Rap1 signaling is a multistep process involving cAMP-induced liberation of Epac1 from a closed, autoinhibitory to an open conformation, allowing binding of Rap1 to the catalytic CDC25-HD domain and GEF activity (8, 11, 24, 33, 34). Our FRET analysis revealed that in cells, the Epac1 mutant that mimics phosphorylation by GRK2 (Epac1-S108E) undergoes normal agonist-induced conformational changes. In addition, in vitro experiments showed that the ability of WT Epac1 in the presence or absence of GRK2 and of phospho-mimic Epac1 to function as a Rap1-GEF did not differ. Nevertheless, phosphorylation by GRK2 markedly reduces Epac1-mediated Rap1 activation in cells. We propose that the GRK2-mediated inhibition of Rap1 activation results from impaired agonist-induced PM accumulation of phosphorylated Epac1. This model is supported by the reduced 8-pCPT-induced PM accumulation of the phospho-mimic mutant of Epac1, whereas reducing cellular GRK2 levels increases PM accumulation of WT Epac1. Moreover, signaling of the phospho-mimic mutant of Epac1 to Rap1 normalized when it was targeted to the PM by addition of a -CAAX motif. PM phosphatidic acid (PA) functions as the anchor for activated Epac1 in response to cAMP, and this binding requires Arg-82 in the DEP domain of Epac (11, 35, 36). PA-dependent PM anchoring of DEP domain-containing proteins also requires penetration of hydrophobic amino acids into the acyl layer of the PM (37). Given our results and the amino acid sequence of the region surrounding Ser-108 in Epac1, it is conceivable that the negative charge added by phosphorylation of Ser-108 reduces its hydrophobicity and introduces negative charges, thereby inhibiting PM accumulation of phosphorylated Epac1.

It remains to be determined which downstream signaling pathways are responsible for the increased mechanical sensitivity of pain-sensing neurons downstream of Epac1-to-Rap1 signaling. A recent study has shown that Epac1 activation increases mechanocurrents mediated by the mechanosensitive channel Piezo2 (7). We show that inhibition of Epac1 signaling by overexpression of GRK2 prevents Epac-mediated sensitization of Piezo2-mediated mechanocurrents. Moreover, decreasing DRG Piezo2 levels in vivo by using asODN treatment protected against neuropathic pain (7) and, as shown here, inhibited the late phase of CFA-induced mechanical hypersensitivity. Consistent with a previous study (38), however, we did not detect a role for Piezo2 in CFA-induced mechanical hyperalgesia as measured 24 h after CFA administration. These findings indicate that the early (<24 h) and late phases of CFA-induced mechanical hyperalgesia are mediated via different mechanisms. Indeed, a recent study showed that only the early phase of CFA-induced hyperalgesia (<5 h) was reduced by the PKA inhibitor PKAi, whereas inhibition of the Epac1 target PKC- ϵ reduced CFA-induced hyperalgesia during the later phase (39). In addition, there is evidence that CFA-induced inflammation leads to a shift in cAMP signaling from a PKA to a PKC- ϵ -mediated pathway (40). On the basis of these earlier findings, we propose that Epac1-mediated sensitization of neuronal Piezo2 contributes to the later phase of CFA-induced hyperalgesia, whereas PKA-mediated Piezo2-independent pathways are operative early after CFA injection. Epac1 signaling could contribute to pain via Piezo2-independent pathways as well. For example, Epac activation leads to closure of potassium channels in pancreatic β cells (41), whereas many antinociceptive drugs work by opening potassium channels in sensory neurons (42). In addition, Piezo2 is expressed in non-neuronal cells, including astrocytes and endothelial cells (43, 44), and we cannot exclude that Piezo2 asODN treatment inhibits CFA-induced hyperalgesia via reducing Piezo2 levels in these cells.

In conclusion, we propose that Epac1 plays a critical role in inflammatory pain via a mechanism involving PM Epac1-to-Rap1 signaling and sensitization of Piezo2-mediated mechanocurrents. We also propose that constitutive GRK2-mediated phosphorylation of Epac1 on Ser-108 represents an essential control mechanism for cAMP-Epac1-dependent functions including mechanical hyperalgesia. Epac1 is known to contribute to multiple processes within and outside the nervous system, including neuronal excitability, long-term potentiation, axon guidance, muscle excitation and contraction, cell migration and adhesion, learning, regulation of inflammation, and energy homeostasis (15, 45–47). It is likely that the GRK2-mediated phosphorylation of Epac1 leading to reduced Epac1-to-Rap1 signaling that we uncovered here regulates these processes as well.

