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Phospho-substrate profiling of Epac-dependent protein kinase C activity

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Abstract

Exchange protein directly activated by cAMP (Epac) and protein kinase A are effectors for cAMP with distinct actions and regulatory mechanisms. Epac is a Rap guanine nucleotide exchange factor that activates Rap1; protein kinase C (PKC) is a major downstream target of Epac-Rap1 signaling that has been implicated in a variety of pathophysiological processes, including cardiac hypertrophy, cancer, and nociceptor sensitization leading to chronic pain. Despite the implication of both Epac and PKC in these processes, few downstream targets of Epac-PKC signaling have been identified. This study characterized the regulation of PKC activity downstream of Epac activation. Using an antibody that recognizes phospho-serine residues within the consensus sequence phosphorylated by PKC, we analyzed the 1-dimensional banding profile of PKC substrate protein phosphorylation from the Neuro2A mouse neuroblastoma cell line. Activation of Epac either indirectly by prostaglandin PGE2, or directly by 8-pCPT-2-O-Me-cAMP-AM (8pCpt), produced distinct PKC phospho-substrate protein bands that were suppressed by co-administration of the Epac inhibitor ESI09. Different PKC isoforms contributed to the induction of individual phospho-substrate bands, as determined using isoform-selective PKC inhibitors. Moreover, the banding profile after Epac activation was altered by disruption of the cytoskeleton, suggesting that the orchestration of Epac-dependent PKC signaling is regulated in part by interactions with the cytoskeleton. The approach described here provides an effective means to characterize Epac-dependent PKC activity.

Keywords

signal transduction; phosphorylation; prostaglandins; cytoskeleton

Introduction

G protein-coupled receptors (GPCRs) coupled to $G_{\alpha}s$ allow environmental stimuli to modulate cellular physiology by activating adenylyl cyclase (AC) to produce cyclic adenosine monophosphate (cAMP)[1]. cAMP is a ubiquitous second messenger that regulates diverse biologic functions; cAMP effector proteins include protein kinase A (PKA) [2], cyclic nucleotide-gated ion channels [3] and exchange protein directly activated by

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cAMP (Epac)[4, 5]. PKA and Epac can act independently, synergistically, or in some cases antagonistically [6–8].

Epac is a guanine nucleotide exchange factor (GEF) for Rap, a member of the Ras family of GTPases 1[4, 5]. Some of the biological consequences of Epac activation in diverse cell types, including peripheral sensory neurons [9] and cardiac myocytes [10], have been reported to require the action of protein kinase C (PKC) downstream of Rap1. Epac-dependent activation of PKC occurs through Rap1 activation of phospholipase C epsilon (PLCε) [11], leading to production of diacylglycerol (DAG) and inositol trisphosphate (IP3) and the subsequent release of ER calcium stores through IP3 receptors [12]. PLC activation represents a canonical pathway for activation of conventional PKC isoforms (PKCα,β,γ) which require both DAG and calcium for activation[13], as well as novel isoforms (PKC δ, ε, η, and θ), which require DAG but are insensitive to cytoplasmic calcium fluctuations [14]. In contrast to the Epac signaling pathway, Gq/11-coupled receptors act through PLC isoforms[15].

Despite the evidence implicating PKC as a downstream effector of Epac, few substrates of Epac-dependent PKC signaling have been identified [16]. In order to gain a better understanding of Epac-dependent PKC signaling, we have used an antibody that recognizes phospho-serine residues within the consensus recognition sequences for PKC to develop a profile of PKC substrate phosphorylation downstream of Epac. In the Neuro 2A (N2A) mouse neuroblastoma cell line, we used the Gs-coupled receptor agonist prostaglandin E2 (PGE2) to provide a physiological stimulus for activation of Epac. PGE2 is a major inflammatory mediator that provides a significant source of G s signaling in cardiac, neuronal and immune cells. With this approach, we identified a subset of PKC phospho-substrate bands that were consistently induced by PGE2 and suppressed by the Epac inhibitor ESI09. Different PKC isoforms contributed to the induction of individual phospho-substrate bands, as determined using isoform-selective PKC inhibitors. Moreover, several Epac-dependent PKC phospho-substrate bands, but not all, were sensitive to cytoskeletal disruption. The approach described here provides an effective means to characterize Epac-dependent PKC activity.

Materials and methods

Cell culture and reagents

The mouse Neuro-2a (N2A) neuroblastoma cell line was purchased from ATCC (ATCC Number: CCL-131). Cells were propagated and cultured at 37° C/5% CO2 in complete media (Minimal Essential Media, GlutaMAX™ (MEM: Gibco-ThermoFisher, Waltham, MA) supplemented with 10% heat inactivated Fetal Bovine Serum (Gibco-ThermoFisher, Waltham, MA) and 1% Penicillin-Streptomycin Solution (Corning, Tewksbury, MA)). Passaging cells were performed by aspirating the complete media and washing the cells with 1x PBS. Cells were incubated for 30 seconds at 37° C with 0.5% Trypsin-EDTA (1X) (Gibco-ThermoFisher, Waltham, MA) to enzymatically detach cells from the bottom of the culture dish. Complete media was added to stop the reaction and cells were seeded in a new tissue culture dish.

In vitro analyses

N2A cells were cultured until 80% confluency in 6-well flat bottom plates (Midsci, Valley Park, MO). Cells were washed with 1x PBS and serum-starved in HBSS (Gibco-ThermoFisher, Waltham, MA) with 1.4mM Ca^{2+} and 0.9mM Mg^{2+} ($HBSS^{+/+}$) for four hours. Cells were stimulated with either 10μM PGE2 (Tocris #2296, Minneapolis, MN), 25μM 8-pCPT-2-O-Me-cAMP-AM (Tocris #4853, Minneapolis, MN) for 30 seconds, or 1μM phorbol 12-Myristate 13-acetate (PMA; LC Laboratories #P-1680) for 20 minutes. PKC inhibitors (20μM GF109203X, α, β1, δ, ε and ζ isoforms, Tocris #0741; 20μM CGP53353, PKCβII, Tocris #2442; 50μM HBDDE, PKCα and PKCγ inhibitor, Abcam #ab141573) were added to cells in complete media for two hours, then media was replaced with serum-free $HBSS^{+/+}$ with PKC inhibitors for another two hours. The long incubation times were used to allow time for basal phosphorylation to be reversed as well as to block stimulated phosphorylation. 10μM SC19220 (a selective EP1 receptor antagonist; Tocris #1206) and 25μM ESI09 [17] (a selective Epac inhibitor; Tocris #4773) were added for the last 30 minutes of the 4-hour $HBSS^{+/+}$ incubation. Cytoskeleton inhibitors (2μM GSK 429286, a selective Rho-kinase inhibitor, Tocris #3726; 10μM Cytochalasin D, a disruptor of actin filaments, Tocris #1233, 5μM Nocodazole, a microtubule inhibitor, Tocris #1228) were added for the last 2 hours of the 4-hour $HBSS^{+/+}$ incubation.

Intracellular delivery of the CH1 antibody and PKC inhibitor peptide

Intracellular delivery of the function-blocking tropomyosin antibody (1μg CH1, Developmental Studies Hybridoma Bank, Iowa City, IA [RRID:AB_2205770]) and PKCε inhibitor peptide (500ng εV1–2, Cayman Chemical [#17476], Ann Arbor, Michigan) was achieved using the Chariot™ Protein Delivery Reagent (Active Motif, Carlsbad, CA), using the manufacturer's protocol: chariot, a 2843 dalton peptide, was incubated with 1μg of CH1 at room temperature for 30 minutes. After the non-covalent chariot-CH1 complex formed, the macromolecular complex was added to N2A cells in the absence of serum for 2 hours at 37°C.

