Epac activation sensitizes rat sensory neurons through activation of Ras

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ABSTRACT

Guanine nucleotide exchange factors directly activated by cAMP (Epacs) have emerged as important signaling molecules mediating persistent hypersensitivity in animal models of inflammation, by augmenting the excitability of sensory neurons. Although Epacs activate numerous downstream signaling cascades, the intracellular signaling which mediates Epac-induced sensitization of capsaicin-sensitive sensory neurons remains unknown. Here, we demonstrate that selective activation of Epacs with 8-CPT-2′-O-Me-cAMP-AM (8CPT-AM) increases the number of action potentials (APs) generated by a ramp of depolarizing current and augments the evoked release of calcitonin gene-related peptide (CGRP) from isolated rat sensory neurons. Internal perfusion of capsaicin-sensitive sensory neurons with GDP-βS, substituted for GTP, blocks the ability of 8CPT-AM to increase AP firing, demonstrating that Epac-induced sensitization is G-protein dependent. Treatment with 8CPT-AM activates the small G-proteins Rap1 and Ras in cultures of sensory neurons. Inhibition of Rap1, by internal perfusion of a Rap1-neutralizing antibody or through a reduction in the expression of the protein using shRNA does not alter the Epac-induced enhancement of AP generation or CGRP release, despite the fact that in most other cell types, Epacs act as Rap-GEFs. In contrast, inhibition of Ras through expression of a dominant negative Ras (DN-Ras) or through internal perfusion of a Ras-neutralizing antibody blocks the increase in AP firing and attenuates the increase in the evoked release of CGRP induced by Epac activation. Thus, in this subpopulation of nociceptive sensory neurons, it is the novel interplay between Epacs and Ras, rather than the canonical Epacs and Rap1 pathway, that is critical for mediating Epac-induced sensitization.

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1. Introduction

Exchange proteins directly activated by cAMP (Epacs) are key downstream signaling effectors of cAMP that function as guanine nucleotide exchange factors (GEFs) for the Ras superfamily of small GTPases (Holz et al., 2006). Recently, an important role of Epacs in mediating pain hypersensitivity in animal models of chronic inflammation has emerged. This hypersensitivity is a consequence of increased excitability of sensory neurons that convey noxious information to the spinal cord (Gold and Gebhart, 2010; Richardson and Vasko, 2002; Woolf and Ma, 2007), a process termed peripheral sensitization. Although much work has described the signal transduction cascades in sensory neurons that mediate sensitization under acute inflammation (Basbaum et al., 2009; Hucho and Levine, 2007; Richardson and Vasko, 2002), less is known about signaling that maintains sensitization under chronic inflammatory conditions. Recent evidence suggests, however, that the transition from acute to persistent hypersensitivity is linked to a switch in signaling pathways that mediate sensitization (Villarreal et al., 2009). For example, acute exposure to prostaglandin E2 (PGE$_2$) produces hyperalgesia and an increase

in the excitability of sensory neurons, which are both mediated by increases in cAMP and activation of protein kinase A (PKA) (Aley and Levine, 1999; England et al., 1996; Hingtgen et al., 1995; Lopshire and Nicol, 1998; Sachs et al., 2009). With inflammation, or when sensory neurons are maintained in the presence of the inflammatory mediator, nerve growth factor (NGF), the sensitization induced by subsequent administration of PGE$_2$ shifts from using PKA as the primary effector to activation of Epacs (Eijkelkamp et al., 2013; Hucho et al., 2005; Vasko et al., 2014; Wang et al., 2007).

The question remains, however as to the downstream signaling by Epacs that contributes to persistent sensitization of sensory neurons. Activation of the Ras family of GTPases by Epac1 (RapGEF3) or Epac2 (RapGEF4) (Bos, 2003; de Rooij et al., 1998) is linked to a number of downstream signaling effectors that may contribute to persistent hypersensitivity, including but not limited to phospholipase Cϵ (PLCϵ), phospholipase D (PLD), mitogen-activated protein kinases (MAPKs), and phosphatidylinositol 3-kinase (PI3K) (Baviera et al., 2010; Holz et al., 2006; Schmidt et al., 2013; Yano et al., 2007). Although Epacs were initially discovered as GEFs for Rap1, controversy exists, however, as to whether Epacs may also act as GEFs for Ras and whether Ras mediates some of the physiological effects regulated by activation of Epacs. (Li et al., 2006; Lopez De Jesus et al., 2006; Zheng and Quilliam, 2003).

