



Exchange protein activated by cAMP (EPAC) controls migration of vascular smooth muscle cells in concentration and time-dependent manner

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Abstract

Background: Cyclic AMP influences many facets of signaling in vascular smooth muscle cells (VSMCs) including those mediated by protein kinase A (PKA) and those operating through exchange proteins activated by cAMP (EPACs). Activation of EPACs has been suggested to promote VSMC migration and to increase arterial neointima development; however, the molecular mechanisms of EPACs in VSMCs still remain unclear. The goal of this study was to identify mechanisms of action of EPACs related to vascular growth, and the hypothesis was that EPACs operate via distinct, kinase-mediated mechanisms to control VSMC migration and proliferation.

Methods and results: Using a wounding scrape injury on confluent rat primary VSMCs, the direct EPAC activator CPT transiently stimulated migration after 6 hours followed by inhibition of migration after 16 hours which persisted through 22 hours. This prolonged and stable inhibition of cell migration by EPAC was potentiated by pharmacologic blockade of MAPK, PI3K, Rac, PKC or PKG. In-Cell Western analysis on intact adherent cells was then used to identify differential phosphorylation of the actin-regulatory protein vasodilator-activated serum phosphoprotein (VASP), as VASP_{Ser157} and VASP_{Ser239} have been suggested as respective readouts of PKA and PKG and to have capacities to regulate cell growth. CPT (10 μ M) alone had no effect on either phosphoVASP species, but in the presence of a PKA inhibitor VASP_{Ser239} phosphorylation was significantly elevated. Involvement of focal adhesion proteins implicated in cell motility (focal adhesion kinase (FAK), paxillin (PAX)), were then assessed with/without EPAC stimulation. CPT increased PAX expression but failed to markedly alter FAK_{Tyr397} phosphorylation. Finally, CPT alone had minimal effects on VSMC proliferation yet along with blockade of Rac, PI3K or MAPK or following direct activation of PKA proliferation was significantly decreased.

Conclusions: These data confirm that PKA and EPAC work cooperatively to primarily inhibit VSMC migration likely through mechanisms largely independent of VASP. Multiple kinases appear to be involved in this EPAC regulation of VSMC migration. Collectively these data suggest that EPAC regulates migration of VSMCs in time- and concentration-dependent manner, yet its precise mechanisms of action and its full influence on cellular proliferation remain to be fully characterized.

Keywords: CPT, cyclic AMP, EPAC, migration, MAPK, PI3K, PKA, PKC, PKG, proliferation, Rac, VASP

Introduction

Vascular growth is an important feature in the normal development of blood vessels and in many forms of homeostatic vascular adaptation and maintenance. Vascular smooth muscle cells (VSMCs) play critical roles in blood vessel growth and in proper vessel function in order to regulate blood flow and vessel tone. During pathophysiological conditions, however, abnormal VSMC growth can lead to lumen occlusion, vessel wall remodeling and dysfunction foundational to cardiovascular diseases.

Cell migration is a necessary process during normal vessel growth and reparative wound repair yet also serves as an integral component of pathologic tissue growth. In order for cells to migrate there has to be interactions between cell-to-cell junctions, cytoskeletal proteins and elements of the extracellular matrix (ECM) [1]. The assembly/disassembly of the actin cytoskeleton along with the formation and dissolution of focal adhesions and other intercellular contacts must be highly coordinated for appropriate cellular movement [2]. In

this light the GTPases Rho, Rac and cdc42 play critical roles: Rho mediates cell contractility, Rac regulates protrusions of lamellipodia and filopodia and cdc42 regulates directionality [3]. Other focal adhesion proteins such as focal adhesion kinase (FAK), Src and paxillin (PAX) also play key roles in this process [2,4]. Focal adhesion kinase is an intracellular protein tyrosine kinase (PTK) that is recruited to and activated at focal adhesion (FA) sites and that acts downstream of multiple ECM components [5]. Focal adhesion kinase exists in an auto-inhibited state and autophosphorylation at Tyr₃₉₇ promotes FAK-Src complex formation resulting in complete FAK activation [6]. The recruitment of FAK to FA is linked to FA turnover; therefore, signals promoting FAK localization have been connected to increased FA turnover and a hyperdynamic cellular state [7]. PAX is an adaptor protein at the interface of the cytoskeleton and plasma membrane that acts as a scaffold for FAK/Src-mediated protein recruitment [8,9]. Another adaptor protein that interacts with the cytoskeleton and is important in FA formation is vasodilator-stimulated serum phosphoprotein (VASP), which also serves as a marker for active protein kinase signaling [10]. In this regard we and others have proposed that VASP is essential for migration of VSMCs [11,12]. Indeed, these multiple players involved in the control of cellular migration present not only redundant layers of precision and accuracy but also a challenge for its therapeutic regulation under pathological conditions.

Cyclic nucleotide signaling is at the forefront of our understanding of the complex regulation of VSM growth due to its numerous downstream targets capable of growth control [13]. Cyclic AMP-mediated activation of the guanine nucleotide exchange factor (GEF), exchange protein activated by cAMP (EPAC), activates Rap [14], an adhesion protein and regulator of cell junctions and integrin- and cadherin-mediated cell migration [14-16]. It has been demonstrated that activation of EPAC/Rap inhibits epithelial cell migration by modulation of FA dynamics [14]. Interestingly, activation of EPAC has been shown to either stimulate or inhibit migration depending on cell type and local micro-environment [14,17,18]. Thus, precise mechanisms of EPAC on vascular cell migration have not been fully defined.