Protein extraction and Western blot analysis

Cells were briefly washed with HBSS and proteins were precipitated with trichloroacetic acid (TCA; [#T0699] Sigma, Burlington, MA). Cell lysates were solubilized in 1x Laemmli without bromophenol blue and quantified by EZQ Protein Quantification kit (ThermoFisher, Waltham, MA). SDS-PAGE was performed with Bolt Bis-Tris 12% gels (ThermoFisher, Waltham, MA) using MOPS-SDS running buffer (500mM MOPS, 500mM Tris base, 1mM EDTA, and 1% (w/v) SDS). Proteins were transferred to Immobilon-FL PVDF Membrane (Millipore, #IPFL00010, Burlington, MA) using Towbin buffer with SDS (250mM Tris base, 1.92M Glycine, 1% (w/v) SDS, 10% methanol). PVDF membrane was blocked with 2.5% BSA in TBS (2.5mM Tris-HCl, 7.5mM NaCl, pH 4.0) at room temperature, rocking for 30 minutes. PVDF membranes were incubated overnight at 4 C with 1:500 Phospho-(Ser) PKC Substrate Antibody (Cell Signaling Technology, #2261, Danvers, MA[RRID:AB_330310]) and 1:10000 anti-tubulin antibody [YL1/2] (Abcam, ab6160, Cambridge, MA[RRID:AB_305328]). PVDF membrane was washed 3 times for 10 minutes each in TBS-Tween (25mM Tris-HCl, 25mM NaCl, 0.1% Tween 20, pH4). PVDF

membrane was incubated with 1:2000 donkey anti-rabbit Cy5 (Jackson ImmunoResearch #711–175-152) and donkey anti-rat Cy3 secondary antibodies for 2 hours at room temperature (Jackson ImmunoResearch #712–165-153). PVDF membranes were imaged using a GE Typhoon 9600 laser scanner (GE Healthcare Life Sciences, Pittsburgh, PA).

Real-Time PCR

For the quantification of E-prostanoid (EP) receptor expression levels, mRNA from N2A cells was isolated using the RNAspin Mini Kit (GE Healthcare Life Sciences, Pittsburgh, PA) and the cDNA was synthesized with Superscript II RT (ThermoFisher, Waltham, MA). Promega Sybr® Green PCR Dye was used for the real-time PCR reaction with the ABI real-time thermal cycler (ThermoFisher, Waltham, MA), performed and quantified essentially as described previously[18]. Each reaction contained 10μl of SYBR green, 1μl of 5μM of both forward and reverse primers, 1μl (121ng) of cDNA and 7μl of DNase/RNase free water for a total of 20μl and was run for 40 cycles. Primers for PTGER1, PTGER2, and PTGER3 (Table 1) were generated by Integrated DNA Technologies based on the sequences from [19]. Other primers were designed using Primer Blast NCBI, and generated by Integrated DNA Technologies. Relative changes in mRNA levels were calculated using the Ct method using Gapdh as the reference gene. Data were plotted as the Ct value for each gene +/− standard deviation. Selective amplification of a single amplicon was confirmed by plotting a dissociation curve against melting temperature after the reaction was complete.

Western Blot Quantification and Statistics

Band intensities were quantified using AutoQuant Imaging software. The intensity of the bands was normalized to tubulin, and the percent inhibition was calculated to determine the contribution of Epac and specific PKC isoforms. Statistical significance was determined by paired t-test and standard error of the mean (SEM) was used to generate error bars. Graphs were generated in Graphpad Prism and assembled in Adobe Photoshop.

Results

Epac-dependent PKC signaling in N2A cells

PGE2 is a versatile lipid mediator that is widely produced in response to inflammation [20] and tissue destruction [21]. The four prostaglandin E receptors, EP1-EP4, differ in signal transduction pathways and tissue distribution [22]: EP2 and EP4 couple to G_s, EP1 couples to $G_{q/11}$, and EP3 couples to $G_{i/o}$ [22]. We used real-time Sybr Green PCR to determine EP receptor mRNA levels in N2A cells (Figure 1). Both EP1 and EP4 were highly expressed, whereas expression of EP2 and EP3 transcripts was quite low. These results indicate that prostaglandins are likely to induce signaling in N2A cells through both G_s activation of adenylyl cyclase and $G_{q/11}$ activation of PLCβ.

To assess PKC activity in N2A cells and provide a 1-dimensional profile of PKC phospho-substrate proteins by molecular weight, we used an antibody selective for phospho-serine residues within the consensus recognition sequence for PKC isoforms. This antibody recognizes phospho-serine residues surrounded by arginine or lysine at the −2 and +2 positions, with a hydrophobic residue at the +1 position [23]. Faint staining of multiple

bands was visible in baseline unstimulated conditions, indicating some basal PKC activity. PMA (1.0μM), used to indiscriminately activate PKC isoforms to demonstrate antibody selectivity for PKC phospho-protein, induced strong staining of a highly reproducible pattern of bands, including those labeled at baseline as well as additional bands. Application of a broad-spectrum cocktail of PKC inhibitors (20μM GF109203X (Bisindolylmaleimide I) for α, β1, δ, ε and ζ isoforms; 20μM CGP53353 for PKCβII) reduced staining of all bands induced by PMA to a level below baseline, confirming that these bands were dependent upon activation of PKC (Figure 2a). Notably, 10μM PGE2 induced a consistent profile of increased staining for a subset of the bands induced by PMA (Figure 2b). We were able to resolve 15 distinct bands in samples stimulated with PGE2, which were individually quantified; 9 of these bands showed a significant and reproducible change in intensity in response to PGE2 (Figure 2c).

To determine the extent to which PKC phospho-substrate staining induced by PGE2 was mediated by Epac signaling, we applied a selective Epac inhibitor, ESI09, to cells before and during application of PGE2, and measured the impact on staining intensity for all 15 identified bands. Only 4 bands were significantly altered by Epac inhibition, bands 5, 6, 13 and 14. ESI09 almost completely eliminated band 13, suppressing staining by 94% ± 1.6%. Band 5 was only partially reduced (by 36% ± 11.5%). In contrast, bands 6 and 14 were consistently increased: band 6 by 19% ± 12.6, and band 14 by 60% ± 12.6 (Figure 2d). Figure 2e illustrates the changes in band intensities in response to ESI09 for each individual experiment.

Given that EP1 was also expressed in our N2A cells and couples to $G_{q/11}$, we examined whether activation of EP1 might be increasing the intensity of ESI09-insensitive bands by applying a selective EP1 antagonist, SC 199220 (10μM), with PGE2. SC 199220 reduced band 1 staining intensity by 28% ± **12.02** and band 7 by 27% ± **8.44** (Figure 3a–b). SC 199220 had no effect on the intensity of any of the ESI09-sensitive bands (5, 6, 13, and 14), supporting the conclusion that $G_{q/11}$-PLC signaling and Epac-RAP-PLC signaling are distinct signaling pathways resulting in different PKC substrate profiles (Figure 3a–b).

PGE2 was used as a stimulus for most experiments in order to restrict our analysis to endogenous receptor-mediated activation of Epac/PKC signaling. However, 8pCpt, a direct activator of Epac, was used to confirm that Epac activation was sufficient to induce PKC substrate phosphorylation. Direct stimulation of Epac with 8pCpt effectively reproduced the PKC phospho-substrate staining profile induced by PGE2 (Figure 4a).

