Published in final edited form as:

Mol. Carcinog. 2015 March ; 54(3): 203–215. doi:10.1002/mc.22091.

Cyclic AMP regulates the migration and invasion potential of human pancreatic cancer cells

Noah P. Zimmerman^{*1,3}, John Roy^{*1}, Andrew D. Hauser^{2,3}, Jessica M. Wilson^{2,3}, Carol L. Williams^{2,3}, and Michael B. Dwinell^{1,3}

¹Department of Microbiology and Molecular Genetics, the Medical College of Wisconsin Cancer Center, 8701 Watertown Plank Road, Milwaukee, WI 53226

²Department of Pharmacology and Toxicology, the Medical College of Wisconsin Cancer Center, 8701 Watertown Plank Road, Milwaukee, WI 53226

³Department of Microbiology and Molecular Genetics, the Medical College of Wisconsin Cancer Center, 8701 Watertown Plank Road Milwaukee, WI 53226

Abstract

Aggressive dissemination and metastasis of pancreatic ductal adenocarcinoma (PDAC) results in poor prognosis and marked lethality. Rho monomeric G protein levels are increased in pancreatic cancer tissue. As the mechanisms underlying PDAC malignancy are little understood, we investigated the role for cAMP in regulating monomeric G protein regulated invasion and migration of pancreatic cancer cells. Treatment of PDAC cells with cAMP elevating agents that activate adenylyl cyclases, forskolin, protein kinase A, 6-Bnz-cAMP, or the cyclic nucleotide phosphodiesterase inhibitor cilostamide significantly decreased migration and Matrigel invasion of PDAC cell lines. Inhibition was dose-dependent and not significantly different between forskolin or cilostamide treatment. cAMP elevating drugs not only blocked basal migration, but similarly abrogated transforming-growth factor- β -directed PDAC cell migration and invasion. The inhibitory effects of cAMP were prevented by the pharmacological blockade of protein kinase A. Drugs that increase cellular cAMP levels decreased levels of active RhoA or RhoC, with a concomitant increase in phosphorylated RhoA. Diminished Rho signaling was correlated with the appearance of thickened cortical actin bands along the perimeter of non-motile forskolin or cilostamide-treated cells. Decreased migration did not reflect alterations in cell growth or programmed cell death. Collectively these data support the notion that increased levels of cAMP specifically hinder PDAC cell motility through F-actin remodeling.

Correspondence: Michael B. Dwinell, PhD, Department of Microbiology and Molecular Genetics, Cancer Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226 USA, Tel: 414 225-7427, FAX: 414 955-6535, m.dwinell@mcw.edu.
*these authors contributed equally to this work

Competing Interests: The authors declare that they have no competing interests to report.

Authors' Contributions: NPZ: conceived of the study, designed and completed the experiments on F-actin signaling, participated in data analysis and interpretation, prepared figures and wrote the manuscript. JR: designed and completed the experiments to measure cellular migration, invasion, proliferation and apoptosis of the cells, maintained the cells in culture, completed the data analysis and interpretation, made the final figures, wrote and edited the manuscript. ADH: worked in the design and completion of Rho GTPase experiments, assisted in data analysis and writing and editing the manuscript. JMW: completed quantification of cortical F-actin experiments. CLW: participated in data analysis and interpretation, as well as editing the manuscript. MBD: conceived of the study, participated in the design and coordination of the experiments, analyzed the data, wrote and edited the manuscript, and provided the space and reagents for completing the work. All authors read and approved the final manuscript.

Keywords

pancreas; phosphodiesterase; protein kinase A; Rho; carcinoma metastasis

Background

Pancreatic cancer is the most deadly malignancy with a 5-year survival rate of only 6% (1). The preponderance of pancreatic cancer is classified as pancreatic ductal adenocarcinoma (PDAC). The vast majority of patients with PDAC have inoperable disease due to either extensive local invasion or distant organ metastasis. Compared with treatment regimens for other common cancers, the current standard of care drugs available for PDAC are relatively ineffective in prolonging survival or decreasing morbidity. Thus, patients with unresectable or recurrent PDAC are unable to receive treatment regimens tailored to their disease burden. Furthermore, there is a paucity of drugs in clinical use that specifically target and effectively interrupt tumor metastasis. The absence of effective drugs is made worse by poor understanding of the underlying mechanisms controlling invasion and metastasis of malignant PDAC cells. The only recognized marker of PDAC malignancy and progression towards metastasis is the loss of the expression of the gene *SMAD4* (2), the product of which is a downstream effector of transforming growth factor (TGF)- β . A known regulator of epithelial-to-mesenchymal transition (3), TGF- β is a well-recognized chemo-attractant that induces cell migration (4). The biochemical or molecular mechanisms linking TGF- β signaling and *SMAD4* inactivation with regulation of the migration potential of PDAC cancer cells remains largely uncharacterized. Thus, studies into the regulatory mechanisms underpinning PDAC metastasis would reveal novel pathways and therapeutic targets with the potential to significantly improve treatment regimens and increase progression-free survival.

Cell migration is a complex physiological process requiring the coordination of multiple pathways to regulate the actin cytoskeleton. Members of the Rho family of monomeric GTPases are key regulators of the membrane associated actin cytoskeleton (5-7). Rho is activated following the exchange of GDP for GTP, leading to its subsequent interactions with, and activation of, downstream effector proteins (6). A major effector of Rho, Rho-associated protein kinase (ROCK) induces the formation of stress fibers and focal adhesions leading to actin-mediated cell migration (8, 9). Posttranslational modifications of small GTPases, such as prenylation, enhance Rho interactions with the cell membrane and define localization of active Rho within distinct cellular compartments (10, 11). Localization of active Rho can be altered through phosphorylation events. The specific isoform RhoA can be phosphorylated at ser188, making the GTPase recognizable by Rho guanine nucleotide dissociation inhibitors, which then prevents interaction with the cell membrane and inhibits cell migration (12-14). Signaling through the RhoA molecular switch is known to be regulated by the second messenger cAMP (12), a potent regulator of cell migration (15). In cancer, RhoA has been identified as an effector of global lamellipodia formation, with preferential regulation of 2D cell migration. In contrast RhoC is known to direct lamellipodia extension and preferentially regulate 3D cell invasion (16). Levels of RhoC, more so than RhoA, were shown to be elevated in PDAC patient specimens suggesting bio

monomeric GTPase may have roles in directed tumor cell invasion and migration (17). RhoC has also been linked with increased migration and invasion of PDAC (18). Additionally, activation of RhoA has been linked with migration and liver metastasis of PDAC (19), while the epidermal growth factor receptor inhibitor gefitinib was shown to inhibit tumor proliferation, in part through decreased RhoA levels in human pancreatic cancer cell lines (20).