In this study we investigated the mechanisms of action of EPAC on migration in rat primary VSMCs. Using an established *in vitro* approach for evaluating cell migration, results demonstrate that EPAC inhibits migration via multiple kinase signals and through increases in PAX and modulation of FA proteins and that these occur in time- and concentration-dependent fashion.

Methods

Rat primary VSMC culture

Following procedures established in our lab [19], thoracic aorta VSMCs were harvested from male Sprague-Dawley rats (100-125 grams) and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and Primocin

(100mg/L) at 37°C in 95% air/5% CO₂. Cells were split and propagated through passage 6 unless otherwise specified. All studies abided by the guidelines of the ECU Animal Care & Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health, Publication No. 85-23, revised 1996).

In-cell western blotting

Evaluation of VASP, FAK and PAX was performed using an established assay for monitoring kinase signaling in intact adherent cells [11,20,21]. VSMCs were seeded (~20,000 cells/well) in 96-well plates and once adhered, pretreated with either vehicle or select kinase inhibitors (30 min) after which they were stimulated with the cAMP analog 8pCPT-2'OMecAMP (CPT; 30 min; Tocris). The selective inhibitors used for In-Cell Westerns were: PKI (for cAMP-dependent protein kinase; Enzo), DT-2 (for cGMP-dependent protein kinase; Sigma-Aldrich), calphostin C (CALC; for PKC; Enzo), PD 98059 (PD; for MAPK; Tocris), LY 294002 (LY; for PI3K; Sigma Aldrich), EHT1864 (EHT; for Rac; Tocris), and PP2a (for Src kinase; Sigma Aldrich). After incubation for various times, media was removed and cells were fixed with 4% formalin in PBS for 20 min. Cells were washed and permeabilized with PBS containing 0.1% Triton X-100, followed by 1X PBS+0.1% Tween-20. Cells were blocked with IR blocking solution (Rockland Biochemicals) for 3 hours and then incubated with primary antibodies directed against VASP_{Ser239} (1:500; Cell Signaling), VASP_{Ser157} (1:500; Cell Signaling), cyclin A (1:500; Santa Cruz), p21 (1:500; Santa Cruz), p27 (1:500; Santa Cruz), PAX (1:1000; Cell Signaling), FAK_{Tyr397} (1:1000; Cell Signaling), or α -tubulin (1:1000; Sigma) overnight at 4°C. Cells were washed with PBS+0.1% Tween-20 followed by incubation with two secondary antibodies: IRDye 800CW (1:500; LiCor) and Alexa Fluor 680 for 1 hour. Cells were washed with PBS containing 0.1% Tween-20 followed by plain PBS. The plate was dried overnight and scanned using the appropriate channels for detection with the Odyssey Imager (LiCor).

Scratch wound migration assay

Per our recent descriptions [11], VSMCs were seeded in 24-well plates and grown to confluence. Cells were injured with a standardized 1 mm scrape and the wells were washed with PBS. In separate cohorts cells were treated with PKI, DT2, CALC, LY, EHT or vehicle for 30 min prior to stimulation with CPT at the time of scraping. Images were captured at time zero and at varying time points up through 22 hours, followed by measurements of the distances migrated by each cell wave front. Data for cell migration are presented as "% regrowth", calculated as the distance the cell wave fronts migrated for the treatment group divided by the distance the wave fronts migrated for the vehicle control (CON) group x100.

MTT assay

VSMCs were seeded in 96-well plates and pretreated with LY or vehicle for 30 min prior to stimulation with CPT for 24hrs.

After treatment media was removed a colorimetric MTT cell proliferation assay kit (ATCC) was used with MTT added to each well and incubated for 4 hours at 37°C. The MTT was removed, MTT solvent was added, and absorbance was read at 570 nm after 15 min.

BrdU assay

VSMCs were seeded in 96-well plates and pretreated with LY, EHT or vehicle for 30 min prior to stimulation with CPT for 24hrs. After treatment media was removed and a colorimetric BrdU ELISA was performed (Roche) using BrdU labeling solution and 3 hour incubation at 37°C. The labeling solution was removed, cells were fixed and the anti-BrdU antibody added for 90 min. Cells were washed, substrate was added, and absorbance was read at 370 nm after 30 min.

Hemocytometry assay

VSMCs were seeded in 24-well plates (40,000 cells/well) and after adherence were quiesced overnight in 0.2% FBS. Cells were pretreated with PKI, DT2, LY, PD, a selective activator of PKA (BnzcAMP; Sigma) or vehicle for 30 min prior to stimulation with CPT for 72 hrs, after which cells were trypsinized and cell counts estimated using a hemocytometer at 10X magnification.

Data analysis

Statistical significance between experiments was determined using an analysis of variance (ANOVA) or paired t-test. In the event that the *F* ratio indicated a significant change occurred, a post-hoc test was performed to identify individual differences. Results are reported as mean±standard error of the mean (SEM). Statistical significance was determined by a *p* value<0.05.