Tonic Epac-dependent PKC signaling in N2A cells

Because some PKC phospho-substrate staining was visible in samples from unstimulated cells and reduced by PKC inhibitors (Figure 4a), we examined whether Epac signaling contributes to this basal PKC activity. Application of ESI09 to unstimulated N2A cells largely eliminated band 13 (86% inhibition). In contrast, ESI09 also increased the intensity of bands 9, 10, and 12 by 31% ± **21.6**, 42% ± **14.41**, and 22% ± **12.62** respectively (Figure 4a–b), suggesting that Epac active under basal conditions may maintain the activity of a phosphatase, or suppress the activity of a discrete pool of PKC. The change in staining

intensity for each band in response to Epac inhibition is shown for each replicate of this experiment in Figure 4c.

Epac-dependent PKCα/γ signaling in N2A cells

The inhibitory effect of ESI09 on band 13 in PGE2-stimulated cells occurred in a dose-dependent manner. In this set of experiments, maximum inhibition reached 94% ± 1.66 (Figure 5a). Band 13 was completely eliminated by broad inhibition of PKC with GF109203X plus CGP53353. PKCα and PKCε have been reported previously to be activated downstream of Epac [9, 24, 25]. Because the PKCα inhibitor (HBDDE) also blocks PKCγ, we used real-time Sybr Green PCR to determine mRNA levels of PKC α, γ, and ε in N2A cells (Figure 5b). N2A cells expressed high levels of all three PKC isoforms, with PKCε having the highest expression (Figure 5b). PKC inhibitors selective for either PKCα/γ (HBDDE) or PKCε (εV1–2) were used to provide greater discrimination as to which PKC isoform(s) were responsible for the induction of individual bands. Strikingly, only HBDDE suppressed PKC phospho-substrate staining: of the 15 identified phospho-protein bands, 9 were significantly inhibited by HBDDE. These included bands 5 and 13, the two bands induced by Epac activation and suppressed by ESI09, as well as bands 9 and 10, which were enhanced by ESI09 in unstimulated cells. In addition, band 15, which was not affected by PGE2, was strongly induced by HBDDE.

Surprisingly, εV1–2 did not inhibit staining, but instead caused a consistent increase in the intensity of 3 bands, 7, 14, and 15. Bands 6 and 12 were not significantly affected by either inhibitor, suggesting that other PKC isoforms were responsible for these phospho-substrate proteins (Figure 5c–d). Together, these results suggest that PKC and/or PKC play a major role in Epac-dependent PKC signaling, but that additional signaling interactions are also involved.

Dependence of Epac-PKC signaling on the cytoskeleton

Epac reportedly connects cAMP signaling to cytoskeletal dynamics[26]. In chronic pain models, PGE2 activates Epac-PKCε in nociceptive sensory neurons, and this signaling pathway is sensitive to cytoskeletal disruption[27]. To determine whether the Epac-dependent PKC phosphorylation of substrate proteins in N2A cells is dependent upon intact cytoskeletal components, we stimulated the cells in the presence of several functionally-distinct cytoskeletal disruptors. We first tested cytochalasin D, a disruptor of actin filaments, and nocodazole, a microtubule inhibitor. Epac-dependent bands were not altered by nocodazole (Figure 6a), but were differentially modulated by cytochalasin D: of the 7 bands that were sensitive to ESI09 either at baseline or after PGE2, band 5 was inhibited by 48% ± **15.53**, but the others were unaffected (Figure 6b).

We next tested an inhibitor of Rho kinase, GSK 429286. Rho kinase mediates a range of cellular processes related to cytoskeletal assembly and reorganization[28–30], including regulation of actin remodeling and the organization of specialized membrane domains through ERM (ezrin, radixin, myosin) proteins, which link transmembrane proteins to the underlying cytoskeleton[31][32]. GSK 429286 inhibited the Epac-dependent PKC

phosphorylation of band 13 by 37% ± **4.89**. Band 9, which was enhanced in the presence of ESI09, was inhibited 41% ± **13.38** by GSK 429286 (Figure 6c).

Finally, we used the function-blocking tropomyosin (TPM) antibody CH1to inhibit TPM binding to actin[33]. TPM stabilizes actin filaments by wrapping around actin oligomers[34, 35], and is known to bind PKCα [36]. Intracellular delivery of CH1 using the Chariot™ protein delivery reagent in N2A cells inhibited the phosphorylation of Epac-dependent band 5 by 30% ± 6.54 and band 9 by 37% ± 16.90 (Figure 6d). In addition, band 3, which was not Epac-dependent but was increased by PGE2, was inhibited by 37% ± 9.04, respectively. Surprisingly, band 13, the band most sensitive to inhibition by ESI09, was increased by 50% ± 6.54 in the presence of CH1.

Collectively, these data demonstrate that activation of Epac drives the activation of specific isoforms of PKC. In N2A cells, Epac acts primarily through PKCα/γ, which phosphorylate a discrete subset of substrate proteins, including several that require specific cytoskeletal components for efficient phosphorylation. The physical interactions of Epac and PKC isoforms with cytoskeletal components are clearly complex and are likely to be multi-factorial.

Discussion

Signaling through cAMP and PKC are often considered to be independent pathways. PKC signaling is canonically induced downstream of $G_{q/11}$-coupled receptors through activation of PLC and the resulting increase in intracellular Ca^{2+} and DAG [37, 38]. cAMP signaling induced by G_s-coupled receptor activation of AC is canonically mediated by PKA[2], and in some cell types through cAMP-gated ion channels[39]. The discovery of Epac as an alternate cAMP effector expanded the repertoire of cAMP signaling and provided a mechanism for Gs activation of PKC.

In this study, we examined the profile of substrate proteins phosphorylated by PKC as an assay to characterize the activation of PKC by Epac. For this purpose, we used an antibody that recognizes phosphorylated phospho-serine residues within the consensus sequence recognized for phosphorylation by PKC isoforms. Using the prostaglandin PGE2 as a well-characterized pro-inflammatory agonist for Gs-coupled receptors, we found that, in N2A cells, PGE2 induced several discrete phospho-substrate bands for PKC in a manner that required functional Epac. A role of the $G_{q/11}$-coupled prostaglandin receptor EP1 in the Epac-dependent staining was ruled out using a selective EP1 receptor antagonist. A low level of Epac-dependent PKC signaling was also present in the absence of stimulation. The activation of PKC by Epac was PKC isoform-selective, given that Epac-dependent bands induced by PGE2 were primarily inhibited by HBDDE, a selective inhibitor of PKCα and γ (both of which were expressed in N2A cells). This result suggests that most biological actions of PGE2-Epac signaling mediated by PKC in N2A cells utilize one or both of these isoforms. Finally, we found that the Epac-dependent phosphorylation of several PKC substrate proteins, but not all, was modulated by interactions with the cytoskeleton. Table 2 provides a summary of the results.

The PKC phospho-substrate antibody used in this study was generated against a series of unique phospho-serine peptides recognized for phosphorylation by PKC isoforms[23]. Our control experiments demonstrated that bands labeled by this antibody were induced by PMA and suppressed in the presence of PKC inhibitors, indicating that the antibody preferentially labels proteins phosphorylated by PKC. The antibody is unlikely to be wholly specific for PKC-mediated phospho-serine, because the AGC kinases (a family of 60 serine/threonine kinases that most notably include PKA, PKG, and PKC) recognize similar consensus phosphorylation sequences and some overlap may exist, e.g., some residues are phosphorylated by both PKC and PKA. To increase confidence in our interpretation of Western banding profiles, we used this antibody in combination with pharmacological tools to selectively identify phospho-protein bands that are dependent on activation of Epac and sensitive to inhibitors of PKC. This approach provides the ability to identify Epac-dependent PKC phosphorylation events downstream of AC activation.