Cyclic AMP is produced from ATP by a family of adenylyl cyclase enzymes. Cellular cAMP interacts with the downstream effectors protein kinase A (PKA), exchange protein directly activated by cAMP, as well as cyclic nucleotide gated ion channels. Signaling by cAMP is regulated by a family of cyclic nucleotide phosphodiesterase (PDE) enzymes which sever the 3'5' phosphodiester bond, yielding AMP, and cessation of the signal. Therefore levels of cAMP can be increased either by stimulating adenylyl cyclase proteins or by inhibiting PDE-mediated degradation. Some heterotrimeric G-protein coupled receptors, such as the nicotinic acetylcholine receptors, beta adrenergic receptors, or adenosine receptors, are known to activate subsets of adenylyl cyclase enzymes, leading to increased cAMP production (21, 22), which alongside activation of additional mediators and several downstream effectors, can evoke migration of numerous cell types, including pancreatic cancer cell lines (21). However, other agents that directly activate adenylyl cyclase, or inhibit PDE bypass heterotrimeric receptor-coupled activation of signaling pathways and specifically elevate cAMP concentrations. Direct elevation of cAMP concentrations has inhibitory effects on the migratory potential of many cell types including leukocytes, endothelial cells, and epithelium (12, 15, 23). Theophylline, green tea, caffeine, xanthine, and other small molecules act as either non-selective or selective inhibitors of PDE activity, with a concordant elevation of cAMP, and have been interrogated for therapeutic use in inflammatory, cardiovascular, pulmonary, neurological and psychological disorders (24).

In this work, we analyzed the potential for increased cytosolic levels of cAMP to inhibit RhoA and RhoC activity and in turn pancreatic cell migration and invasion, two distinct functions critically involved in cancer metastasis. Through the use of forskolin, an activator of adenylyl cyclase enzymes, or PDE inhibitors, a significant inhibition of migration of three pancreatic cancer cell lines was detected. Activation of the cAMP effector PKA decreased Rho activity. An accumulation of cortical actin occurred after stimulation with forskolin or cilostamide, with cAMP elevating agents blocking both baseline as well as TGF- β -induced, migration and invasion.

Methods

Chemicals

The adenylyl cyclase activator, forskolin, the PDEs inhibitor, cilostamide, and the cell permeable myristoylated PKA inhibitor PKI were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). 6-Bnz-cAMP was purchased from BioLog Life Science Institute (Axxora LLC, San Diego, CA, USA). The competitive nonselective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) [IC_{50} =2-50 μ M] and the intrinsic apoptosis inducing nucleoside analog gemcitabine were obtained from Sigma-Aldrich. Y27632 was purchased

from EMD Millipore (Calbiochem, San Diego, CA, USA). Growth-factor-reduced Matrigel Basement Membrane Matrix was acquired from BD Biosciences (San Jose, CA, USA).

Cell culture

Human Panc1 (CRL-1469) and MiaPaCa2 (CRL-1420) pancreatic cancer cells were purchased from the ATCC (Manassas, VA, USA) and grown in Dulbecco's Modified Eagles Medium (Invitrogen, San Diego, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). A third human pancreatic cancer cell line, HPAFII (CRL-1997), was grown in Modified Eagles Medium with 10% (v/v) heat-inactivated FBS and in the absence of antibiotics. Cells were maintained at 37°C in atmospheric oxygen and 5% CO₂.

Transwell migration and invasion assays

2D migration assays were performed using transwell plates (8 µm pore size polycarbonate membrane, 6.5-mm diameter) (Corning Inc., Corning, NY), coated with 15 µg/mL collagen IV for 30 minutes at 37°C as we have documented previously for human breast and colon cancer cells (25-27). 3D invasion assays were performed with the 8 µm pore size transwell plates coated with Matrigel Basement Membrane Matrix diluted 1:10 in growth medium. Cells were serum-starved 2 hours prior to treatment. Treatments were given in the upper well in an admixture with 100,000 cells, while serum-free medium was placed in the bottom well. Cell migration assays were completed in serum-free medium in order to mitigate confounding effects of proliferation. The duration of the migration assay was optimized and was 22 hours for Panc1 and HPAFII cells and 6 hours for MiaPaCa2 cells. The duration of the invasion assay for Panc1 cells was 72 hours. For the invasion assay, medium in the top and bottom chambers of the transwell plate were exchanged every 24 hours, including appropriate inhibitors or stimulants. For either the invasion or migration assay, cells remaining on the top of the membrane were removed with a sterile swab and the cells on the bottom-side were fixed and stained with DAPI. Six evenly spaced pictures were taken for each membrane, the number of DAPI positive cells counted and the average cell number for each experiment calculated.

Proliferation and apoptosis studies

Cells were placed in 6-well plates and treated for the indicated times, at the end of which cells were detached in enzyme-free EDTA buffer, washed with PBS, and fixed using 100% ethanol. Fixed cells were then stained using propidium iodide and analyzed using the Guava Flow Cytometer (Millipore, Billerica, MA, USA). Prior to fixation, a 10µL aliquot was removed, mixed with trypan blue (Invitrogen, Grand Island, NY, USA) and the number of phase bright cells that excluded trypan were counted to determine the relative number of live cells.

To measure apoptosis, cells were placed in 6-well plates and treated for the indicated times. Cells were detached from the culture plate using EDTA and a 50µL aliquot removed and incubated with the caspase-3/7 glow reagent (Promega, Madison, WI, USA) as defined previously (28, 29). Luciferase activity, as a measure of caspase-catalyzed apoptosis, was then quantified by luminescence spectroscopy (Victor3 Wallac Perkin-Elmer Life and Analytical Sciences, Waltham, MA, USA).

Monomeric G protein activity

RhoA and RhoC activity was measured using RhoA Activation Assay Biochem Kit (Cytoskeleton Inc., Denver, CO, USA). In addition, RhoA activity was quantified by immuno-dot blot and G-LISA. RhoC activity was assessed by pulldown-immunoblot analysis as there is no specific solid-phase G-LISA available. Briefly, cells were plated in 60mm tissue culture dishes and grown to 60-80% confluence in full growth medium. Medium was aspirated, the cells washed and treated for 20 minutes with forskolin (10 μ M), 6-Bnz-cAMP (100 μ M), cilostamide (100 μ M), or UPA (1 μ g/mL). Cells were quickly lysed on ice, scraped and lysates cleared by centrifugation and snap frozen until analysis by the solid-phase G-LISA pulldown kit.