Results

Effect of the direct activator of EPAC (CPT) on cell migration

It was previously reported that EPAC activation by CPT promotes migration in rat aortic VSMCs [18]; however, in that study only a single time point (4 hours) was used and a mechanism was not described. In our current work we sought to determine the mechanisms of action of CPT and its ability to control migration in rat primary VSMCs. Using concentration- and time-dependent approaches and an established [11] *in vitro* wound model, results show that CPT at 30µM and 50µM significantly increased cell migration compared to vehicle controls after 6 hours (Figure 1A), in agreement with the aforementioned report [18]. However, in order to evaluate longer-term persistent migration longer time points were used and results show that after 16 and 22 hours these concentrations of CPT failed to markedly alter cell migration yet a lower concentration (10µM) significantly inhibited migration compared to vehicle control cells (Figure 1B). This trend (*p*=0.394) towards a reduction in migration with 10µM CPT was still evident after 22 hours compared to control cells (Figure 1C).

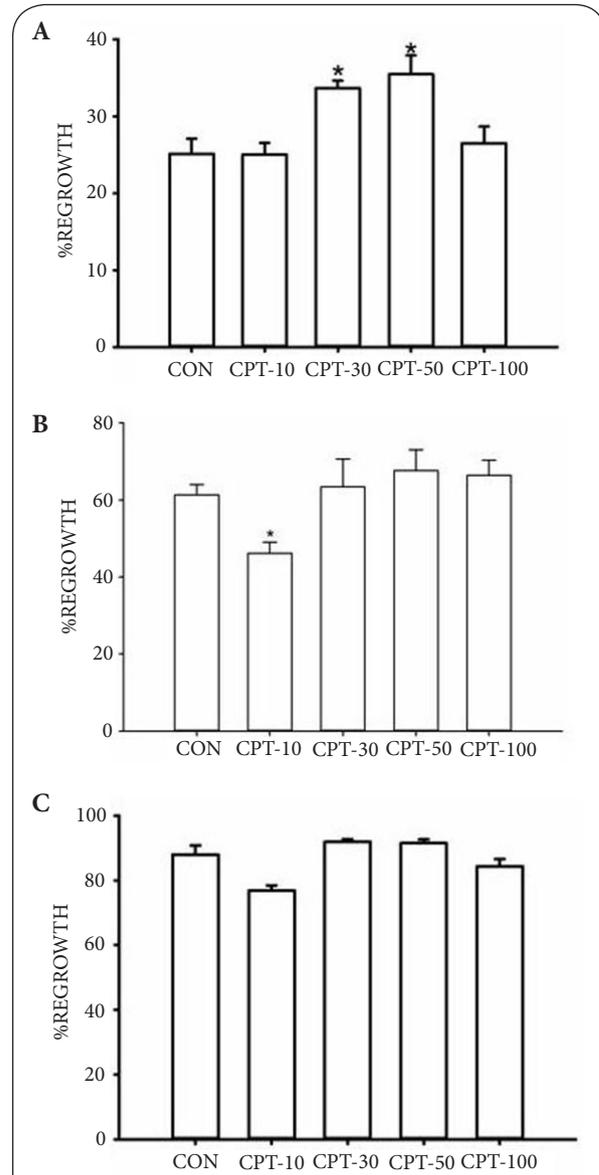


Figure 1. Effect of the EPAC stimulator 8pCPT-2'OMecAMP (CPT) on cell migration. (A) Treatment of rat primary VSMCs with vehicle (controls; CON) or CPT (10-100µM) for 6 hours (n=16). (B) Treatment of cells with vehicle (CON) or CPT (10-100µM) for 16 hours (n=6). (C) Treatment of cells with vehicle (CON) or CPT (10-100µM) for 22 hours (n=3). **p*<0.05 versus CON.

The primary effector protein of EPAC is Rap [22], and Rap can activate either PI3K [23] or Rac [24], both previously implicated in the control of cell migration [25,26]. In our initial investigation into potential mechanisms of action of CPT on cell migration we used a pharmacologic inhibitor of PI3K (LY294002 (LY); 1µM) or Rac (EHT1864 (EHT); 1µM) in the presence or absence of CPT (10µM) and performed the wounding scrape injury for up to 22 hours. After 6 hours we observed no significant changes in cell migration with CPT

compared to vehicle controls (Figure 2A). Although the effects of LY alone at this time point closely matched those observed with CPT alone, concomitant LY and CPT significantly inhibited cell migration compared to controls (Figure 2A). After 16hrs, EHT, LY and an inhibitor of MAPK, (PD98059 (PD); 10µM) alone inhibited cell migration compared to vehicle controls (Figure 2B). CPT (10µM) exerted no additional effects on cell migration in the presence of these inhibitors compared to the inhibitors alone. Interestingly, after 22 hours CPT (10µM) in combination with EHT significantly reduced migration compared to controls (Figure 2C).

Cyclic AMP is a known inhibitor of cell migration [14,27,28]. In addition, we have previously demonstrated cAMP-directed protein kinase involvement in VSMC migration [11]. Therefore, we examined the effect of pharmacologic kinase blockade in the presence of CPT. Interestingly, none of these kinase inhibitors (PKI to inhibit PKA; calphostin C for PKC; DT-2 for PKG) had any effect on cell migration after 6 hours compared to CPT alone (data not shown). However, at 16 hours CALC augmented the inhibitory effect of CPT (Figure 2D) and the effect was still evident after 22 hours (Figure 2E). Additionally, at 22 hours DT2 with/without CPT also inhibited migration compared to vehicle or CPT alone (Figure 2F).