Several downstream targets and functional consequences of Epac-PKC signaling have now been reported, but it is likely that a broad diversity of targets remain to be discovered. In this study, we identified several phospho-protein bands representing targets of regulation by Epac-dependent PKC signaling in N2A cells. Band 13 was almost completely suppressed by ESI09 (94% ± 1.6% inhibition), suggesting that this band is most likely a single PKC substrate protein phosphorylated downstream of Epac. In contrast, band 5 was only partially inhibited by ESI09 (36% ± 11.52 inhibition) suggesting either that this band contains multiple proteins, with only some species regulated by Epac, or that other kinases such as PKA may phosphorylate the same serine residue as PKC.

We did not discriminate between the two isoforms of Epac (Epac1 and Epac2) in this study, because the drugs used here to manipulate Epac function (8pCpt and ESI09) act on both isoforms[4, 5, 40]. Both Epac1 and Epac2 can drive activation of PKC through Rap1 activation of PLC. Epac1 expression is fairly ubiquitous and has been investigated more extensively. Epac2 shows a more restricted distribution, with highest levels in brain, pancreas and testes. Both Epac isoforms have been implicated in neurite outgrowth, protection of cardiac cells from ischemia/reperfusion injury, and cancer cell proliferation and metastasis, through diverse actions on signal transduction cell division, cytoskeletal morphology, and adhesion[41, 42]. Distinct roles for Epac1 and Epac2 have also been identified, including sensitization of mechanical transduction in sensory neurons, promotion of vascular inflammation mediated by IL-6 (Epac1)[16; 41], and release of insulin from pancreatic beta cells (Epac2)[46].

In addition to the Epac-dependent bands that were induced in response to PGE2, we were surprised to find 5 bands that were consistently enhanced by the inhibition of Epac, either in unstimulated cells or in the presence of PGE2. Application of ESI09 with PGE2 increased the phosphorylation of bands 6 (19% ± 12.62) and 14 (60% ± 12.62), whereas application of ESI09 to unstimulated cells increased bands 9, 10 and 12. This unexpected result suggests that Epac signaling in N2A cells maintains the activity of a phosphatase, or suppresses the activity of a kinase, to suppress phosphorylation of those proteins under basal conditions. Epac has been shown previously to activate protein phosphatase 2A in the PCCL3 follicular thyroid cell line[43], providing a precedent for this regulatory mechanism. Our data indicate

that constitutive Epac signaling negatively regulates the phosphorylation state of a restricted subset of PKC substrates (bands 9, 10 and 12), while other substrate proteins are PKC-phosphorylated in response to acute Epac activation (bands 5 and 13).

One question we sought to address was whether the activation of PKC by Epac was isoform-selective, as both conventional and novel PKC isoforms can potentially be activated downstream of PLC activation by Rap1; both PKCα and PKCε have been separately implicated as effectors of Epac signaling [25][44]. Application of the PKCα/γ inhibitor HBDDE suppressed the induction of both Epac-dependent phospho-protein bands (5 and 13) by PGE2. Interestingly, HBDDE also strongly inhibited both bands that were sensitive to the EP1 antagonist (1 and 7). In contrast, the PKCε inhibitor V1–2 did not suppress any of the PGE2-induced bands. Instead, V1–2 increased the intensity of several Epac-independent bands (7, 14 and 15). HBDDE also strongly enhanced band 15. These increases in staining were consistent and significant across replicates. One possibility is that the increased staining indicates cross-regulation of other PKC isoforms in response to the inhibitors, as previously reported[45], since all identified bands were nearly eliminated by non-specific inhibition of PKC (Figure 2).

Previous studies have reported biological effects of constitutive Epac activity, including modulation of insulin secretion and leptin signaling [46]. We found evidence of constitutive Epac activity in N2A cells, indicated by the changes in PKC phospho-substrate profiles in response to inhibition of Epac under basal conditions. Strikingly, the major bands visible in unstimulated cells (#9, 10, and 12) were enhanced in response to ESI09, suggesting that basal Epac activity either promotes phosphatase activity or suppresses kinase activity. Inhibition of Epac under basal conditions reduced the phosphorylation of band 13 by 87% ± 4.04, which was largely dependent on PKC.

Numerous papers report that biological actions of Epac signaling are closely associated with cytoskeletal remodeling, including regulation of vascular permeability, receptor trafficking and cell adhesion [47, 48][49][50] [51]. To dissect the contributions of different cytoskeletal components to Epac signaling in N2A cells, we tested the impact of several different cytoskeletal disruptors on Epac-dependent PKC phospho-protein profiles. We used nocodazole to inhibit microtubule polymerization, cytochalasin D to disrupt the polymerization of actin filaments, and CH1, a function-blocking antibody against TPM [33]. TPM binds to F-actin and stabilizes the actin cytoskeleton, preventing actin severing, which is required for actin remodeling in response to intracellular signals[52]. We also tested an inhibitor of Rho kinase, which is a key regulator of cytoskeletal dynamics. Using these tools, we found that PGE2-induced PKC phosphorylation of band 5 was sensitive to both cytochalasin D and CH1. These data indicate that the Epac-dependent phosphorylation of band 5, which is mediated by PKCα/γ, is dependent upon actin remodeling. In contrast, phosphorylation of band 13, which was also mediated largely by PKCα/γ, was insensitive to cytochalasin D treatment, and was enhanced by CH1 (PKCα/γ phosphorylation of band 13 was increased by 50% ± 10.06). These results indicate a complex interplay between Epac signaling and cytoskeletal remodeling that may be permissive for Epac activation, or may regulate the interaction between Epac and PKCα/γ. It is worth noting that PKCα can bind directly to tropomyosin[36] and filamin[53], another actin-associated protein, although a

recent report indicates that the translocation of PKCα to the plasma membrane is independent of the actin cytoskeleton[54].

Rho kinase is a key effector of cytoskeletal dynamics and has been suggested to regulate the subcellular localization of Epac. The exact mechanism that anchors Epac to the plasma membrane has not been identified, but ERM proteins have been implicated in Epac translocation and local function at the membrane. Phosphorylation of inactive, cytoplasmic ERM proteins by Rho kinase induces the active conformation required for translocation to the plasma membrane[31]. For example, Epac1 enhances cell adhesion in HEK cells in a manner that requires interaction with ERM proteins and translocation to the plasma membrane[50]. In mouse peripheral sensory neurons, translocation of Epac1 to the plasma membrane leads to modulation of Piezo2 ion channel function, although the involvement of ERM proteins has not been evaluated[16]. Inhibition of Rho kinase may prevent the anchoring of Epac to the plasma membrane by ERM proteins, or may act more indirectly by preventing cytoskeletal remodeling. In the present study, Epac-dependent PKC phospho-substrate bands 9 and 13 were suppressed by Rho kinase inhibition, suggesting that ERM-Epac scaffolding could regulate access of Epac-activated PKC to domain-specific substrates. This interpretation is consistent with the idea that the consequences of Epac activation are highly context-dependent, based on the subcellular localization of Epac orchestrated by scaffolding proteins. Profiling of Epac-dependent PKC substrate bands provides an assay for the evaluation of mechanisms targeting Epac to functionally-discrete domains in different cell types.

cAMP signaling is tightly regulated spatially and temporally through scaffolding of adenylyl cyclases and associated signaling proteins, including Epac, kinases, and phosphatases, among others[55]. Understanding the unique interactions between Epac and PKC isoforms will help to clarify the role of cAMP signaling through Epac in various pathological processes, such as ischemic stroke due to endothelial barrier dysfunction, chronic pain due to nociceptor sensitization, cardiac hypertrophy, and neuroblastoma differentiation due to microtubule-stabilizing factors[56]. Here, we provide an approach for the characterization of Epac-dependent PKC signaling that allows the separation of discrete phospho-protein substrate bands by molecular weight. Furthermore, the unbiased identification of downstream targets for Epac-PKC signaling poses a significant challenge that can be approached using this technique. Future experiments in our laboratory will combine the approach used here with mass spectrometry to identify putative Epac-PKC targets and investigate their roles in the physiological consequences of Epac signaling. Elucidation of substrate proteins for Epac-dependent PKC activity should provide novel therapeutic targets for evaluation in a number of diverse pathological conditions.