Materials and Methods

We used Epac1 $^{-/-}$ mice (17, 48) and their WT control littermates in a C57BL/6 background at an age of 12–14 wk. All procedures were performed in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (49) and were approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center. Mechanical hyperalgesia was measured by using von Frey hairs, and heat withdrawal latency times were determined by using the Hargreaves (IITC Life Science). The 8-pCPT and 8-pCPT-acetoxymethyl ester (AM) (Axora) were dissolved in DMSO. ESI-09 was dissolved in ethanol (5 mg/mL) followed by dilution in corn oil (1:1) and speed vacuum to remove ethanol. GTP-bound Rap1 was pulled down by using Ral-GDS beads (18), and Rap1 was quantified by Western blotting. Currents were recorded as described (7) by using Axopatch 200B and Multiclamp 700 amplifiers (Axon Instruments, Molecular Devices) from HEK293 cells. In vitro Epac1 GEF activity was measured by using a similar protocol as described (28). Cells were imaged by using a DeltaVision Deconvolution Microscope (Applied Precision) or a SPE Leica Confocal Microscope (Leica Microsystems) at 37°C . Data are expressed as mean \pm SEM and were analyzed by using two-tailed Student's t test or one- or two-way ANOVA.

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- Simon LS (2012) Relieving pain in America: A blueprint for transforming prevention, care, education, and research. *J Pain Palliat Care Pharmacother* 26(2):197–198.
- Wei F, et al. (2002) Genetic elimination of behavioral sensitization in mice lacking calmodulin-stimulated adenylyl cyclases. *Neuron* 36(4):713–726.
- Eijkelkamp N, Singhamar P, Heijnen CJ, Kavelaars A (2015) Sensory neuron cAMP signaling in chronic pain. *Cyclic Nucleotide Signaling, Methods in Signal Transduction Series*, ed Cheng X (CRC, Boca Raton, FL), pp 113–134.
- Taiwo YO, Levine JD (1991) Further confirmation of the role of adenylyl cyclase and of cAMP-dependent protein kinase in primary afferent hyperalgesia. *Neuroscience* 44(1):131–135.
- Aley KO, Levine JD (1999) Role of protein kinase A in the maintenance of inflammatory pain. *J Neurosci* 19(6):2181–2186.
- Hucho TB, Dina OA, Levine JD (2005) Epac mediates a cAMP-to-PKC signaling in inflammatory pain: An isolectin B4(+) neuron-specific mechanism. *J Neurosci* 25(26):6119–6126.
- Eijkelkamp N, et al. (2013) A role for Piezo2 in EPAC1-dependent mechanical allodynia. *Nat Commun* 4:1682.
- de Rooij J, et al. (2000) Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. *J Biol Chem* 275(27):20829–20836.
- de Rooij J, et al. (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 396(6710):474–477.
- Kawasaki H, et al. (1998) A family of cAMP-binding proteins that directly activate Rap1. *Science* 282(5397):2275–2279.
- Ponsioen B, et al. (2009) Direct spatial control of Epac1 by cyclic AMP. *Mol Cell Biol* 29(10):2521–2531.
- Vliem MJ, et al. (2008) 8-pCPT-2'-O-Me-cAMP-AM: An improved Epac-selective cAMP analogue. *ChemBioChem* 9(13):2052–2054.
- Mei FC, et al. (2002) Differential signaling of cyclic AMP: Opposing effects of exchange protein directly activated by cyclic AMP and cAMP-dependent protein kinase on protein kinase B activation. *J Biol Chem* 277(13):11497–11504.
- Schmidt M, et al. (2001) A new phospholipase-C-calcium signalling pathway mediated by cyclic AMP and a Rap GTPase. *Nat Cell Biol* 3(11):1020–1024.
- Breckler M, et al. (2011) Rap-linked cAMP signaling Epac proteins: Compartmentation, functioning and disease implications. *Cell Signal* 23(8):1257–1266.
- Eijkelkamp N, et al. (2010) Low nociceptor GRK2 prolongs prostaglandin E2 hyperalgesia via biased cAMP signaling to Epac/Rap1, protein kinase Cepsilon, and MEK/ERK. *J Neurosci* 30(38):12806–12815.
- Wang H, et al. (2013) Balancing GRK2 and EPAC1 levels prevents and relieves chronic pain. *J Clin Invest* 123(12):5023–5034.
- van Triest M, Bos J (2004) Pull-down assays for guanosine 5'-triphosphate-bound Ras-like guanosine 5'-triphosphatases. *Methods Mol Biol* 250:97–102.
- Pitcher JA, et al. (1999) Feedback inhibition of G protein-coupled receptor kinase 2 (GRK2) activity by extracellular signal-regulated kinases. *J Biol Chem* 274(49):34531–34534.
- Dinudom A, et al. (2004) The kinase Grk2 regulates Nedd4/Nedd4-2-dependent control of epithelial Na⁺ channels. *Proc Natl Acad Sci USA* 101(32):11886–11890.
- Rehmann H, Das J, Knipscheer P, Wittinghofer A, Bos JL (2006) Structure of the cyclic-AMP-responsive exchange factor Epac2 in its auto-inhibited state. *Nature* 439(7076):625–628.
- Rehmann H, et al. (2003) Structure and regulation of the cAMP-binding domains of Epac2. *Nat Struct Biol* 10(1):26–32.