kinase-mediated phosphorylation of VASP is required for its activation and this has been documented to occur at discrete serine (Ser)/threonine (Thr) sites [10,30]. Therefore, in order to determine if the effects of EPAC on cell migration occur through modulation of VASP and to gain insights into potential kinase-mediated mechanisms, VSMCs were pretreated with PKI (10µM), CALC (100nM) or LY (1µM), respective inhibitors of PKA, PKC and PI3K, with/without CPT (10µM). CPT failed to noticeably alter expression of the reported PKG-sensitive VASP_{Ser239}, but in the presence of the PKA inhibitor PKI, CPT significantly increased VASP_{Ser239} compared to vehicle controls and CPT alone (Figure 3A). Also, CPT in the presence of the other kinase inhibitors (LY, CALC) had no effect on VASP_{Ser239} expression compared to CPT alone (Figure 3A). In a parallel line of investigation VSMCs were pretreated with the inhibitors PKI (10µM), CALC (100nM) or DT-2 (10µM), inhibitors of PKA, PKC and PKG, respectively, in addition to inhibitors of PI3K (LY) and MAPK (PD) with/without CPT (10µM). Neither CPT alone or in the presence of any of the kinase inhibitors had an effect on expression of the reported PKA site VASP_{Ser157} (Figure 3B). Interestingly, the Rac inhibitor (EHT 1µM) alone significantly increased VASP_{Ser157} but it was not effective in the presence of CPT (Figure 3C).

Effect of EPAC on VASP phosphorylation

VASP is a cytoskeletal and focal adhesion protein proposed to be involved in migration of VSMCs [11,29]. Additionally,

Effect of EPAC on PAX and focal adhesion kinase (FAK)

To further evaluate the mechanisms of action of EPAC on migration, we examined the expression of the cytoskeletal

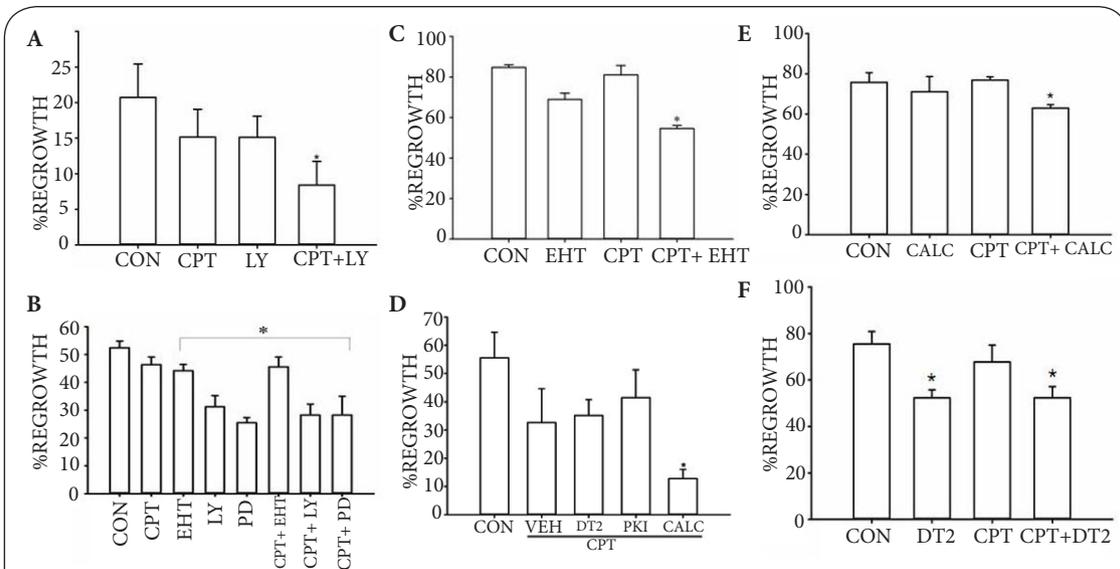


Figure 2. Effect of kinase blockade on cell migration in the presence of CPT. (A) Pretreatment with an inhibitor of PI3K (LY; 1µM; 30 min) with/without CPT (10µM) for 6 hours (n=6). (B) Pretreatment with inhibitors of Rac (EHT; 1µM; 30 min), MAPK (PD; 10µM; 30 min) or PI3K (LY; 1µM; 30 min) with/without CPT (10µM) for 16 hours (n=16). (C) Pretreatment with EHT (1µM; 30 min) with/without CPT (10µM) for 22 hours (n=6). (D) Pretreatment with inhibitors of PKG (DT2; 10µM), PKA (PKI; 10µM), or PKC (CALC; 100nM) for 30 min prior to treatment with CPT (10µM) for 16 hours (n=6). (E) Pretreatment with CALC (100nM) for 30 min prior to treatment with CPT (10µM) for 22 hours (n=3). (F) Pretreatment with DT2 (10µM) for 30 min prior to treatment with CPT (10 µM) for 22 hours (n=6). For all experiments appropriate vehicle controls (CON) were used, and *p<0.05 versus CON.

