A Novel Epac-Rap-PP2A Signaling Module Controls cAMP-dependent Akt Regulation*

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Rap1b has been implicated in the transduction of the cAMP mitogenic signal. It is phosphorylated and activated by cAMP, and its expression in models where cAMP is mitogenic leads to proliferation and tumorigenesis. Akt is a likely downstream effector of cAMP-Rap1 action. cAMP elevation induced a rapid and transient Akt inhibition that required activated and phosphorylated Rap1b. However, the mechanism(s) by which cAMP-Rap regulates Akt remains unclear. Here we show that (i) upstream regulators, PIK and PDK1, are not the target(s) of the cAMP inhibitory action; (ii) constitutively active Akt and calyculin A-stimulated Akt are resistant to cAMP inhibition, suggesting the action of a phosphatase; (iii) cAMP increases the rate of Akt dephosphorylation, directly implicating an Akt-phosphatase; (iv) Epac- and protein kinase A (PKA)-specific analogs synergistically activate Akt; (v) Rap1b has been implicated in the transduction of the cAMP mitogenic signal. It is phosphorylated and activated by cAMP, and its expression in models where cAMP is mitogenic leads to proliferation and tumorigenesis. Akt is a likely downstream effector of cAMP-Rap1 action. cAMP elevation induced a rapid and transient Akt inhibition that required activated and phosphorylated Rap1b. However, the mechanism(s) by which cAMP-Rap regulates Akt remains unclear. Here we show that (i) upstream regulators, PIK and PDK1, are not the target(s) of the cAMP inhibitory action; (ii) constitutively active Akt and calyculin A-stimulated Akt are resistant to cAMP inhibition, suggesting the action of a phosphatase; (iii) cAMP increases the rate of Akt dephosphorylation, directly implicating an Akt-phosphatase; (iv) Epac- and protein kinase A (PKA)-specific analogs synergistically inhibit Akt, indicating the involvement of both cAMP-dependent effector pathways; (v) H89 and dominant negative Epac 279E block cAMP-inhibitory action; (vi) Epac associates in a complex with Akt and PP2A, and the associated-phosphatase activity is positively modulated by cAMP in a PKA- and Rap1-dependent manner; (vii) like its action on Akt inhibition, PKA- and Epac-specific analogs synergistically activate Epac-associated PP2A; and (viii) dominant negative PP2A blocks cAMP-inhibitory action. Thus, we uncovered a novel cAMP-Epac/PKA-Rap1b-PP2A signaling module involved in Akt regulation that may represent a physiological event in the process of cAMP stimulation of thyroid mitogenesis.

Akt is activated by several agonists that stimulate phosphatidylinositol 3-kinase activity, which catalyzes the formation of the D3 phosphoinositides phosphatidylinositol 3,4,5-triphosphate and phosphatidylinositol 3,4-bisphosphate (7). These membrane lipids bind to the Akt pleckstrin homology (PH) domain (8, 9), leading to a stable interaction of Akt with cellular membranes. This membrane recruitment primes Akt for activation by phosphorylation. Full activation of Akt requires a PDK1 (phosphoinositide-dependent kinase 1)-dependent phosphorylation of Thr308 in the activation loop of Akt (10, 11) and a second phosphorylation event at Ser473 in the hydrophytic C-terminal domain of Akt (12). Although Akt can be autophosphorylated (13), several kinases with the ability to phosphorylate Akt Ser473 have been reported (14–17). Akt phosphorylation at steady state is tightly regulated, representing a balance between kinase-activating and phosphatase-inactivating events. Although efforts were originally mainly focused on the mechanism(s) of Akt activation, a role for agonist-mediated Akt inactivation is being uncovered. Several protein phosphatases, including canonical PP1 (18, 19) and PP2A (20–31) as well as newly identified Akt phosphatases (32), were recently reported to bind and dephosphorylate Akt in an agonist-dependent manner.

cAMP can either stimulate (33–43) or inhibit (44–50) Akt activity. We have previously reported that cAMP inhibited Akt activity in PCCCL3 thyroid cells via the GTP-binding protein Rap1b (47) and suggested that it played a role in TSH mitogenic action in these cells. cAMP rapidly promotes activation of Rap1b via the guanine exchange factor Epac (3) and phosphorylation of Rap1b-Ser79 through PKA (52). Active and phosphorylated Rap1b is strictly required for TSH stimulation of DNA synthesis (2, 3, 53) as well as inhibition of Akt activity (47). However, the molecular mechanisms by which cAMP inhibits Akt activity remain largely undefined. Here we report that cAMP-dependent inhibition of Akt in PCCCL3 thyroid cells is mediated by PP2A, the identification of a novel and stable Epac-PP2A signaling complex, and the modulation of its phosphatase activity by Rap-GTP and PKA.