Immunoblot analysis

Panc1 cells were grown to 80% confluence and serum-starved 2 hours before treatment. Cells were solubilized in modified RIPA buffer [50 mM Tris•HCl, pH 7.4, 150 mM NaCl, 0.25% (v/v) sodium deoxycholate, 1.0% (v/v) NP-40, 0.1% (v/v) SDS and 1 mM EDTA] supplemented with Protease Inhibitor Cocktail Set III (EMD Biosciences) and the phosphatase inhibitors 10 mM sodium orthovanadate, 40 mM glycerolphosphate, and 20 mM sodium fluoride as phosphatase inhibitors. Lysates were centrifuged at 550 rpm for 5 minutes at 4°C to pellet nuclei. Protein concentration was determined using a Bradford protein assay (BCA kit, Pierce Biotechnology, Rockford IL, USA) and 10 μ g of protein size-separated using reducing SDS-PAGE. Proteins were electrophoretically transferred to PVDF (Immobilon-P, Millipore) for immunoblot analysis. Detection of total RhoA was performed using a mouse anti-RhoA monoclonal antibody (Cytoskeleton Inc.). Rabbit anti-RhoC polyclonal antibody (ab64659) was obtained from AbCam (Cambridge, MA, USA). Phosphorylated RhoA was detected using a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Densitometric analysis was performed using a FluorChem HD2 (Cell Biosciences, Santa Clara, CA, USA) and Alphaview 3.2.4.0 software to quantify protein levels.

Actin quantification

Panc1 cells were grown to 80% confluence and wounded using a 200 μ L pipette tip, washed twice, treated for 6 hours, and fixed with 4% (v/v) paraformaldehyde. Cells were washed with PBS; permeabilized using 0.1% (v/v) Triton X-100, and F-actin visualized using Alexa-fluor-594-labeled phalloidin (Invitrogen). Images of cells moving into the denuded surface made by the pipet tip were taken on a Nikon Eclipse Ti inverted microscope. An investigator, blinded to the treatment, analyzed the images to determine whether cells displayed primarily stress fiber actin consistent with cell migration or peripheral cortical actin. As a second quantification strategy, the thickness of the fluorescently labeled peripheral actin bands was measured using NIS Elements software (Nikon Instruments, Melville, NY, USA). Briefly, a one pixel line was drawn perpendicular to the actin band encompassing the thickness of the phalloidin stained actin. This was repeated at seven randomly selected spots around the cell membrane of twenty different cells from 3 individual staining assays. The length of each line was then calculated by NIS Elements software (Nikon) and converted to mean width in microns.

Statistical analyses

Differences between unstimulated control and experimental samples were analyzed by unpaired Student's *t*-test using GraphPad Prism 4 (La Jolla, CA, USA). Multiple comparisons between groups were analyzed using a two-way ANOVA and a Tukey post-hoc analysis used to identify pairwise differences (GraphPad Prism 4). Statistical significance was defined as $P \leq 0.05$.

Results

Elevated levels of cAMP decrease pancreatic cancer cell migration and invasion

We first sought to investigate the potential for cAMP elevation to either increase or decrease migration of human pancreatic cancer cells. We therefore used a transwell assay to model single cell migration characteristic of malignant pancreatic tumors. As quantified in Figure 1A and shown in representative fluorescence images (Figure 1B), we determined that forskolin-mediated adenylyl cyclase activation led to a dose-dependent decrease in migration of Panc1 pancreatic carcinoma cells. In Panc1 cells, 22-hour treatment with 10 μ M forskolin was sufficient to arrest cellular migration compared to cells without treatment (Figure 1A). This response was not restricted solely to Panc1 cells as the optimal 10 μ M dose of forskolin similarly blocked constitutive migration of MiaPaCa2 (Figure 1C) and HPAFII (Figure 1D) pancreatic cancer cell lines to levels below that of untreated control cells.

Cellular cAMP is enzymatically hydrolyzed to AMP by PDE enzymes, inhibitors of which are used pharmacologically to increase levels of cAMP. Therefore, the ability of PDE inhibitors to decrease pancreatic cancer cell migration was next tested. As predicted, blocking the degradation of cAMP with the pan-PDE inhibitor IBMX significantly decreased migration of Panc1 cells compared to unstimulated control cells (Figure 2A, 2C). Furthermore, inhibition of the specific PDE3 isoform, an enzyme shown to be highly expressed in the pancreas (30), using cilostamide, resulted in decreased migration of both Panc1 (Figure 2A, 2C) and MiaPaCa2 (Figure 2B, 2D) cells compared to untreated controls cells. The level of inhibition using PDE inhibitors was comparable to that evoked by the 10 μ M dose of forskolin (Figure 1).

We next determined the ability of cAMP to affect cytokine-directed migration of PDAC tumor cells. The addition of TGF- β to the bottom chamber during a transwell migration assay demonstrated a significant increase in migration of Panc1 cells toward the motility stimulus. However, treatment with forskolin and cilostamide decreased TGF- β directed migration (Figure 3A). Given the importance of pancreatic cancer cell invasion in the early stages of metastasis, we next tested the effects of elevating cAMP on pancreatic cancer cell invasion. Adapting the transwell assay for extracellular basement membrane matrix (Matrigel)-dependent invasion, we found that treatment with forskolin or cilostamide significantly diminished basal levels of Panc1 cell 3D invasion (Figure 3B, 3C). Further, treatment with either forskolin or cilostamide potently inhibited TGF- β -directed invasion of Panc1 cells into the Matrigel plug (Figure 3B, 3C). Cumulatively, these data suggest that increasing the production of cAMP with adenylyl activators, such as forskolin, or blocking its degradation with PDE inhibitors, such as cilostamide, interrupts pancreatic cancer cell

migration along a collagen-coated substrate and invasion into a parenchymal extracellular matrix. Moreover, cAMP elevating agents not only decreased basal motility and invasion, but also evoked a braking effect strong enough to override the TGF- β stimulatory signal.

cAMP alters migration through activation of PKA

To investigate the mechanism by which cAMP alters cellular migration and invasion, Panc1 and MiaPaCa2 monolayers were treated with the PKA specific activator 6-Bnz-cAMP. Cells treated with 6-Bnz-cAMP demonstrated a significant decrease in transwell migration of Panc1 (Figure 4A, 4C) and MiaPaCa2 (Figure 4B, 4D) cells compared to untreated control cells. The relative abrogation of migration was comparable in both Panc1 and MiaPaCa2 cell lines and was comparable to the inhibition evoked by either forskolin (Figure 1) or cilostamide (Figure 2). Importantly, we found that PKA-mediated inhibition of migration (Figure 4A) returned fully to baseline levels when cells were pretreated with PKI, an inhibitory peptide specific for PKA. Importantly, pretreatment with PKI also prevented migration blockade driven by elevated levels of cAMP produced from forskolin or cilostamide (Figure 4E). Together these data indicate that PKA is a key effector of cAMP's inhibitory effects on pancreatic carcinoma cell migration.