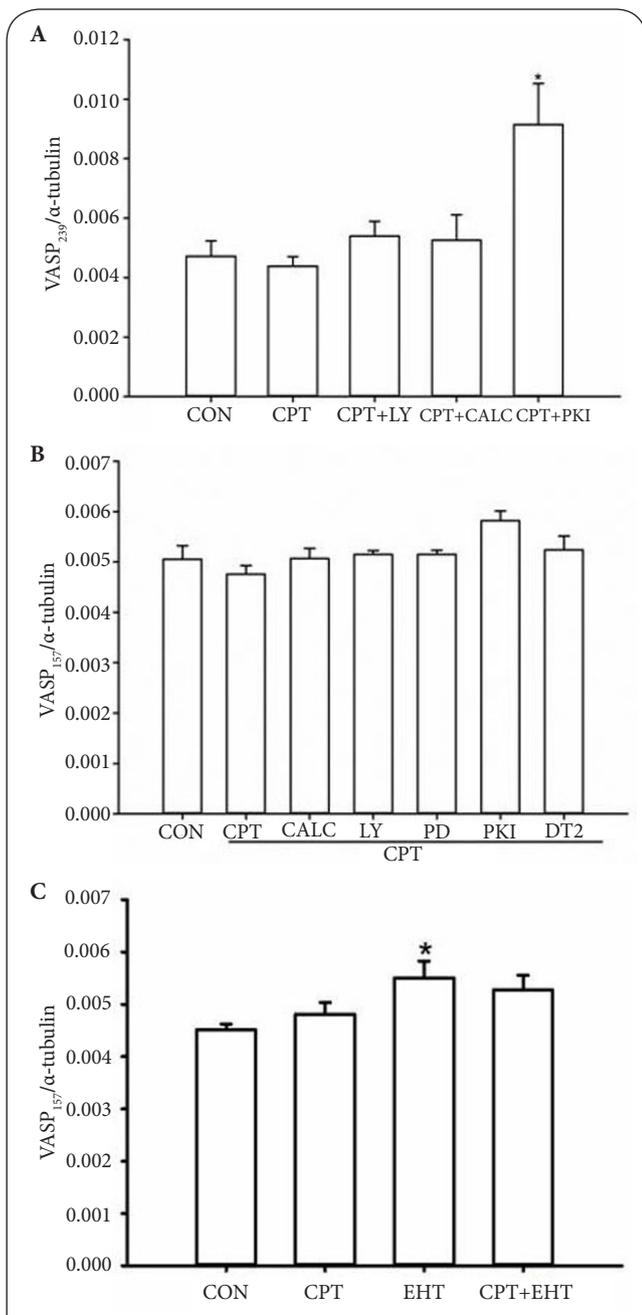


Figure 3. Effect of kinase blockade on VASP phosphorylation in the presence of CPT.
 (A) Phosphorylation of VASPSer239 in the presence of CPT. Cells were pretreated with LY (1 μ M), CALC (100nM) or PKI (10 μ M) for 30 min prior to treatment with CPT (10 μ M) for 30 min (n=8). (B) Phosphorylation of VASPSer157 in the presence of CPT. Cells were pretreated with LY (1 μ M), CALC (100nM), DT2 (10 μ M), PD (10 μ M) or PKI (10 μ M) for 30 min prior to treatment with CPT (10 μ M) for 30 min (n=8). (C) Phosphorylation of VASPSer157. Pretreatment with EHT (1 μ M) for 30 min prior to treatment with CPT (10 μ M) for 30 min (n=16). VASP expression was normalized to α -tubulin expression in each experiment. For all experiments appropriate vehicle controls (CON) were used, and *p<0.05 versus CON.

proteins PAX and FAK. Activation of EPAC via CPT significantly increased expression of PAX (100 μ M) after 30 minutes compared to vehicle control cells (Figure 4A). Treatment with the Rac inhibitor EHT alone had no effect on PAX expression (data not shown), but in the presence of 10 μ M CPT EHT blunted the increase observed with CPT alone (Figure 4B). Regarding FAK, CPT alone had no effect on FAK_{Tyr397}, but in the presence of the Src inhibitor PP2 (1 μ M) FAK_{Tyr397} was slightly increased (p=0.067) compared to vehicle controls (Figure 4C).

Effect of EPAC on cell proliferation

In addition to cell migration, cell proliferation is an integral component of growth. In order to examine the effect of EPAC on cell proliferation we used three different approaches: the MTT assay, BrdU labeling and hemocytometry. Using MTT after 24 hours, CPT (10 μ M) alone had no significant effect on cell proliferation; however, in the presence of the PI3K inhibitor LY (1 μ M), CPT significantly inhibited proliferation compared to controls (Figure 5A). We also treated the cells with the other kinase inhibitors examined in this study; however, none of these agents had any effect on proliferation with/without CPT (data not shown). BrdU staining after 24 hours also indicated that CPT (10 μ M) alone had no significant effect; however, CPT (50 μ M) significantly increased proliferation at this time point compared to controls (Figure 5B). In the presence LY (1 μ M) or the Rac inhibitor EHT (1 μ M), cell proliferation was significantly inhibited yet there was no further reduction in proliferation in the presence of CPT (Figure 5C). Hemocytometry after 72 hours demonstrated that CPT (10 μ M) alone had no effect on proliferation; however, in the presence of either a selective activator of PKA (BnzcAMP, 10 μ M), LY or PD cell proliferation was decreased (Figure 5D). Interestingly, EHT in the presence of CPT significantly increased proliferation at this experiment. It was previously determined that cAMP is an inhibitor of cell proliferation and together with EPAC the effect is augmented [13]. We confirmed these results and also show that inhibition of Rac stimulates proliferation of rat primary VSMCs.

Effect of EPAC on cell cycle proteins

Lastly, we investigated the cell cycle inhibitory proteins p21 and p27 along with cell cycle regulatory cyclin A to determine if they are involved in the mechanisms of CPT on cell growth. CPT did not affect any of these cell cycle regulatory proteins compared to respective vehicle controls after 30 minutes (data not shown).