EXPERIMENTAL PROCEDURES

Materials—Forskolin, isobutylmethylxanthine, and H89 were obtained from Calbiochem. Phosphatase inhibitors were obtained from Alexis. Phosphorylation-specific (Ser473, Thr308) and phosphorylation state-independent rabbit polyclonal anti-Akt antibodies were purchased from New England Biolabs (Beverly, MA). Monoclonal antibodies against HA (HA.11) and Myc (9E10) were from Covance Research Products. [γ-32P]ATP (3000 Ci/mmol) and P32 ATP (100 Ci/mmol) were obtained from Amersham Biosciences.
Epac-Rap1b and PKA, via PP2A, Inhibit Akt

Ci/mmol) and [32P]orthophosphoric acid (carrier-free) were from MP Biomedicals. Cross-tide and anti-phospho-syntheticosine antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). Protein G- and GSH-Sepharose were from GE Healthcare. Phosphatidylinositol, ATP, cAMP, and digitonin were from Sigma. DiFMU was obtained from Invitrogen.

Cell Culture—PCCL3 cells were cultured as previously described (3) in Coon’s modified Ham’s F-12 medium supplemented with 5% fetal bovine serum and four hormones: TSH (1 milliunits/ml), insulin (10 μg/ml), transferrin (5 μg/ml), and hydrocortisone (1 nM) at 37 °C in an atmosphere of 5% CO2 in air. HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum.

Plasmids and Transfection—All tagged Rap1 plasmids have been described elsewhere (52). Akt constructs were a gift from Dr. Toker (Beth Israel Deaconess, Boston, MA). FLAG-tagged PDK1 constructs were a gift from Dr. Liu (University of Texas Health Science Center), wild type and dominant negative PP2A-L309A were provided by Dr. J. Gotz (University of Zurich, Switzerland), and HA-GRP1-PH was a gift from Dr. J. Klarlund (University of Pittsburgh). PCCL3 cells, plated in 6-well plates, were transfected with the indicated plasmids (1.0–1.5 μg of DNA/well) using Fugene 6 reagent according to the supplier’s protocol (Roche Molecular Biochemicals). After a 24-h recovery, cells were starved in Coon’s medium containing 0.2% bovine serum albumin and transferrin (5 μg/ml) for 16 h before treatment and harvest.

Immunoprecipitation and Western Blotting—For immunoprecipitation, extracts were prepared in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 10 mM Na2PO4, 2 mM sodium orthovanadate, 100 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1% Triton X-100, followed by a centrifugation step for 30 min at 4 °C. For total lysates, cells were directly harvested in SDS sample buffer. Samples were resolved by SDS-PAGE followed by transfer onto polyvinylidene difluoride membranes. After blocking in 5% nonfat milk, membranes were probed with the appropriate primary antibodies. Immunoreactive proteins were detected by a chemiluminescence detection kit (Pierce), using horseradish peroxidase-conjugated secondary antibodies. Specific bands were quantified by image analysis using Bio-Rad Molecular Dynamics software.

Immunofluorescence Staining and Confocal Microscopy—Cells were cultured on coverslips in 6-well plates and transfected with the indicated constructs. For immunofluorescent staining, cells were rinsed with phosphate-buffered saline (PBS; pH 7.4), fixed in 3.7% formaldehyde in PBS for 10 min at room temperature, permeabilized in 0.5% Triton X-100 in PBS for 20 min, and washed in 0.1 M glycine in PBS for 10 min. The coverslips were washed five times for 5 min with PBS and blocked with 1% bovine serum albumin, 1% goat serum in PBS for 30 min. The cells were incubated with fluorescein isothiocyanate-coupled HA antibody or Cy3-coupled Myc antibodies for 60 min at room temperature and washed five times in 1% bovine serum albumin in TBS for 10 min. The coverslips were mounted in PermaFluor (Thermo Electron Corp.), and samples were examined under confocal microscopy.

RESULTS

Upstream Akt Regulatory Elements Are Not the Target(s) for cAMP-mediated Inhibition—In PCCL3 thyroid cells, elevation of cAMP transiently inhibits basal and insulin-mediated Akt phosphorylation and activation (47). To start dissecting the
confirm that cAMP levels did not have an effect on PIK activity, GRP1-PH was used as a highly specific phosphatidylinositol 1,4,5-trisphosphate probe (56) under different conditions of stimulation. As shown in Fig. 1C, translocation of both Myc-Akt and HA-GRP1-PH to a membrane compartment was observed by immunofluorescence upon insulin stimulation. However, although PIK inhibitor wortmannin blocked this translocation event, forskolin stimulation did not have any effect. Thus, the combined results of Fig. 1 clearly establish that cAMP inhibitory action on Akt lies downstream of the receptor-PIK signaling module.