Proliferation and apoptosis were unaffected by elevated cAMP

Proliferation and programmed cell death were next measured to test if the decrease in migrated cells resulted from alterations in cell number. Propidium iodide staining revealed little, if any, significant difference in cellular DNA content between unstimulated cells and those treated with forskolin, cilostamide, or 6-Bnz-cAMP (Figure 5A). The lack of significant changes was measured through 72-hours, a duration 3-times longer than the migration assays. Furthermore, cell counts, using the trypan blue exclusion assay revealed a similar lack of effect on cell survival over the same 72-hour time course (Figure 5B). To determine if the decrease in transwell migration reflected alterations in programmed cell death, levels of caspase-3/7 activity as an indicator of apoptosis were next measured. Neither of the cyclic AMP elevating agents, forskolin or cilostamide, nor the analog 6-Bnz-cAMP, significantly increased apoptosis of Panc1 cells over the 72-hour time course of the experiment (Figure 5C). These findings suggest that the lack of transwell migration reflected alterations in cellular motility.

cAMP decreases RhoGTPase activity

We next interrogated the ability of cAMP to modulate the actin cytoskeletal rearrangements needed for cell migration and invasion. Given their elevated levels in PDAC specimens (17), known roles in regulating cellular migration (16) and linkage with PKA signaling (12, 31), we focused our studies on RhoA and RhoC. Our immunoblot analyses indicated the consistent protein expression of RhoA, RhoB and RhoC in Panc1 PDAC cells (not shown). Active monomeric GTPase levels were detected and quantified using complementary approaches. First using a semi-quantitative Rhotekin-pulldown assay, we found that increasing cAMP with forskolin or cilostamide lead to a consistent, albeit statistically insignificant, decrease in levels of active RhoA-GTP (Figure 6A) or RhoC-GTP (Figure 6B). In order to quantify and statistically analyze this decrease we next turned to a solid-phase pulldown assay. As shown in Figure 6C, forskolin, cilostamide, and 6-Bnz-cAMP

treatment lead to a statistically significant decrease in active RhoA levels. The concentrations of cAMP-elevating agents shown to decrease levels of active RhoA were the same doses shown to effectively block cellular migration. As a positive control, lyso-phosphatidic acid which we have previously used to increase Rho-dependent intestinal epithelial cell migration (32) significantly increased active RhoA levels. A comparable assay for RhoC is not available and was therefore not completed. Consistent with the decrease in active RhoGTP, immunoblot analyses of RhoA demonstrated a significant increase in RhoA phosphorylation at ser188 after 20 minute stimulation with forskolin, cilostamide, or 6-Bnz-cAMP (Figure 6D). Levels of phosphorylated RhoA were undetectable in control, no stimulation, or positive control cells. Taken together, these data indicate that cAMP elevating agents forskolin, cilostamide, and 6-Bnz-cAMP consistently decrease levels of active Rho. Phosphorylation of ser188 is consistent with Rho-guanine nucleotide dissociation inhibitor-mediated inactivation of the RhoA.

Elevated cAMP inhibits Rho-ROCK-F-actin signaling

To further evaluate the role of Rho signaling in the function of elevated cAMP, we tested the absolute capacity to arrest migration through this pathway by inhibiting the downstream effector ROCK. Alone, the ROCK inhibitor Y27632 [10 μ M] significantly decreased Panc1 cell migration (Figure 7A). Notably, the level of inhibition evoked by Y27632 was equal to that elicited by forskolin or cilostamide alone. When used in combination, we found no additional or combinatorial effect of Y27632 on migration in the presence of forskolin or cilostamide (Figure 7A).

Next, phalloidin staining of polymerized F-actin was assessed as the final downstream measure of Rho regulation of the migration machinery. During cellular migration the elongation of the cell is accompanied by RhoA and RhoC signaling and the accumulation of stress fiber actin in lamellipodia, the filaments of which run parallel to the direction of cell movement (6, 16, 33). In contrast, non-migratory cells maintain cortical actin cords which run along the periphery of cells. Highlighted in Figure 7B, treatment with forskolin or cilostamide resulted in the marked appearance of cortical actin bundles at the leading edge and the membranes of adjoining cells at the migration front. Untreated cells were notable for the presence of stress fiber actin organization in long filaments (Figure 7B, arrowhead). In the forskolin (Figure 7B, arrow) or cilostamide-treated cells, we observed complete reorganization of actin into cortical bundles. In contrast to cells treated with cAMP elevating agents, cells stimulated with a migration inducing positive control had minimal cortical actin, with actin arranged instead in lamellipodial extensions (not shown). Quantification of phalloidin fluorescence confirmed that forskolin and cilostamide increased both the percentage and width of cortical actin (Figure 7C, 7D). Compared to untreated control cells, the thickness of the cortical actin band was significantly increased in cells treated with the cAMP elevating agents forskolin or cilostamide (Figure 7D). Taken together, these data are consistent with the notion that elevating cAMP with forskolin or cilostamide decreases pancreatic cell migration through inactivation of RhoGTPase-ROCK signaling, leading in turn to reorganization of the F-actin cytoskeleton.

Discussion

Pancreatic cancer remains extremely lethal due to its high incidence of tumor invasion and metastasis. While the initiating events in PDAC tumorigenesis are well described (34-37), the driving mechanisms whereby PDAC tumor cells emigrate to distal tissues remain poorly understood. In this study, we establish that cAMP elevating agents decrease migration in three pancreatic cancer cell lines. A significant decrease in cell migration and invasion was achieved by two different means, through activation of adenylyl cyclase enzymes using forskolin, as well as by PDE inhibition using either IBMX or cilostamide. A similar significant decrease in migration was induced through activation of PKA with 6-Bnz cAMP. Blockade of migration by forskolin, cilostamide, or 6-Bnz cAMP was reversed by blocking PKA through PKI administration, identifying a specific signaling mechanism for the observed inhibition. Further, cAMP levels appeared to impact the migration regulatory effect of RhoA and potentially even RhoC, on the actin cytoskeleton. Our data show that excess cAMP induced by forskolin or cilostamide abrogates cell migration and invasion in vitro by reduced signaling and actin remodeling mediated by over-expressed RhoA and RhoC.

These data support a model wherein cellular cAMP disrupts two separate stages of metastasis – the initial stage of cancer cell invasion into surrounding stroma; and the migratory phase required for circulating tumor cells to emigrate from the primary tumor environment and form micro-metastases at distant tissues. Equally impactful was the finding that cAMP elevating agents were effective in blocking both constitutive as well as TGF- β inducible invasion and migration. Our findings here expand upon prior reports linking TGF- β activation of PKA by SMAD4 (38, 39). Interestingly, alterations in TGF- β signaling have been associated with widespread metastatic disease (40). Our results suggest that cAMP can reactivate the tumor suppressive functions of PKA whereby it acts as a molecular brake on PDAC cancer cell migration. Alternatively, inhibition of PKA signaling has been tied to changes in the proliferative and apoptotic status of pancreatic cancer cell lines (41, 42). In contrast to those reports, we found using forskolin or cilostamide to increase cytoplasmic levels of cAMP and, in turn, activate PKA had little, if any effect, on cellular proliferation or programmed cell death. Indeed, we demonstrate a potent effect for cAMP and active PKA in regulation of pancreatic cancer cell migration. One possibility for these disparate findings is differences in cell lines used or perhaps the levels of cAMP induced by the varying treatment regimens. Indeed, cAMP stimulated by heterotrimeric G protein coupled receptors appears to increase cell migration (21, 22). Our work herein used drugs which by-pass G protein coupled receptors to elevate cAMP, suggesting that excess levels of cAMP mediates cellular migration arrest.