Discussion

In this report results demonstrate that activation of the cAMP target EPAC exerts concentration- and time-dependent control over growth of rat primary VSMCs. Findings show that in the migratory process, a significant and critical mechanism underlying many vascular growth disorders, higher concentrations of the EPAC activator CPT acutely yet transiently stimulate migration (Figure 1A) while a lower

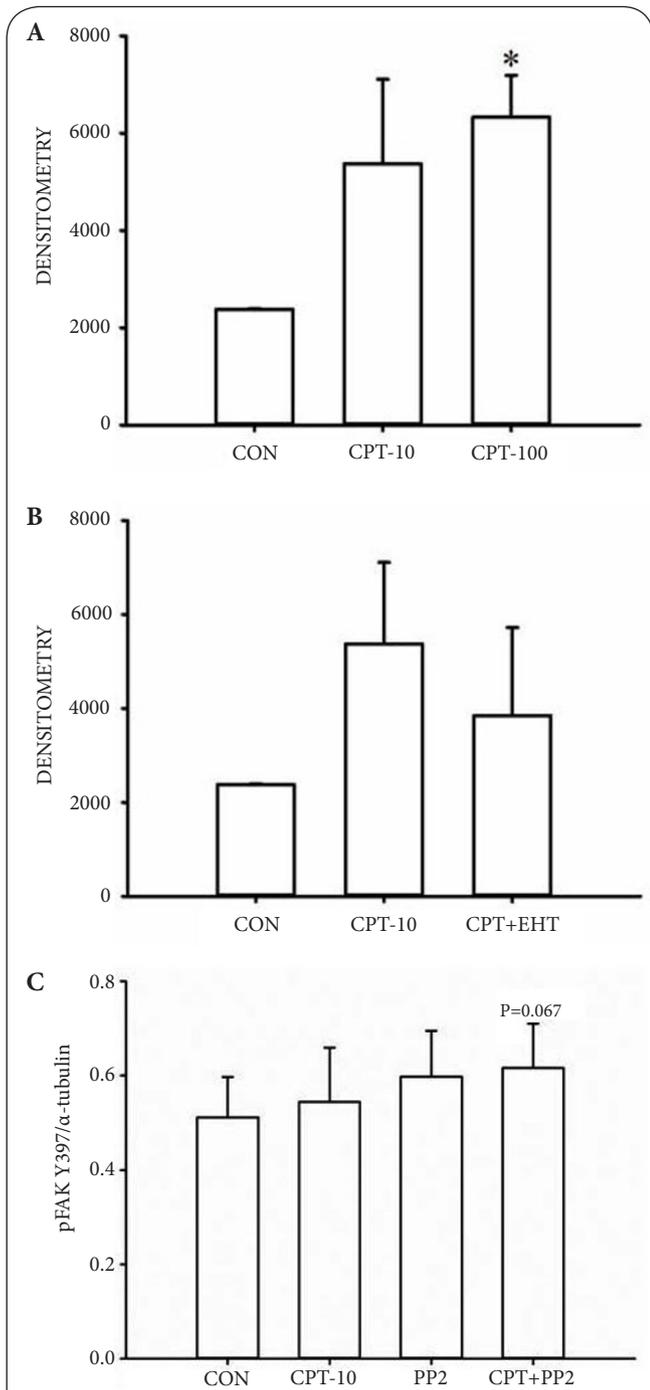


Figure 4. Effect of CPT on paxillin and FAK expression. (A) Cells were treated with CPT (10µM, 100µM) or vehicle for 30 min (n=3). (B) Cells were pretreated with EHT (1µM) for 30 min prior to treatment with CPT (10µM) or vehicle for 30 min (n=3). Densitometry for the band(s) of interest was performed using ImageJ software (NIH). (C) Effect of kinase inhibitors on FAK phosphorylation in the presence of CPT. Cells were pretreated with PP2 (1µM) for 30 min prior to treatment with CPT (10µM) for 30 min (n=8). For all experiments appropriate vehicle controls (CON) were used, and *p<0.05 versus CON.

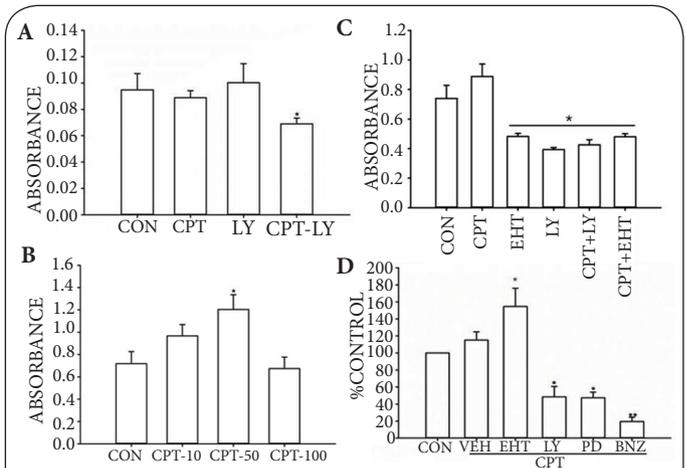


Figure 5. Effect of CPT on cell proliferation.