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Therefore, determining the downstream signaling pathways that mediate sensitization of nociceptive sensory neurons after activation of Epacs is of high importance in understanding signaling in chronic pain. Consequently, we investigated whether activation of the canonical Epac target Rap1, or the less conventional target, Ras were necessary for the sensitizing actions of Epacs on capsaicin-sensitive sensory neurons. We demonstrate that exposing sensory neuronal cultures to the Epac agonist, 8-CPT-2′-O-Me-cAMP-AM (8CPT-AM) enhances the activity of the small G proteins, Rap1 and Ras. Epac activation with 8CPT-AM also augments the number of action potentials generated by a ramp of depolarizing current and the evoked release of immunoreactive calcitonin gene-related peptide (iCGRP) from sensory neurons. Surprisingly, these actions are not attenuated by inhibition of Rap1, but rather by inhibition of Ras. Our findings demonstrate that Ras is a critical downstream effector of Epac-induced sensitization in capsaicin-sensitive sensory neurons, suggesting a unique signaling pathway for maintaining sensitization of these neurons during chronic inflammation.

2. Methods

2.1. Materials

Tissue culture supplies were obtained from Invitrogen (Carlsbad, CA) and normocin from Invivogen (San Diego, CA). Capsaicin, potassium chloride, 1-methyl-2-pyrrolidinone (MPL), poly-D-lysine, laminin, and routine chemicals were purchased from Sigma-Aldrich (St. Louis, MO). MPL was the vehicle used to prepare water-insoluble drugs. The maximal concentration of MPL used was 0.01%. Nerve growth factor was purchased from Harlan Bioproducts for Science (Indianapolis, IN). The $G_{\beta\gamma}$ inhibitor gallein (catalog # 3090) was purchased from Tocris Biosciences (Bristol, UK). The Epac agonist, 8-pCPT-2′-O-Me-cAMP-AM (8CPT-AM, catalog # NC9940185), was purchased from ThermoFisher Scientific (Fremont, CA), whereas the phosphate trisacetoxymethyl ester (PO4-AM3, catalog # BLG-P030-003) and N6-Benzoyladenosine-3′, 5′-cAMP-AM (6BNZ-AM catalog # BLG-P079-01) were purchased from Axxora (San Diego, CA). The Rap1 shRNA lentiviral particles (LV) (catalog # sc-270358), control shRNA LV (catalog # sc-108080), rabbit anti-GAPDH antibody for Western blotting (catalog # sc-25778), and HRP-conjugated goat anti-rabbit secondary antibody for Western blotting (catalog # sc-2030) were purchased from Santa Cruz Biotechnology (Dallas, TX). The antibodies used for internal perfusion into sensory neurons were also purchased from Santa Cruz Biotechnology: rabbit anti-Rap1 antibody (catalog # sc-65), normal rabbit IgG (catalog # sc-2027), and normal rat IgG (catalog # sc-2026). HRP-conjugated goat anti-mouse antibody (catalog # 170-6516) was purchased from Bio-Rad (Hercules, CA). The following were purchase from EMD Millipore (Billerica, MA): the Y13-259 rat anti-Ras antibody for internal perfusion into sensory neurons (catalog # OP01A), the rabbit anti-Rap1 antibody for Western blotting (catalog # 07-916), the Ras antibody for Western blotting (catalog # 5-516), the Rap1 activation kit (catalog # 17-321) and the Ras activation kit (catalog # 17-218). The CGRP antibody was a generous gift from Dr. M. Iadarola at the NIH. The Animal Care and Use Committee at Indiana University School of Medicine, Indianapolis, IN approved all procedures used in these studies.