- Selvaratnam R, Akimoto M, VanSchouwen B, Melacini G (2012) cAMP-dependent allostery and dynamics in Epac: an NMR view. *Biochem Soc Trans* 40(1):219–223.
- DiPilato LM, Cheng X, Zhang J (2004) Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. *Proc Natl Acad Sci USA* 101(47):16513–16518.
- Tsalkova T, Mei FC, Cheng X (2012) A fluorescence-based high-throughput assay for the discovery of exchange protein directly activated by cyclic AMP (EPAC) antagonists. *PLoS One* 7(1):e30441.
- Zhu Y, et al. (2015) Biochemical and pharmacological characterizations of ESI-09 based EPAC inhibitors: Defining the ESI-09 "therapeutic window". *Sci Rep* 5:9344.
- Almahariq M, et al. (2013) A novel EPAC-specific inhibitor suppresses pancreatic cancer cell migration and invasion. *Mol Pharmacol* 83(1):122–128.
- Tsalkova T, Blumenthal DK, Mei FC, White MA, Cheng X (2009) Mechanism of Epac activation: structural and functional analyses of Epac2 hinge mutants with constitutive and reduced activities. *J Biol Chem* 284(35):23644–23651.
- Vasko MR, et al. (2014) Nerve growth factor mediates a switch in intracellular signaling for PGE2-induced sensitization of sensory neurons from protein kinase A to Epac. *PLoS One* 9(8):e104529.
- Eijkelkamp N, et al. (2010) GRK2: A novel cell-specific regulator of severity and duration of inflammatory pain. *J Neurosci* 30(6):2138–2149.
- Kleibeker W, et al. (2007) A role for G protein-coupled receptor kinase 2 in mechanical allodynia. *Eur J Neurosci* 25(6):1696–1704.
- Ferrari LF, et al. (2012) Transient decrease in nociceptor GRK2 expression produces long-term enhancement in inflammatory pain. *Neuroscience* 222:392–403.
- Ponsioen B, et al. (2004) Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator. *EMBO Rep* 5(12):1176–1180.
- Rehmann H, et al. (2008) Structure of Epac2 in complex with a cyclic AMP analogue and RAP1B. *Nature* 455(7209):124–127.
- Consonni SV, Glierich M, Spanjaard E, Bos JL (2012) cAMP regulates DEP domain-mediated binding of the guanine nucleotide exchange factor Epac1 to phosphatidic acid at the plasma membrane. *Proc Natl Acad Sci USA* 109(10):3814–3819.
- Qiao J, Mei FC, Popov VL, Vergara LA, Cheng X (2002) Cell cycle-dependent subcellular localization of exchange factor directly activated by cAMP. *J Biol Chem* 277(29):26581–26586.
- Shin JJ, Loewen CJ (2011) Putting the pH into phosphatidic acid signaling. *BMC Biol* 9:85.
- Ranade SS, et al. (2014) Piezo2 is the major transducer of mechanical forces for touch sensation in mice. *Nature* 516(7529):121–125.
- Huang WY, Dai SP, Chang YC, Sun WH (2015) Acidosis mediates the switching of Gs-PKA and Gi-PKC: dependence in prolonged hyperalgesia induced by inflammation. *PLoS One* 10(5):e0125022.
- Wang C, Gu Y, Li GW, Huang LY (2007) A critical role of the cAMP sensor Epac in switching protein kinase signalling in prostaglandin E2-induced potentiation of P2X3 receptor currents in inflamed rats. *J Physiol* 584(Pt 1):191–203.
- Zhang Y, et al. (2015) P2Y purinergic receptor-regulated insulin secretion is mediated by a cAMP/Epac/Kv channel pathway. *Biochem Biophys Res Commun* 460(3):850–856.
- Tsantoulas C, McMahon SB (2014) Opening paths to novel analgesics: The role of potassium channels in chronic pain. *Trends Neurosci* 37(3):146–158.
- Coste B, et al. (2010) Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. *Science* 330(6000):55–60.
- Ferrari LF, Bogen O, Green P, Levine JD (2015) Contribution of Piezo2 to endothelium-dependent pain. *Mol Pain* 11(1):65.
- Almahariq M, Mei FC, Cheng X (2014) Cyclic AMP sensor EPAC proteins and energy homeostasis. *Trends Endocrinol Metab* 25(2):60–71.
- Parnell E, Palmer TM, Yarwood SJ (2015) The future of EPAC-targeted therapies: Agonism versus antagonism. *Trends Pharmacol Sci* 36(4):203–214.
- Schmidt M, Dekker FJ, Maarsingh H (2013) Exchange protein directly activated by cAMP (epac): A multidomain cAMP mediator in the regulation of diverse biological functions. *Pharmacol Rev* 65(2):670–709.
- Suzuki S, et al. (2010) Differential roles of Epac in regulating cell death in neuronal and myocardial cells. *J Biol Chem* 285(31):24248–24259.
- Committee on Care and Use of Laboratory Animals (1996) *Guide for the Care and Use of Laboratory Animals* (Natl Inst Health, Bethesda), DHHS Publ No (NIH) 85-23.
- Poobalan AS, et al. (2001) Chronic pain and quality of life following open inguinal hernia repair. *Br J Surg* 88(8):1122–1126.
- Wang H, et al. (2011) GRK2 in sensory neurons regulates epinephrine-induced signalling and duration of mechanical hyperalgesia. *Pain* 152(7):1649–1658.