Our results support a prior report showing that inhibition of RhoA signaling leads to decreased pancreatic cancer cell invasion and migration (20). In that work, it was subsequently shown that pancreatic cells genetically modified to have decreased RhoA activity had decreased metastatic burden in xenograft models. Though previous reports have shown mixed results with regard to the expression of RhoA in human pancreatic cancers, high RhoC expression has been linked with poor prognosis, and increased metastasis in pancreatic cancer patients (17). RhoA and RhoC are both known to regulate cell motility through parallel pathways, though very little is known regarding cross-talk between these

two partnered effectors (43). We found that increasing cAMP not only decreased levels of active RhoA, but lead to a similar trend in declination of active RhoC-GTP levels. RhoA and RhoC work cooperatively to regulate cell movement, with RhoA coordinating lamellipodia formation and stress fiber formation and RhoC orchestrating lamellipodial spread, F-actin bundle reorganization, and directional movement. Consistent with the pronounced cytostatic effect of forskolin, cilostamide or 6-Bnz-cAMP, we found that stimulation with cAMP elevating agents decreased activation of both RhoA and RhoC monomeric G proteins. The alteration in actin cytoskeletal re-arrangement observed in our work is not dissimilar to that observed in cells treated with the pan-Rho inhibitor exoenzyme C3 (44). It is not clear from our work if cAMP and PKA are working exclusively through RhoA and RhoC, or if the strong decrease in invasion and migration reflects a more global effect on other effectors regulating the actin cytoskeleton. Alternatively, it is possible the negative effect we observed reflects alterations on RhoGTPase localization or other changes in Rho regulating proteins.

Cellular cAMP has recognized roles in inflammation (15, 45, 46). The favorable effects of cAMP on leukocytes include decreased production of proinflammatory cytokines (47, 48), diminished leukocyte adhesion to endothelial cells (49-51), and disruption of small GTPase signaling (12, 25, 31). In the pancreas, in vivo elevation of cAMP following intravenous administration of pan-PDE inhibitors improved inflammation in rat models of cerulein or taurocholate-induced pancreatitis (47, 52, 53), a precursor and risk-factor for pancreatic cancer. In our cell culture systems, we determined that increased cAMP signaling also decreased the 2D migration and 3D invasion of pancreatic cancer cell lines. Accordingly, it is plausible that the benefit of PDE inhibitors observed in the pancreatitis model may reflect decreased tumor cell growth, invasion and metastasis. The response of pancreatic carcinoma cells to elevated cAMP suggests that both PKA as well as PDE3 may provide promising targets for treatment of both early and late stage pancreatic diseases such as pancreatitis and pancreatic ductal adenocarcinoma. In light of the fact that there are a plethora of PDE inhibitors available, it is tempting to speculate that those therapeutics could reduce both migration and invasion of pancreatic cancer cells. Taken together, our work indicates that agents that elevate cellular cAMP levels and activate PKA markedly diminish the migration and Matrigel invasion of human pancreatic cancer cells. Inhibition of migration and invasion was correlated with decreased Rho activity, and shifted F-actin from lamellipodial stress fibers to concrete cortical bands.

Acknowledgments

We thank Qing Lai for expert technical assistance.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA: A Cancer Journal for Clinicians*. 2012; 62(1):10-29. [PubMed: 22237761]
2. Blackford A, Serrano OK, Wolfgang CL, Parmigiani G, Jones S, Zhang X, et al. SMAD4 gene mutations are associated with poor prognosis in pancreatic cancer. *Clin Cancer Res*. 2009 Jul 15; 15(14):4674-9. [PubMed: 19584111]
3. Lagna G, Hata A, Hemmati-Brivanlou A, Massague J. Partnerships between DPC4 and SMAD proteins in TGF-beta signalling pathways. *Nature*. 1996 Oct 31; 383(6603):832-6. [PubMed: 8893010]

Mol Carcinog. Author manuscript; available in PMC 2015 March 01.

4. Hwang S, Zimmerman NP, Agle KA, Turner JR, Kumar SN, Dwinell MB. E-cadherin is critical for collective sheet migration and is regulated by the chemokine CXCL12 protein during restitution. *Journal of Biological Chemistry*. 2012 Jun 22; 287(26):22227–40. [PubMed: 22549778]
5. Aspenstrom P, Fransson A, Sjöström J. Rho GTPases have diverse effects on the organization of the actin filament system. *Biochem J*. 2004 Jan 15; 377(Pt 2):327–37. [PubMed: 14521508]
6. Ridley AJ. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol*. 2005 Oct; 16(10):522–9. [PubMed: 16949823]
7. Wennerberg K, Der CJ. Rho-family GTPases: It's not only rac and rho (and I like it). *J Cell Sci*. 2004 Mar 15; 117(Pt 8):1301–12. [PubMed: 15020679]
8. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nafuku M, et al. Regulation of myosin phosphatase by rho and rho-associated kinase (rho-kinase). *Science*. 1996 Jul 12; 273(5272):245–8. [PubMed: 8662509]
9. Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, et al. Phosphorylation and activation of myosin by rho-associated kinase (rho-kinase). *J Biol Chem*. 1996 Aug 23; 271(34):20246–9. [PubMed: 8702756]
10. Chenette EJ, Mitin T, Der CJ. Multiple sequence elements facilitate chp rho GTPase subcellular location, membrane association, and transforming activity. *Mol Biol Cell*. 2006 Jul; 17(7):3108–21. [PubMed: 16641371]
11. Berg TJ, Gastonguay AJ, Lorimer EL, Kuhlmann JR, Li R, Fields AP, et al. Splice variants of SmgGDS control small GTPase prenylation and membrane localization. *J Biol Chem*. 2010 Nov 12; 285(46):35255–66. [PubMed: 20719748]
12. Lang P, Gesbert F, Delespigne-Carmagnat M, Stancovski R, Fouchet M, Bertoglio J. Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. *EMBO J*. 1996 Feb 1; 15(3):515–9. [PubMed: 8599934]
13. Michalson D, Silletti J, Murphy G, D'Eustachio P, Rush M, Phillips MR. Differential localization of rho GTPases in living cells: Regulation by hypervariable regions and RhoGDI binding. *J Cell Biol*. 2001 Jan 8; 152(1):11–26. [PubMed: 11149925]
14. DerMardirossian C, Bokoch GM. GDIs: Central regulatory molecules in rho GTPase activation. *Trends Cell Biol*. 2005 Jul; 15(7):556–63. [PubMed: 15921909]
15. Lorenovics MJ, Fernandez-Borja M, Hordijk PL. cAMP signaling in leukocyte transendothelial migration. *Arterioscler Thromb Vasc Biol*. 2007 May; 27(5):1014–22. [PubMed: 17347487]
16. Ridley AJ. RhoA, RhoB and RhoC have different roles in cancer cell migration. *J Microsc*. 2013;n/a–n/a.
17. Suwa H, Ohshio G, Imamura T, Watanabe G, Arai S, Imamura M, et al. Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas. *Br J Cancer*. 1998; 77(1):147–52. [PubMed: 9452160]
18. Dietrich KA, Schwarz P, Liska M, Grass S, Menke A, Meister W, et al. Specific induction of migration and invasion of pancreatic carcinoma cells by RhoC, which differs from RhoA in its localisation and activity. *Biol Chem*. 2009 Oct; 290(10):1065–77. [PubMed: 19612867]
19. Li Z, Chang Z, Chiao LJ, Kang Y, Xia Q, Zhu C, et al. TrkB^{WT} induces liver metastasis of pancreatic cancer cells by sequestering rho GTP dissociation inhibitor and promoting RhoA activation. *Cancer Research*. 2009 Oct 01; 69(19):7351–9. [PubMed: 1973448]
20. Kusama T, Mukai M, Eide H, Ishikawa O, Tatsuta M, Nakamura H, et al. Inactivation of rho GTPases by p190 RhoGAP reduces human pancreatic cancer cell invasion and metastasis. *Cancer Sci*. 2006 Sep; 97(9):848–53. [PubMed: 16776775]
21. Schuller HM, Al-Wadei H. Neurotransmitter receptors as central regulators of pancreatic cancer. *Future Oncology*. 2010 Feb 01; 6(2):221–3. 2013/08. [PubMed: 20146581]
22. Ntantie E, Gonyo P, Lorimer EL, Hauser AP, Schulman M, McAllister D, et al. An adenosine-mediated signaling pathway suppresses prenylation of the GTPase Rap1B and promotes cell scattering. *Sci Signal*. 2013 May 28; 6(277):ra39. [PubMed: 23716716]
23. Zimmerman NP, Kumar SN, Turner JR, Dwinell MP. Cyclic AMP dysregulates intestinal epithelial cell restitution through PKA and RhoA. *Inflamm Bowel Dis*. 2011 Oct 12.
24. Bender AT, Beavo JA. Cyclic nucleotide phosphodiesterases. Molecular regulation to clinical use. *Pharmacological Reviews*. 2006 Sep 01; 58(3):489–520. [PubMed: 16968949]