(A) MTT assay: cells were pretreated with LY (1µM) for 30 min prior to treatment with CPT (10µM) over a 24 hour period (n=10). (B) BrdU assay: cells were treated with CPT (10-100µM) for 24 hours (n=8). (C) BrdU assay: cells were pretreated with LY (1µM) or EHT (1µM) for 30 min prior to treatment with CPT (10µM) or vehicle for 24 hours (n=6). (D) Hemocytometry: cells were pretreated with EHT (1µM), LY (1µM), PD (10µM) or BnzAMP (10µM) for 30 min prior to treatment with CPT (10µM) for 72 hours (n=6). Per the manufacturer's instructions absorbance was read at specified wavelengths. Using trypan blue exclusion staining no differences in cell viability were observed in any treatment group (data not shown). For all experiments appropriate vehicle controls (CON) were used, and *p<0.05 versus CON.

concentration inhibits migration at extended and more physiologically-relevant time points (Figures 1B and 1C). Notably, at these prolonged times the higher concentrations of CPT failed to noticeably alter migration. Given the complexity of mechanisms that have capacity to control cell migration, these data infer that EPAC (selectively stimulated by CPT) serves biologically important regulatory functions during cell migration in concentration- and time-dependent fashion. These initial studies also provided foundation for subsequent analyses of potential mechanisms underlying regulation of cell migration by EPAC. Based on pharmacologic blockade studies, the mechanisms of action of EPAC on migration appear to be at least partly dependent upon PI3K during early stages (Figure 2A) and Rac, MAPK (Figure 2B), PKC (Figures 2D and 2E) and PKG (Figure 2F) during later stages. Further analyses suggest that EPAC can operate in conjunction with PKA via the cytoskeletal protein VASP_{Ser239} (Figure 3A) and in conjunction with Rac through VASP_{Ser157} (Figure 3C) and possibly involving the FA proteins PAX and FAK (Figure 4). Additionally, these actions of EPAC appear selective for migration as its effects on proliferation appear minimal and inconsistent (Figure 5). In summary, these results show that EPAC, selectively stimulated via CPT, has important growth regulatory properties in VSM particularly on cell migration

and that it operates via complex and discrete, context-specific and largely kinase-mediated signaling mechanisms.

Two major downstream pathways for cAMP include stimulation of PKA through phosphorylative cascades or via EPAC signaling. Cyclic AMP is involved in many facets of cellular signaling and generally exerts anti-migratory actions mediated by its main effector PKA [31]; however, the effect of PKA on cell migration can be inhibitory or stimulatory depending on cell conditions [17,32-34]. Previous reports show that EPAC, activated by CPT at high concentrations (300 μ M) can inhibit migration in human prostate carcinoma cells [35] and at mid-range concentrations (30-100 μ M) can promote migration in VSMCs [18] and melanoma cells [36]. New data shown here (Figure 1A) support these previous observations by showing enhanced migration of primary VSMCs exposed to CPT at 30 and 50 μ M after 6 hours; however, this stimulation is only transient as these concentrations of CPT failed to affect migration after longer time points. Moreover, it has been reported that high concentrations of CPT can be non-selective and may operate through non-physiological, EPAC-alternative avenues [37-39]. Notably, using longer more stable and physiological time points a persistent reduction in cell migration following CPT treatment (at a lower, more selective concentration) was observed (Figures 1B and 1C). Based on these data it is suggested that the previously observed pro-migratory actions of EPAC are only transient and may be due to 'off-target' effects of CPT at higher concentrations, and new data presented here argue that EPAC serves physiologically important actions by inhibiting migration of VSMCs.

Recent studies also suggest that the effects of EPAC can occur in concert, opposite to or independently of the actions of PKA [13,40-43]. In the current studies inhibition of PKA in the presence of CPT had no effect on cell migration (data not shown); however, inhibition of PKA and activation of EPAC together significantly increased phosphorylation of VASP_{Ser239} (Figure 3A), the cytoskeletal protein isoform generally stimulated by PKG and suggested to be involved in migration [10,44]. Interestingly, CPT had no effect on VASP_{Ser157}, an alternate phosphorylation site activated by PKA that has also been suspected to be involved in cell growth [45]. These findings illustrate complex and potentially synergistic effects that EPAC has with protein kinases in the control of cell migration.

In an effort to further understand the inhibitory mechanisms of EPAC on cell migration we examined cytoskeletal FA proteins as they have been suggested to contribute to the control of cellular growth by cyclic nucleotide-directed kinases [46,47] and have prominent roles in cytoskeletal dynamics and adhesion [5,48,49]. New findings show that CPT increases PAX expression and appears to do so in a concentration-dependent manner (Figure 4A). The increase in PAX could be strengthening the focal adhesion contacts [8,50] and cytoskeletal stability as has been observed in epithelial cells [51,52] and migration [9,53], thereby reducing capacity of these cells to move in response to a stimulant. In complement, FAK and Src together

act as a signaling nexus to induce phosphorylation of FA proteins including autophosphorylation of FAK itself, PAX and p130Cas [4,6,54]. Notably, phosphorylation of PAX by FAK is not required for FA localization [55] but is important for regulating their dynamics, which act as docking sites for other proteins to regulate activities of the Rho-GTPases in order to advance migration [8,56,57]. Interestingly, CPT alone has no effect on FAK_{Tyr397}; however, there is a slight increase ($p=0.067$) in FAK_{Tyr397} phosphorylation upon inhibition of Src kinase (Figure 4C). FAK auto-phosphorylates at Tyr₃₉₇ and then recruits Src to phosphorylate other FAK sites including Tyr₅₆₆ and Tyr₅₆₇ [6,58]. Possibly Src is inhibitory for this auto-phosphorylation event in order to prevent dissolution of those FA sites, which would subsequently inhibit migration. Nonetheless, these other phosphorylation sites of FAK possibly involved in the migratory process were not examined in the current study.