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Compliance with Ethical Standards

This article does not contain any studies with animals performed by any of the authors. This article does not contain any studies with human participants performed by any of the authors. All authors certify that they have no conflicts

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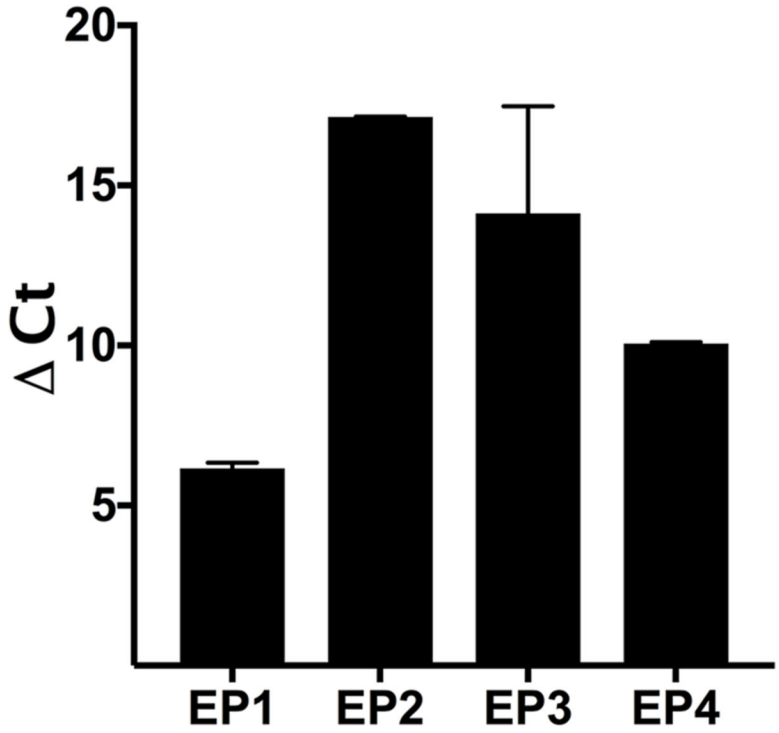

Fig. 1. EP receptor expression in N2A cells

Real-time qPCR on N2A cells revealed much higher expression levels for EP1 and EP4 than for EP2 and EP3. Note that a higher Ct value indicates a lower mRNA level; an increase of 1 unit Ct indicates a 50% decrease in [mRNA]. Bars show the Ct +/− s.d for each receptor normalized to GAPDH.

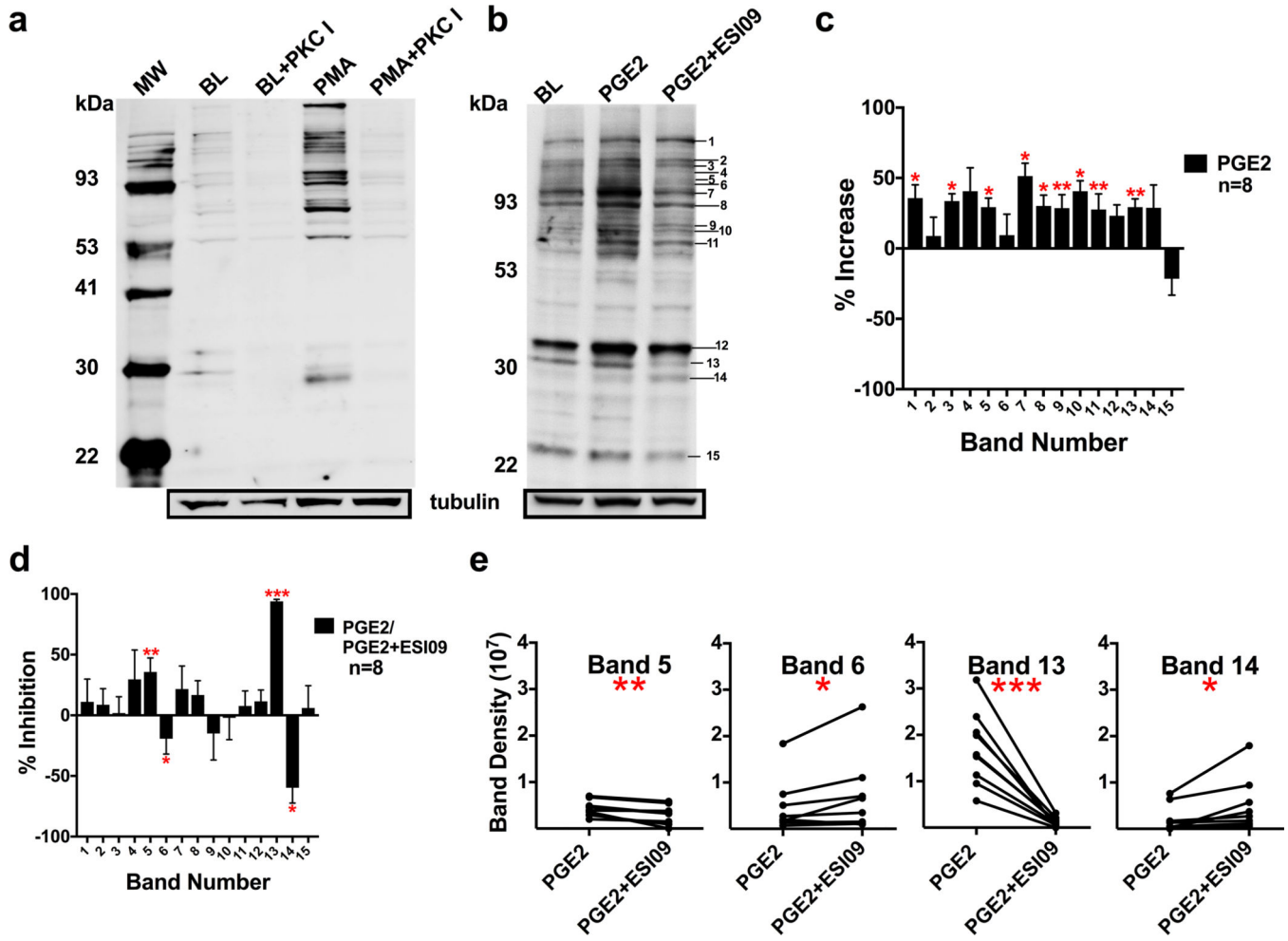

Fig. 2. PGE2 activates Epac-dependent PKC signaling in neuroblastoma N2A cells

Western blot analysis of PKC phospho-substrate profiles of (**2a**) PMA-stimulated (20 minutes) or (**2b**) PGE2-stimulated (30 seconds) N2A cells +/− inhibitors (PKC I= 20μM GF109203X and 20μM CGP53353 for broad-spectrum inhibition of PKC isoforms α, β1, βII, δ, ε, ζ). The graph in **Fig 2c** quantifies the percent increase in band intensity in response to PGE2. **Fig 2d** quantifies the percent inhibition by ESI09 of staining induced by PGE2 for each individual identified band (designated by the number shown on the blot in **2b**). **2e** shows the change in band intensity for each experimental replicate in response to application of ESI09 for the 4 significant bands in **2d**. Band intensities were normalized to tubulin, and percent inhibition of staining was calculated to determine the contribution of Epac signaling to PKC phospho-substrate staining for each band. Statistical significance was determined by paired t-test. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$. Western blots shown are representative examples. PMA=Phorbol myristate acetate; ESI09= Epac inhibitor.