25. Wendt MK, Johannesen PA, Kang-Decker J, Binion DG, Shah V, Dwinell MB. Silencing of epithelial CXCL12 expression by DNA hypermethylation promotes colonic carcinoma metastasis. *Oncogene*. 2006 Aug 17; 25(36):4986–97. [PubMed: 16568088]
26. Wendt MK, Cooper AN, Dwinell MB. Epigenetic silencing of CXCL12 increases the metastatic potential of mammary carcinoma cells. *Oncogene*. 2008 Feb 28; 27(10):1461–71. [PubMed: 17724466]
27. Drury LJ, Ziarek JJ, Gravel S, Velukamp CT, Takekoshi T, Hwang ST, et al. Monomeric and dimeric CXCL12 inhibit metastasis through distinct CXCR4 interactions and signaling pathways. *Proc Natl Acad Sci U S A*. 2011 Oct 25; 108(43):17655–60. [PubMed: 21990345]
28. Wendt MK, Drury LJ, Vongsa RA, Dwinell MB. Constitutive CXCL12 expression induces anoikis in colorectal carcinoma cells. *Gastroenterology*. 2008 Aug; 135(2):508–17. [PubMed: 18558091]
29. Drury LJ, Wendt MK, Dwinell MB. CXCL12 chemokine expression and secretion regulates colorectal carcinoma cell anoikis through beta1-integrin-mediated intrinsic apoptosis. *PLoS One*. 2010 Sep 22; 5(9):e12895. [PubMed: 20877572]
30. Lakics V, Kerman EH, Boess FG. Quantitative comparison of phosphodiesterase mRNA distribution in human brain and peripheral tissues. *Neuropharmacology*. 2010 Nov; 59(6):367–74. [PubMed: 20938871]
31. Qiao J, Huang F, Lum H. PKA inhibits RhoA activation: A protection mechanism against endothelial barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol*. 2003 Jun; 284(6):L972–80. [PubMed: 12588708]
32. Moyer RA, Wendt MK, Johannesen PA, Turner JR, Dwinell MB. Rho activation regulates CXCL12 chemokine stimulated actin rearrangement and restitution in model intestinal epithelia. *Lab Invest*. 2007 Aug; 87(8):807–17. [PubMed: 17572689]
33. Lotz MM, Rabinovitz I, Mercurio AM. Intestinal restitution: Progression of actin cytoskeleton rearrangements and integrin function in a model of epithelial wound healing. *Am J Pathol*. 2000 Mar; 156(3):985–96. [PubMed: 10702414]
34. Hruban RH, Maitra A, Kern SE, Goggins M. Precursors to pancreatic cancer. *Gastroenterol Clin North Am*. 2007 Dec; 36(4):831–49. vi. [PubMed: 17926793]
35. Hingorani SR, Wang L, Murtani AS, Combs C, Deramaudena IB, Hruban RH, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell*. 2005 May; 7(5):469–81. [PubMed: 15894267]
36. Hruban RH, Wilentz KE, Maitra A. Identification and analysis of precursors to invasive pancreatic cancer. *Methods Mol Med*. 2005; 103:1–13. [PubMed: 15542896]
37. Maitra A, Fukushima N, Takaori K, Hruban RH. Precursors to invasive pancreatic cancer. *Adv Anat Pathol*. 2005 Mar; 15(2):81–91. [PubMed: 15731576]
38. Zhang L, Duan CJ, Binkley C, Li G, Uhler MD, Logsdon CD, et al. A transforming growth factor beta-induced Smad3/Smad4 complex directly activates protein kinase A. *Molecular and Cellular Biology*. 2004 Mar 01; 24(5):2169–80. [PubMed: 14966294]
39. Yang H, Lee CJ, Zhang L, Sans MD, Simeone DM. Regulation of transforming growth factor beta-induced responses by protein kinase A in pancreatic acinar cells. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. Jul; 2008 295(1):G170–8. [PubMed: 18467503]
40. Iacobuzio-Donahue CA, Velculescu VE, Wolfgang CL, Hruban RH. Genetic basis of pancreas cancer development and progression: Insights from whole-exome and whole-genome sequencing. *Clinical Cancer Research*. 2012 Aug 15; 18(16):4257–65. [PubMed: 22896692]
41. Farrow B, Rychahou P, Murillo C, O'Connor KJ, Kawamura T, Evers JM. Inhibition of pancreatic cancer cell growth and induction of apoptosis with novel therapies directed against protein kinase A. *Surgery*. 2003 Aug; 134(2):197–205. [PubMed: 12947318]
42. Boucher MJ, Duchesne C, Laine J, Morisset J, Rivard N. cAMP protection of pancreatic cancer cells against apoptosis induced by ERK inhibition. *Biochem Biophys Res Commun*. 2001 Jul 13; 285(2):207–16. [PubMed: 11444427]
43. Wheeler AP, Ridley AJ. Why three rho proteins? RhoA, RhoB, RhoC, and cell motility. *Exp Cell Res*. 2004 Nov 15; 301(1):43–9. [PubMed: 15561444]

44. Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*. 1992 Aug 7; 70(3):401–10. [PubMed: 1643658]
45. Schäfer PH, Parton A, Gandhi AK, Capone L, Adams M, Wu L, et al. Apremilast, a cAMP phosphodiesterase-4 inhibitor, demonstrates anti-inflammatory activity in vitro and in a model of psoriasis. *Br J Pharmacol*. 2010 Feb; 159(2):842–55. [PubMed: 20050849]
46. Videla S, Vilaseca J, Medina C, Mourelle M, Guarner F, Salas A, et al. Selective inhibition of phosphodiesterase-4 ameliorates chronic colitis and prevents intestinal fibrosis. *J Pharmacol Exp Ther*. 2006 Feb; 316(2):940–51. [PubMed: 16254133]
47. Sandoval J, Escobar J, Pereda J, Sacchetto N, Rodriguez JL, Sabater L, et al. Pentoxifylline prevents loss of PP2A phosphatase activity and recruitment of histone acetyltransferases to proinflammatory genes in acute pancreatitis. *J Pharmacol Exp Ther*. 2009 Nov; 331(2):609–17. [PubMed: 19071881]
48. Klemm P, Harris HJ, Perretti M. Effect of rolipram in a murine model of acute inflammation: Comparison with the corticoid dexamethasone. *Eur J Pharmacol*. 1995 Jul 25; 281(1):69–74. [PubMed: 8566119]
49. Lauanna C, Campoell JJ, Butcher EC. Elevation of intracellular cAMP inhibits RhoA activation and integrin-dependent leukocyte adhesion induced by chemoattractants. *J Biol Chem*. 1997 Sep 26; 272(39):24141–4. [PubMed: 9305861]
50. Berends C, Dijkhuizen L, de Monchy JC, Dubois AE, Geurtsen J, Kauffman HF. Inhibition of PAR-induced expression of CD11b and shedding of L-selectin on human neutrophils and eosinophils by the type IV selective PDE inhibitor, rolipram. *Eur Respir J*. 1997 May; 10(5):1000–7. [PubMed: 9163638]
51. Netherton SJ, Sutton JA, Wilson LS, Carter RL, Maurice DH. Both protein kinase A and exchange protein activated by cAMP coordinate adhesion of human vascular endothelial cells. *Circ Res*. 2007 Oct 12; 101(8):769–76. [PubMed: 17473021]
52. Gervet Cambrero L, Camps B, de La Asuncion JG, Cerda M, Tollin A, Pallardo FV, et al. Pentoxifylline ameliorates cerulein-induced pancreatitis in rats: Role of glutathione and nitric oxide. *J Pharmacol Exp Ther*. 2000 May; 293(2):670–6. [PubMed: 10773043]
53. Sato T, Otaka M, Odashima M, Kato S, Jin M, Koriishi N, et al. Specific type IV phosphodiesterase inhibitor ameliorates cerulein-induced pancreatitis in rats. *Biochem Biophys Res Commun*. 2006 Jul 21; 345(1):339–44. [PubMed: 16759642]

List of Abbreviations

FBS	fetal bovine serum
IBMX	3-isobutyl-1-methylxanthine
PDAC	pancreatic ductal adenocarcinoma
PDE	phosphodiesterase
PKA	protein kinase A
ROCK	Rho-associated protein kinase
TGF	transforming growth factor

Figure 1 Adenylyl cyclase activation abrogates constitutive migration of pancreatic cancer cell lines

(A) Panc1 cells were treated with a concentration of curve of forskolin to activate adenylyl cyclase and increase cAMP levels. After 22 hours, migration was measured using a transwell migration assay, with 10% fetal bovine serum serving as a positive control (+). Cells migrated were quantified by taking 6 pictures of each transwell membrane and the number of cells and the number of cells migrated per field counted. Asterisk (*) denotes $P \leq 0.05$ and double asterisk (**) denotes $P \leq 0.01$ compared to unstimulated (NS) control. (B) Representative images of DAPI-stained Panc1 cells that have migrating across the transwell insert at each concentration of forskolin (FSK). Size bar = 100 μ m. (C) Transwell migration assay of MiaPaCa2 cells. Migration was measured after 6 hour 10 μ M forskolin treatment. Asterisk (*) denotes $P \leq 0.05$ and triple asterisk (***) denotes $P \leq 0.001$ compared to unstimulated (NS) control. (D) Transwell migration assays with HPAFII cells. Migration was measured after 22 hour stimulation with 10 μ M forskolin. Asterisk (*) denotes $P \leq 0.05$ and triple asterisks (***) denotes $P \leq 0.001$ compared to unstimulated (NS) control. Values in panels A, C, and D are mean $\pm$ SE, n = 8, 5, or 4, respectively.

Figure 2 Phosphodiesterase inhibition blocks basal migration in pancreatic cancer cell lines
 (A) Panc1 cells were treated with either phosphodiesterase 3 inhibitor cilostamide (Cilo, [100μM]) or the pan-phosphodiesterase inhibitor IBMX [100μM]. After 22 hours, migration was measured using a transwell migration assay. Heat-inactivated fetal bovine serum was the positive control (+). Cells migrated were quantified by taking 6 pictures of each transwell membrane and the number of cells migrated per field counted. (B) MiaPaCa2 cells were treated 6 hours with 100μM cilostamide (Cilo) and migrated cells enumerated per microscopic field and normalized to an un-stimulated (NS) control. Values in panel A are mean ± SE, n=8. Values in panel B are mean ± SE, n=6. Asterisk (*) denotes $P \leq 0.05$ and triple asterisks (***) denotes $P \leq 0.001$ compared to NS controls. Representative images of DAPI-stained Panc1 (C) or MiaPaCa2 (D) cells that have migrated across the transwell. Size bar = 100μm.