2.2. Isolation and cell culture of adult rat sensory neurons

Dorsal root ganglia (DRGs) from male Sprague–Dawley rats (100–200 g, Harlan, Indianapolis, IN) were dissected and sensory neurons were isolated as previously described (Burkey et al., 2004; Zhang et al., 2012). Briefly, the animals were sacrificed via CO_2 asphyxiation and the DRGs from the entire spinal column (for release experiments) or from the lumbar region (for electrophysiology experiments) were collected into an ice-cold solution of Puck's saline and subsequently

trimmed to remove nerve fibers. For release experiments, the Puck's saline was replaced with F-12 medium supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 100 μg/ml normocin-O™, 50 μg/ml penicillin, 50 μg/ml streptomycin, 50 μM 5-fluoro-2′-deoxyuridine, and 150 μM uridine (F-12 growth medium) containing ~3 mg/ml collagenase and the tissues were incubated for 90 min at 37 °C in a 3% CO_2 incubator. For patch clamp electrophysiology experiments, the Puck's solution was replaced with F-12 growth medium containing 10 U/ml of papain and the DRGs were incubated for 10 min at 37 °C, followed by a 50 min incubation at 37 °C with F-12 medium containing 1 mg/ml collagenase and 2.5 mg/ml dispase. After a brief centrifugation (1000 ×g for 1 min), the supernatants from either preparation were aspirated; the DRGs were resuspended in 2 ml of F-12 growth medium containing 30 ng/ml of nerve growth factor (NGF) and dissociated using mechanical agitation. For release experiments, cells were plated at an approximate density of 30,000 cells per well of a 12-well plate precoated with 0.1 mg/ml of poly-D-lysine and 5 μg/ml of laminin. For patch clamp experiments, cells were plated at an approximate density of 7500 cells per well of a 48-well plate containing plastic coverslips precoated with 0.1 mg/ml of poly-D-lysine and 10 μg/ml of laminin. The isolated cells were maintained in culture at 37 °C and 3% CO_2. The F-12 growth medium supplemented with NGF was changed 24 h after plating, and every other day thereafter. Cells were used 3–8 days after plating for patch clamp experiments in order to minimize space clamp issues, and 10–12 days after plating for release experiments in order to optimize the expression of CGRP and thus the ability to measure basal release of the peptide. In all instances, controls for release and electrophysiology experiments were from wells of cells harvested at the same time as those treated with various experimental manipulations.

2.3. Release of immunoreactive calcitonin gene-related peptide (iCGRP) from sensory neurons

Release experiments were performed on sensory neurons as previously described (Vasko et al., 1994). Briefly, the neuronal cultures were washed once with 0.4 ml of HEPES buffer consisting of (in mM): 25 HEPES, 135 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 1 Mg_2Cl_2, 3.3 dextrose, and 0.1% (w/v) bovine serum albumin, pH 7.4. Thereafter, the cells were incubated in 10 min sequential periods in 0.4 ml of the HEPES buffer at 37 °C. In order to determine basal neuropeptide release, the cells were exposed to HEPES buffer alone for 10 min during the first incubation. The second 10 min incubation occurred in HEPES buffer in either the absence or presence of drug to assess the effect of treatment on basal release. The third 10 min incubation occurred in HEPES buffer containing either 30 nM capsaicin or 30 mM KCl (substituted for equimolar NaCl) in the absence or presence of drug. The fourth 10 min incubation was with HEPES buffer alone in order to demonstrate a return to resting levels of release. After each of the incubations, the buffer was removed and aliquoted for iCGRP radioimmunoassay (RIA). At the conclusion of the release protocol, each well of cells was incubated in 0.4 ml of 0.1 N HCl for 10 min, scraped and an aliquot assayed for iCGRP to determine the remaining amount of peptide in the cells.

For the RIA, 300 μl of buffer from the aliquoted samples was incubated with 25 μl of a CGRP antibody (1:70,000 dilution) and 25 μl of [125]I-[Tyr0] CGRP. After 16 h, 0.5 ml of 1% charcoal in 0.1 M phosphate buffer, pH 7.4 was added to each tube. The tubes were centrifuged at 1500 ×g for 20 min and the supernatant containing radiolabeled peptide bound to antibody was decanted into separate tubes. Radioactivity was measured using gamma scintillation spectrometry. The counts generated from a CGRP standard curve were used to calculate the amount of iCGRP in each sample. Total CGRP content was determined by adding the amount released to the total remaining after the release. Release data are presented as percent of total content.