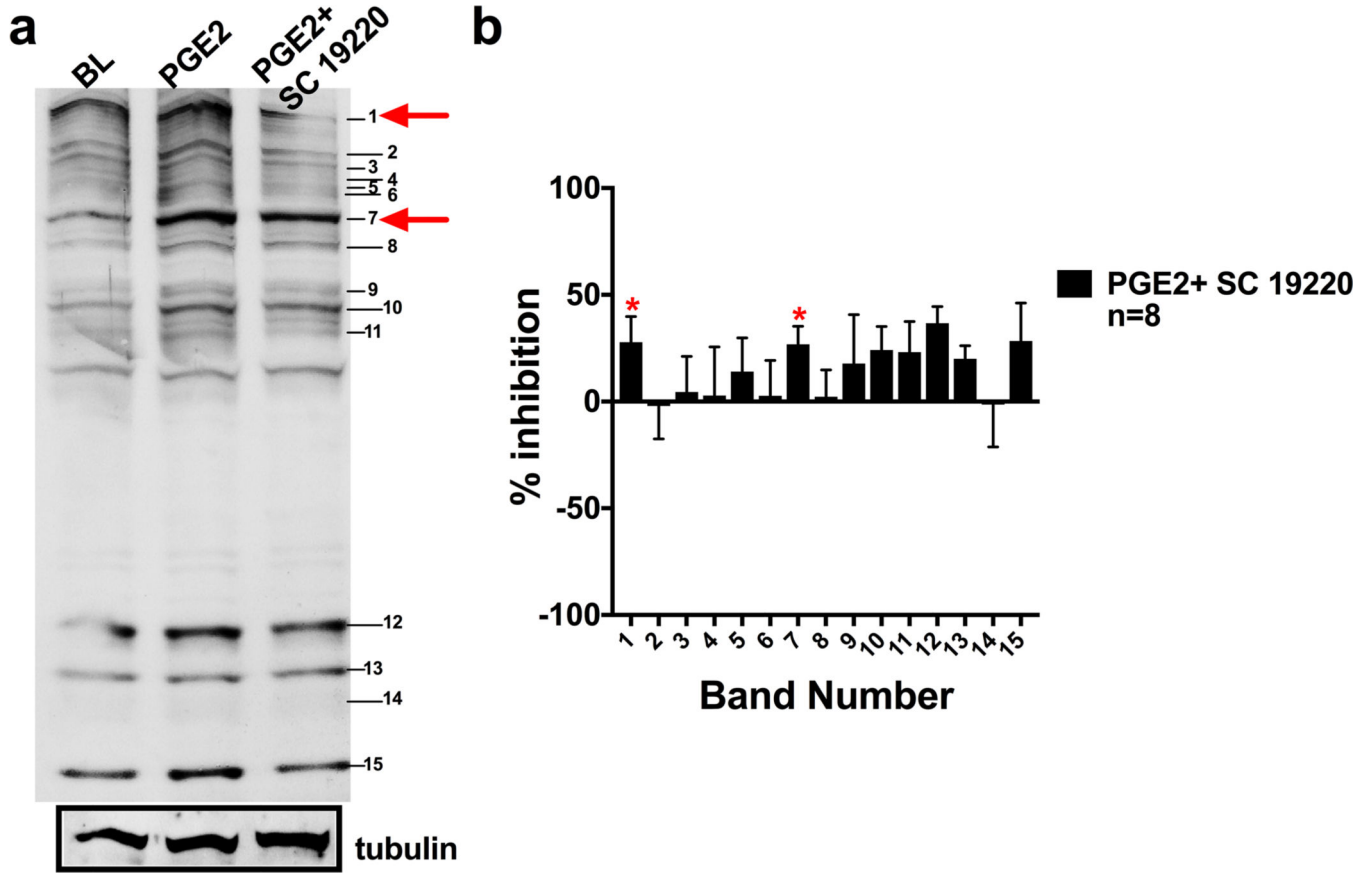

Fig. 3. Contribution of EP1 to PKC phospho-profiles in N2A cells

3a shows PKC phospho-substrate profiles of PGE2-stimulated (30 seconds) N2A cells +/− 10μM EP1 antagonist SC 199220. **3b** quantifies the percent inhibition of PGE2-induced staining of individual identified bands in the presence of the EP1 antagonist.