VASP is an actin binding protein that is associated with FA and membrane structures [59,60]. VASP is phosphorylated by kinases downstream of cAMP and cGMP (PKA, PKG) [61] as well as by PKC/PKD and AMPK [62-64]. VASP has been shown to play important roles in cytoskeletal dynamics and cell migration [10,12,65], and in this regard phosphorylation of VASP_{Ser239} is suggested to be responsible for growth inhibitory effects in VSMCs [45]. In the current study, this could possibly help explain the inhibitory effect EPAC on migration (observed at low concentrations and at longer time points); however, only after PKA is inhibited does significant VASP_{Ser239} phosphorylation occur (Figure 3A). Furthermore, the primary protein responsible for phosphorylation at this site has been reported to be PKG, yet kinase crosstalk occurs as demonstrated by our lab and others [11,21,61], and these data suggest an interaction between PKA and VASP_{Ser239} as well. The cGMP-PKG signaling pathway has been documented to have growth inhibitory effects in VSMCs [11,45]. Moreover, these results also suggest that there is crosstalk between cGMP and Rac as has been shown in adipocytes [66]. Furthermore, PKG was shown to regulate FA sites and migration [47,66] as well as disrupt Rac1-dependent FA formation [67]. EPAC has been shown to activate PKC [68], which in turn can phosphorylate VASP [69], possibly at Ser₂₃₉ in response to CPT. These complex findings suggest that there is a preferential sequence in the phosphorylation/dephosphorylation of VASP as has been previously suggested [61,70,71], and that if one signaling pathway is inhibited another pathway can compensate in order to maintain function.

Due to crosstalk among kinases we examined the effects of EPAC on VASP_{Ser157}, a site known to be involved in proliferation via PKA and PKC phosphorylation [45]. Our data show that CPT has no effect on this site in the presence or absence of broad kinase blockade (Figure 3), suggesting that CPT does not operate through VASP_{Ser157} to control cell migration.

In complement to cell migration we investigated the potential capacity of EPAC to control cell proliferation. Published reports cite that EPAC can affect vascular growth [13,14,18,35], and in

this study we examined multiple indices of cell proliferation following exposure to CPT at multiple time points. Using BrdU incorporation, results show that CPT (50µM) stimulates proliferation (**Figure 5B**). Interestingly, in the presence of inhibitors of PI3K or MAPK or following stimulation of PKA the increase in proliferation is significantly reduced to below control levels (**Figure 5D**). Of note, CPT in the presence of Rac inhibition significantly increased cell numbers over those observed with CPT alone, highlighting the importance of different kinase regulatory proteins at different times (**Figure 5D**). We also examined positive (cyclin A) and negative (p21, p27) cell cycle regulatory proteins, and EPAC had no effect on any of them compared to vehicle controls (data not shown). Overall these results show that the influence of EPAC is predominantly on cell migration with minimal influence over cell proliferation. Also, EPAC in combination with PKA stimulation may have a synergistic effect on vascular growth that could be utilized for potential therapeutic applications.

Conclusion

The observed concentration- and time-dependent effects of EPAC on primary VSMC growth are intriguing and could suggest EPAC as a regulated pivotal switch in the control of VSMC migration and perhaps proliferation. Based on these observations some potential signaling avenues for Epac in its control of VSMC migration are shown in the schematic in **Figure 6**.

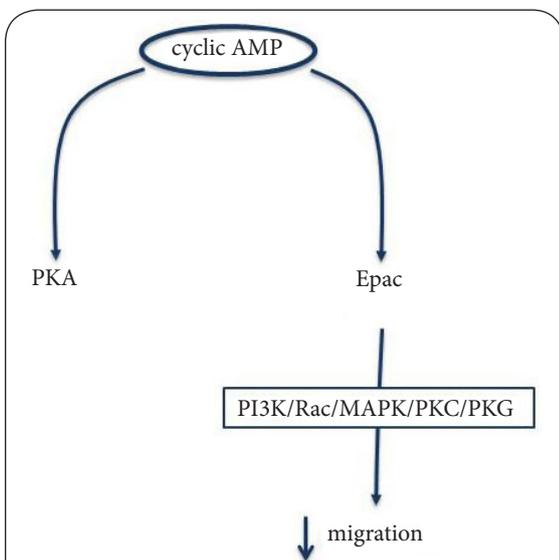


Figure 6. Epac signaling on VSMC migration. Using the Epac activator CPT in rat primary VSMCs, results from this study suggest that Epac operates in highly contextual and regulated fashion to regulate cellular migration. New findings support roles for PI3K, Rac, MAPK, PKC and PKG in helping to reduce migration by Epac. Preliminary observations also lend collaborative roles for the alternate cAMP target PKA and for the focal adhesion elements VASP, PAX and FAK, yet their exact roles in VSM migration control by Epac remain uncertain.

Cumulatively these data suggest EPAC as a physiological relevant signal downstream of cAMP/PKA that has capacity to control cellular growth yet does so in a highly contextual and tightly regulated fashion. These findings also argue for continued work aimed at elucidating cyclic nucleotide- and kinase-driven regulators of cellular function and growth.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	SPA	DNM	DAT
Research concept and design	✓	--	✓
Collection and/or assembly of data	✓	✓	--
Data analysis and interpretation	✓	--	✓
Writing the article	✓	--	✓
Critical revision of the article	✓	--	✓
Final approval of article	✓	✓	✓
Statistical analysis	✓	--	✓

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