Figure 3 cAMP elevating agents block basal and TGF- β mediated migration and invasion of pancreatic cancer cells

Pancreatic cells were treated with forskolin (F, [10 μ M]) or cilostamide (C, [100 μ M]) to elevate cAMP. In separate groups (+TGF- β), the medium on the bottom-side of the transwell contained 1ng/mL of TGF- β to induce cell migration. (A) After 22 hours, migration was measured using a transwell migration assay with 10% fetal bovine serum serving as a positive control (+). Migration was quantified by taking 6 pictures of each transwell membrane and an average number of cells migrated per field quantified. Values are mean $\pm$ SE, n=3. Double asterisks (***) denotes $P \leq 0.01$ compared with non-stimulated (NS) controls. Triple asterisks (***) denotes $P \leq 0.001$ compared with NS. Triple pounds (###) denotes $P \leq 0.001$ compared with the TGF- β alone control (-). (B) After 72 hours, invasion was measured using a transwell assay with growth factor-reduced Matrigel basement membrane matrix at a 1:10 dilution. The positive control (+) was as defined in (A). Cells that invaded the matrix were quantified by taking 6 pictures of each transwell membrane and the average number of cells migrated per field enumerated. Values are mean $\pm$ SE of the averages normalized to NS value in each data set, n=4. Asterisk (*) denotes $P \leq 0.05$, double asterisks (**) denotes $P \leq 0.01$, and triple asterisks (***) denotes $P \leq 0.001$ compared with NS. Triple pounds (###) denotes $P \leq 0.001$ compared with TGF- β alone control (-). (C) Representative images of transwell invasion assays. Cells were not stimulated (NS) or treated with the control (+), forskolin (FSK), cilostamide (Cilo) with or without (-) TGF- β . Size bar = 100 μ m.

Figure 4 Protein kinase A (PKA) signaling regulates pancreatic cancer cell migration

Panc1 cells were treated with 6-bnz-cAMP (6-bnz, [100 μ M]), a PKA activator, and/or the specific PKA inhibitor PKI [10 μ M]. (A) Migration was measured after 22 hours and the average number of cells migrated per field calculated. Values are mean $\pm$ SE, n=8. Triple asterisks (***) denotes $P \leq 0.001$ compared to un-stimulated (NS) control. (B) MiaPaCa2 cells were treated 6 hours with 100 μ M 6-bnz-cAMP (6-bnz) and migration measured. Values are mean $\pm$ SE, n = 6. Asterisk (*) denotes $P \leq 0.05$ compared to NS. Representative images of Panc1 (C) or MiaPaCa2 (D) transwell migrated cells for each treatment in panel A or panel B, respectively. Size bar = 100 μ m. (E) Panc1 cells were treated 22 hours with forskolin (F, [10 μ M]) or cilostamide (C, [100 μ M]). Where indicated (+PKI), cells were also treated with 10 μ M PKI. Values are mean $\pm$ SE, n = 3. Double asterisks (**) denotes $P \leq 0.01$ and triple asterisks (***) denotes $P \leq 0.001$ compared with unstimulated (NS) controls.

Figure 5 Elevated cAMP and PKA activation minimally alter proliferation or apoptosis of Panc1 cells

(A) Panc1 cells were treated with 10 μ M forskolin (FSK), 100 μ M 6-Bnz-cAMP (6-bnz), or 100 μ M cilostamide (Cilo) for 24, 36, 48, and 72 hours. 10% fetal bovine serum served as a positive control (+). Cells were stained with propidium iodide and proliferation measured using flow cytometry. Representative histograms of 4 separate flow cytometry plots are shown. (B) Trypan blue exclusion was measured in parallel to measure total live cells. A 10 μ L aliquot of cells from (A) was reserved at 12, 24, 36, 48, and 72 hours and live cells enumerated using a hemocytometer. Total number of live cells were counted, averaged, and normalized to an un-stimulated (NS) control. Values are mean $\pm$ SE, n=4. Asterisk (*) denotes $P \leq 0.05$ and triple asterisks (***) denotes $P \leq 0.001$ compared to unstimulated (NS) control. (C) Cells were treated with FSK [10 μ M], 6-bnz [100 μ M], or Cilo [100 μ M] for 1, 24, 48, and 72 hours. Unstimulated (0hr) cells were the negative control. As an apoptosis positive control, cells were treated with 100 μ M gemcitabine (+). Apoptosis was measured using the Caspase-3/7 glow assay and luminescence measured by spectroscopy. Luminescence data was normalized to NS control cells at each time point. Values are mean $\pm$ SE, n=3. Asterisk (*) denotes $P \leq 0.05$ and triple asterisks (***) denotes $P \leq 0.001$ compared to NS.

Figure 6 cAMP elevating drugs decrease Rho activity

Panc1 cells were treated with forskolin (FSK, [10 μ M]) or cilostamide (Cilo, [100 μ M]) to elevate cAMP. 6-Bnz cAMP (6-bnz, [100 μ M]) treated was used to specifically activate PKA. (A) Active RhoA was measured by immunoblot (IB) after Rho pulldown with rhotekin beads that specifically bind GTP-bound Rho. Densitometric analysis of active compared to total RhoA levels normalized to the basal active RhoA fraction (NS). A representative immunoblot is shown, n=3 for LPA, Cilo, Cilo+PKI, and PKI, n=4 for NS, FSK, and FSK+PKI. (B) Active RhoC was measured by western blot after Rho pulldown with rhotekin beads that specifically bind GTP-bound Rho. Densitometric analyses of active compared to total RhoC levels were normalized to the basal active RhoC fraction (NS). A representative immunoblot is shown, n=3 for LPA, Cilo, Cilo+PKI, and PKI, n=4 for NS, FSK, and FSK+PKI. (C) Active RhoA was measured using GLISA solid state Rho pulldown. Lysophosphatidic acid (LPA, [1 μ g/ml]) was used as a positive control. Relative active RhoA levels were normalized to un-stimulated (NS) control cells. (D) Phosphorylated (p-RhoA) and total RhoA (RhoA) protein levels were detected using monoclonal antibodies specific for each and assayed by immunoblot and quantified using densitometry. Phosphorylated RhoA levels were normalized to total RhoA levels prior to quantification. A representative immunoblot is shown, n = 5. Values are mean $\pm$ SE. Asterisk (*) denotes $P \leq 0.05$ compared to NS.

Figure 7 cAMP elevating agents induce cortical actin formation

Panc1 cells were treated with forskolin (FSK (F), [10 μ M]) or cilostamide (Cilo (C), [100 μ M]) to elevate cAMP. (A) After 22 hours migration was measured using a transwell migration assay, with 10% fetal bovine serum serving as a positive control (+). Where indicated (+ROCK-I), cells were also treated with the ROCK inhibitor Y27632 [10 μ M]. (B) Representative images of Panc1 non-stimulated (NS) or cells treated with forskolin. Actin was stained with Alexafluor-594 labeled phalloidin. Representative images from three individual experiments were assessed to determine whether the primary form of actin displayed was cortical or stress fiber. Actin organized in long filament stress fibers (arrowhead) compared to bundles of actin re-organized along the cell periphery (arrow) in FSK-stimulated cells. Size bar = 20 μ m. (C) The percent of cells with cortical actin was determined by an investigator blinded to the treatment. An average of 25 cells was analyzed per treatment and treatments were run in triplicate. (D) Width of cortical actin was measured using Nikon Elements software. Seven measurements were made across the actin band of the cell membrane on all cells in the field of view for each of 3 separate experiments. Values in A, C, and D are mean $\pm$ SE, n = 3. Asterisk (*) denotes $P \leq 0.05$, double asterisks (**) denotes $P \leq 0.01$, and triple asterisks (***) denotes $P \leq 0.001$ compared with un-stimulated (NS) controls.