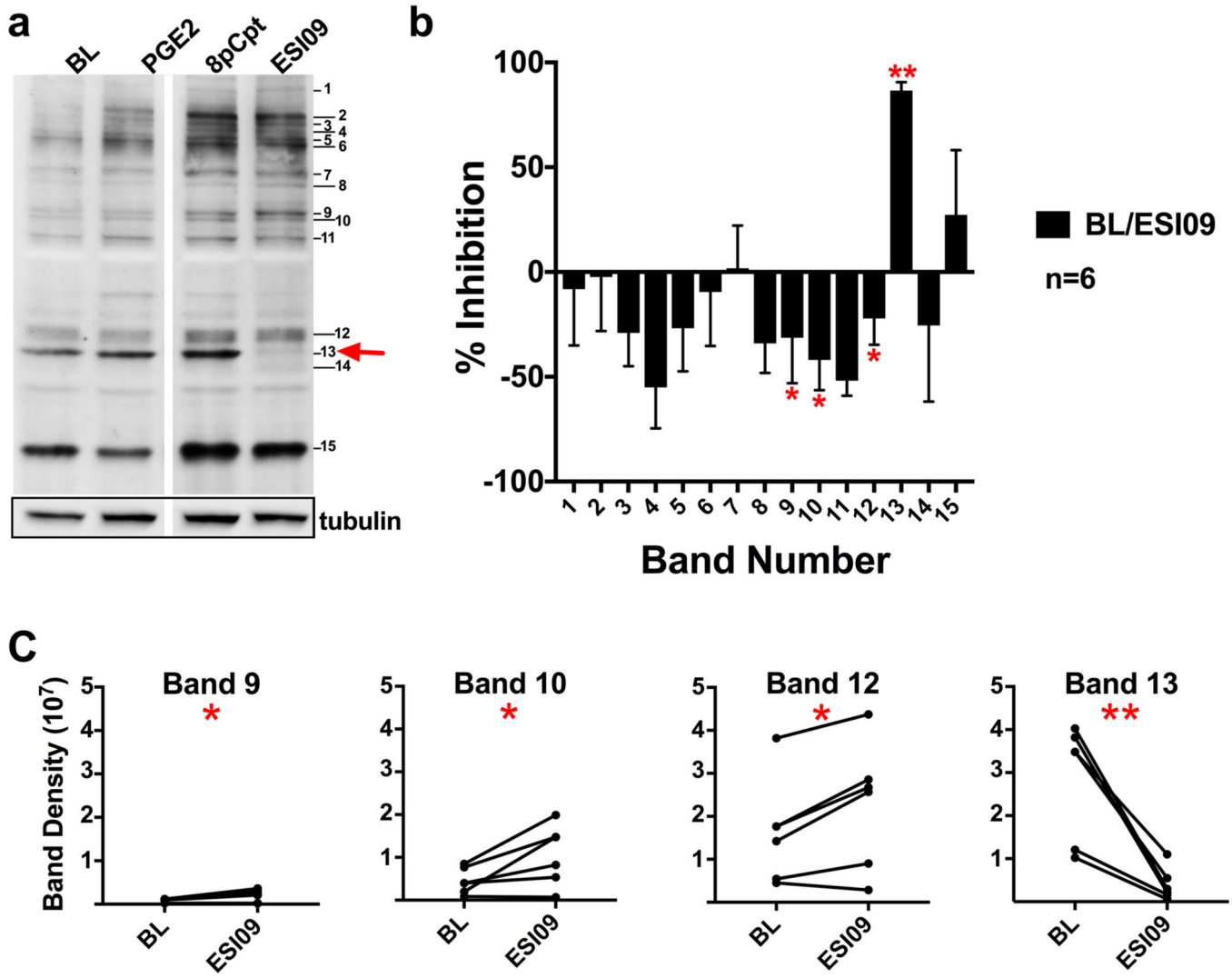

Fig. 4. Epac regulates basal PKC activity

(**4a**) Representative Western blot of PKC phospho-substrate profiles of unstimulated cells (BL), and cells exposed to PGE2 (30 seconds), the Epac activator 8pCpt (30 seconds), or ESI09 alone (30 minutes). **4b** quantifies the impact of ESI09 on the intensity of individual identified bands from unstimulated cells, numbered on the blot (displayed as percent of baseline). **4c** shows the change in individual band intensity for each experimental replicate for each band showing significant inhibition by ESI09. Band intensities were normalized to tubulin and the percent inhibition by ESI09 was calculated to determine the contribution of Epac signaling in unstimulated cells. Statistical significance was determined by paired t-test (n=6). * = $p<0.05$; ** = $p<0.01$. 8pCpt=Epac activator; ESI09= Epac inhibitor.

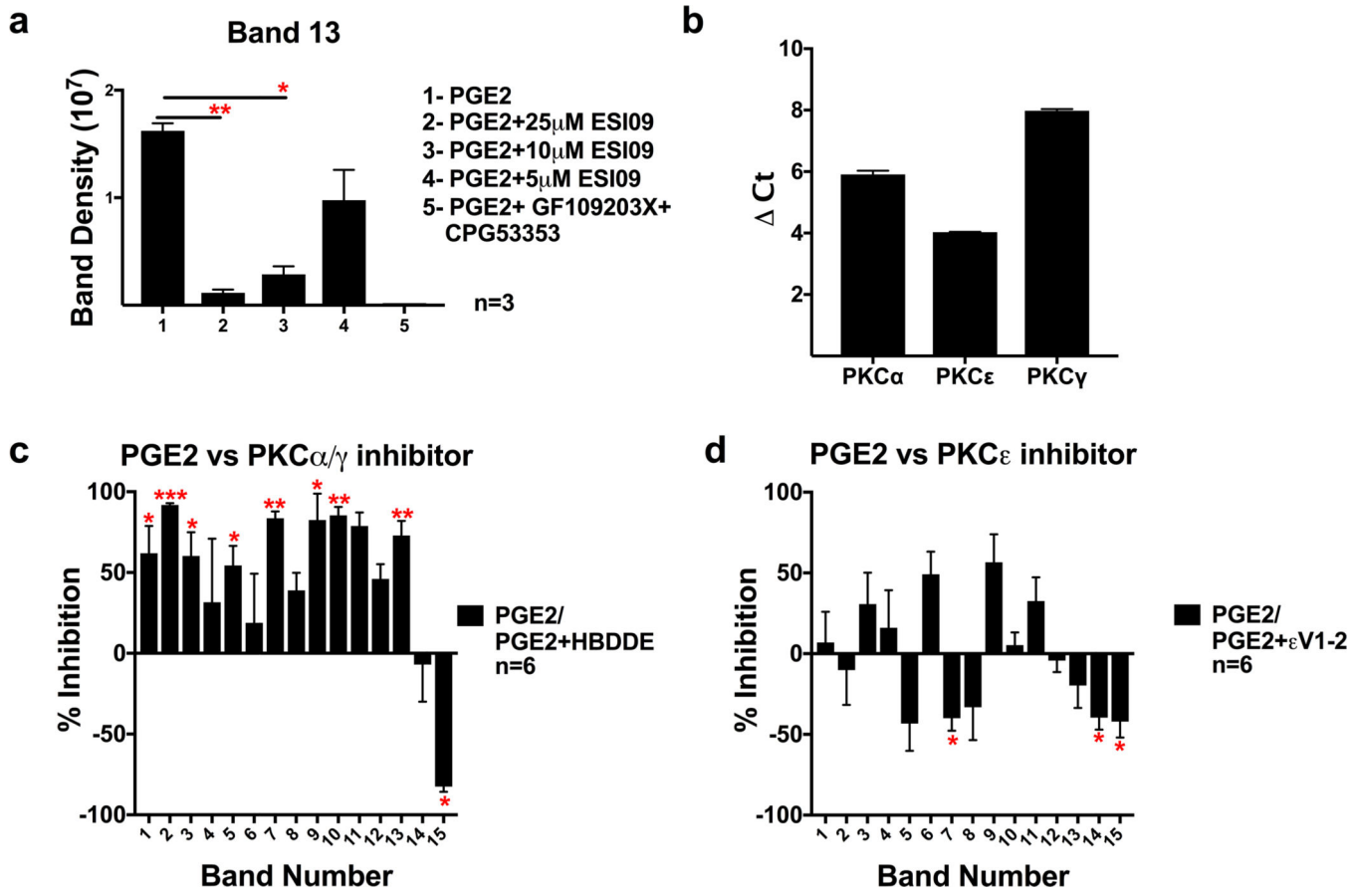

Fig. 5. PKC isoform selectivity of PGE2-induced phospho-protein bands

(**5a**) Graph displays the intensity of band 13 in protein samples from N2A cells that were stimulated with 10μM PGE2 in the presence of increasing concentrations of ESI09. Band 13 was completely eliminated by addition of PKC inhibitors 20μM GF109203X and 20μM CGP53353. (**5b**) Real-time qPCR revealed mRNA levels of PKCα, ε, and γ in N2A cells. Bars show the Ct + s.d for each PKC isoform, perfomed in triplicate and normalized to GAPDH. The percent inhibition of PGE2-induced PKC phospho-substrate staining is shown in the presence of PKCα/γ inhibitor HBDDE (**5c**) or PKCε inhibitor peptide εV1–2 (**5d**). Band intensities were normalized to tubulin and the percent inhibition of staining was calculated to determine the contribution of specific PKC isoforms. GF109203X inhibits PKCα, β1, δ, ε and ζ isoforms; CGP53353 inhibits PKCβII; HBDDE inhibits PKCα and PKCγ isoforms. Statistical significance was determined by paired t-test. * $p<0.05$; ** $p<0.01$.

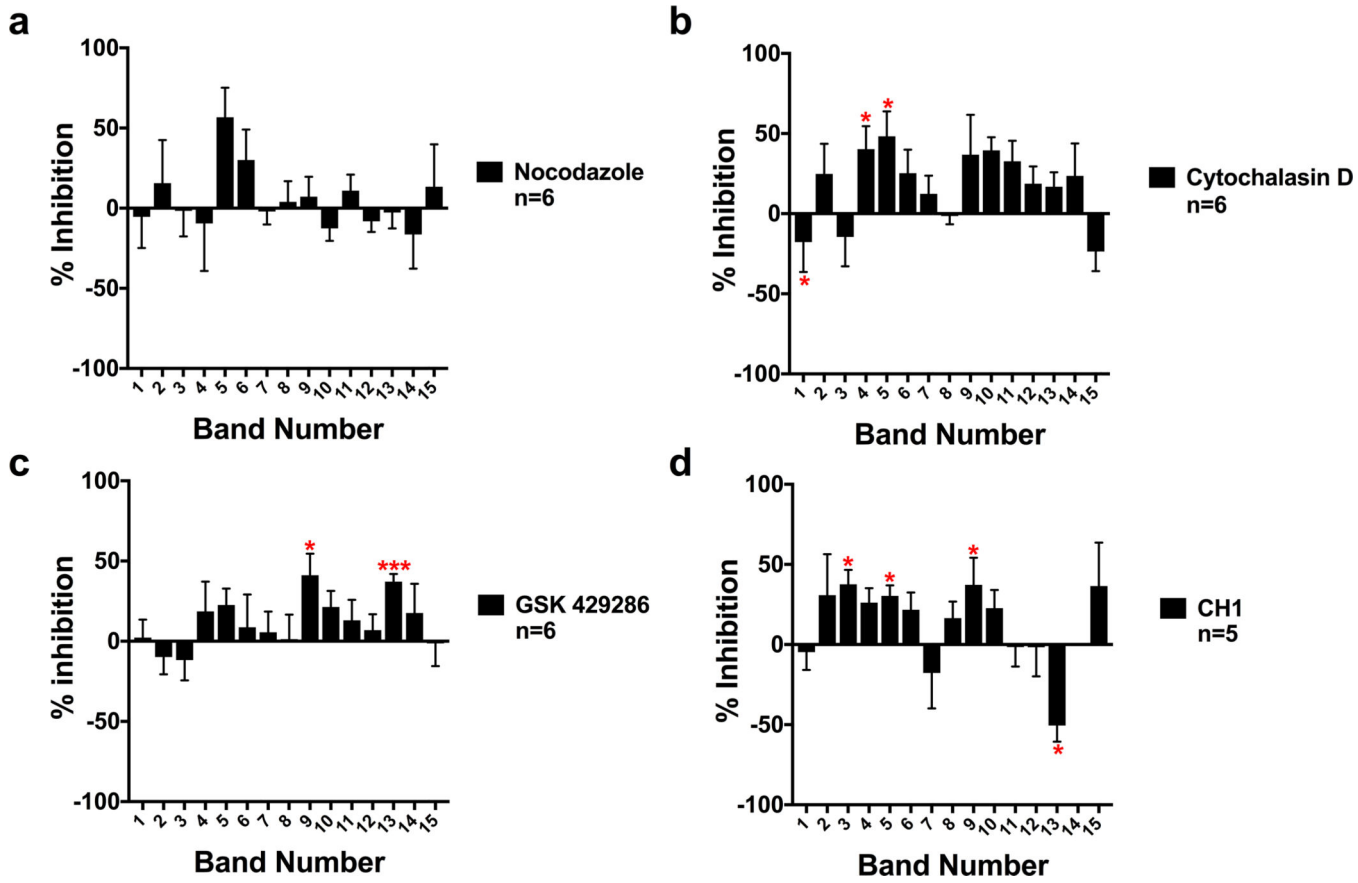

Fig. 6. Cytoskeletal modulators alter Epac-dependent PKC phospho-substrate staining

N2A cells were stimulated for 30 seconds with 10 μM PGE2 in the presence of 5 μM nocodazole (**6a**), 10 μM cytochalasin D (**6b**), 2 μM GSK 429286 (**6c**), or 1 μg TPM monoclonal function-blocking antibody (CH1) (**6d**). Band intensities were normalized to tubulin and the percent inhibition of staining was calculated to determine the contribution of the specific cytoskeletal modulators. Statistical significance was determined by paired t test. * = $p<0.05$; *** = $p<0.001$. GSK 429286 = selective Rho-kinase inhibitor.

Table 1

Real-time PCR primers for murine EP receptors and PKC isoforms

Ptger1 forward	CTA AGA TTC GGG CTC TCC ATA AA
Ptger1 reverse	GTG GGT AGA AGG TGT TGA GAT T
Ptger2 forward	GGC CTC ATT GGA GAA GTG TAA G
Ptger2 reverse	GCA GCC CAA ACT ACC TCA TAA A
Ptger3 forward	GGT TCC TGT GAA GAC TGA AGA C
Ptger3 reverse	AAG GTT CTG AGG CTG GAG ATA
Ptger4 forward	AAT TTG CTT CCA GGT TCG CA
Ptger4 reverse	CTG GGG AAT GTG ACT TGC AG
Prkca forward	CCGAGGGAGAGAGGGATGTC
Prkca reverse	TGCACAAACTGGGGGTTGAC
Prkce forward	GGAGGACGCCATCAAGCAA
Prkce reverse	CAGCATGAGCTCCCTCCAT
Prkcg forward	CGCAGTGGCGAAAACTTTGA
Prkcg reverse	AGAATTCGGACTAGAGAGGGT

Table 2

Summary of PKC-dependent phospho-substrate protein bands in N2A cells

	Pharmacological Manipulation								
			PGE2 vs:						
Band	**PGE2/BL**	**ESI09/BL**	**ESI09**	**SC 199220**	**HBDDE**	***e*V1–2**	**Cytochalasin D**	**GSK 429286**	**CH1**
1	↑ 35.72 +/− 9.4			↓ 27.83 +/− 12.02	↓ 62.00 +/− 16.92		↑ 17.72 +/− 18.64		
2	↑ 8.88 +/− 13.2				↓ 91.81 +/− 1.09				
3	↑ 33.64 +/− 5.2				↓ 60.24 +/− 114.71				↓ 37.6 +/− 9.04
4	↑ 40.57 +/− 16.7						↓ 40.26 +/− 14.34		
5	↑ 29.27 +/− 6.5		↓ 35.74 +/− 11.52		↓ 54.43 +/− 12.11		↓ 48.26 +/− 15.53		↓ 30.42 +/− 6.54
6	↑ 9.35 +/− 14.9		↑ 19.33 +/− 12.62						
7	↑ 51.38 +/− 9.11			↓ 26.8 +/− 8.44	↓ 83.62 +/− 4.24	↑ 39.94 +/− 7.75			
8	↑ 30.21 +/− 1.61								
9	↑ 28.58 +/− 9.56	↑ 31.41 +/− 21.6			↓ 82.45 +/− 16.35			↓ 41.18 +/− 13.38	↓ 37.29 +/− 16.90
10	↑ 40.63 +/− 1.41	↑ 41.93 +/− 14.41			↓ 85.41 +/− 5.25				
11	↑ 27.67 +/− 11.08								
12	↑ 23.16 +/− 7.78	↑ 22.13 +/− 12.62							
13	↑ 29.26 +/− 5.93	↓ 86.6 +/− 4.04	↓ 94.00 +/− 1.66		↓ 72.96 +/− 9.03			↓ 37.11 +/− 4.89	↑ 50.53 +/− 10.06
14	↑ 28.86 +/− 16.18		↑ 59.78 +/− 12.58			↑ 39.64 +/− 7.37			
15	↓ 21.5 +/− 11.68				↑ 82.51 +/− 3.20	↑ 42.10 +/− 9.83			

Table 2 summarizes the effects of cell stimulation and manipulation of signaling with selective inhibitors. Increases or decreases in band intensity are indicated with up or down arrows. In the first column, values in gray did not reach significance. Bold boxes around rows 5 and 13 highlight the two bands that were significantly enhanced by PGE2 and inhibited by ESI09, indicating Epac-dependent PKC substrate phosphorylation. Nocodazole was not included here because it did not affect the intensity of any bands. BL= Baseline (unstimulated); ESI09= Epac inhibitor; SC 199220= EP1 antagonist; HBDDE= PKCα/inhibitor; eV1–2= PKCε inhibitor peptide; GSK429286= Rho kinase inhibitor; CH1= function-blocking tropomyosin mAb.