PDK1, a kinase activated by products of PIK, phosphorylates Akt at Thr308, an event required for its kinase activity and downstream signaling. cAMP has been recently suggested to inhibit Akt under some conditions (49). To address if this represents the inhibitory target of cAMP in PCCL3 cells, a myristoylated PDK1 construct that is constitutively targeted to the plasma membrane was co-transfected with Akt. Similarly, co-transfection with the constitutively active PDK1 516E, was performed to address whether cAMP could inhibit Akt under those conditions. If translocation represents the inhibitory cAMP target or there was a cAMP inhibitory action on PDK1 activity, it is expected that the myristoylated construct will bypass this step, rendering Akt resistant to cAMP. As can be seen in Fig. 2, forskolin effectively inhibited insulin-stimulated Akt under these conditions. Consistent with these results, forskolin stimulation did not affect PDK1 activity, as assessed by an anti-phospho-PDK1 (Ser241) antibody (not shown). Thus, neither PIK nor PDK1 represents the target for the inhibitory action of cAMP on Akt.

**Constitutively Active Akt Is Resistant to cAMP**—Steady-state phospho-Akt represents a balance between its rate of phosphorylation mediated by upstream kinases and its rate of dephosphorylation by phosphatases. Our results (Figs. 1 and 2) indicate that upstream kinase events do not represent the target for cAMP inhibition. Accordingly, a set of experiments was designed to assess the possibility that cAMP impacts the rate of Akt dephosphorylation. Conversion of Ser473 and Thr308 (the activating phosphorylation sites) to the phosphomimetic residues aspartic acid renders a constitutively active Akt protein (57). If cAMP increases the rate of Akt dephosphorylation, this constitutively active S473D/T308D protein (DD-Akt) should become resistant to the inhibitory effects of forskolin. As shown in Fig. 3, although forskolin inhibited both basal and insulino-resistant target site, the effect of forskolin stimulation on the upstream elements in the pathway were investigated. PIK activation represents a receptor-proximal event required for Akt activation (55). An immune complex PIK activity assay was performed using anti-phosphotyrosine antibodies, and the reaction products were analyzed by TLC plates (Fig. 1A). Although insulin stimulated PIK activity, no effect of forskolin was observed even at concentrations that maximally inhibited Akt activity (IC50 ~ 0.1 μM) (49). Moreover, forskolin had an effect on a sample treated with saturating amounts of LY294002, a PIK inhibitor (Fig. 1B). Similar effects were observed on both critical phosphorylation sites, Ser473 and Thr308. To further
stimulated WT Akt activity, no effect was observed on the maximally stimulated constitutively active DD-Akt protein. These results are consistent with a role of the phosphorylation sites as targets for cAMP action and suggest an Akt phosphatase as a molecular player responsible for cAMP-dependent inhibition.

**Calyculin A- and Okadaic Acid-activated Akt Is Resistant to cAMP**—Phosphatase inhibitors (i.e. okadaic acid and calyculin A) increase the steady-state levels of phospho-Akt (58). If an Akt phosphatase is the target for cAMP, inhibition of its activity should render Akt resistant to cAMP inhibition, as observed above for the DD-Akt protein. Treatment of PCCL3 cells with calyculin A for 20 min increased the phosphorylation of both Ser473 and Thr308 (Fig 4A) in a dose-dependent manner, with a concomitant increase in its kinase activity (Fig. 4B). However, although basal Akt activity was inhibited by forskolin, calyculin A-stimulated Akt was completely resistant to the inhibitory effects of forskolin (Fig. 4B). Similar results were observed with foscirecin (not shown) and okadaic acid treatment, at concentrations that specifically inhibited PP2A without affecting PP1 activity (Fig. 4, C and D). Therefore, by abolishing phosphatase activity, the ability of cAMP to inhibit Akt is lost, indicating an Akt phosphatase might represent the cAMP-inhibitory target.

**cAMP Stimulates the Rate of Akt Dephosphorylation**—To assess whether phospho-Akt is a direct substrate of the putative cAMP-stimulated phosphatase activity, a pulse–chase experiment was designed to directly measure the rate of Akt dephosphorylation upon cAMP stimulation. The rationale of the experiment was to radiolabel the pool of Akt upon insulin treatment, followed by a chase phase with 5 mM cold ATP to decrease specific activity and therefore avoid any interference from de novo upstream phosphorylation events. Since ATP is nonpermeable, a digitonin permeabilization protocol was devised, and the effect of 20 μM cAMP on Akt phosphorylation was assessed by measuring 32P-labeled phospho-Akt upon immunoprecipitation. Under basal nonstimulated conditions, digitonin led to a leak out of Akt from the cells (not shown). Therefore, stimulation with insulin before permeabilization was strictly required and served a dual role: phosphorylation of the Akt pool and, most importantly, “trapping” phospho-Akt intracellularly during permeabilization. Under these experimental conditions (Fig. 5, top), the addition of cAMP during the chasing phase induced Akt dephosphorylation with a $t_{1/2}$ of
of calyculin A completely abrogated the cAMP-inhibitory effect (Fig. 5). These results demonstrate that cAMP stimulation directly affects the rate of Akt dephosphorylation in a phosphatase-dependent manner.

Synergistic Action of PKA and Epac on Akt Dephosphorylation—We have previously implicated both cAMP effector branches, PKA and Epac, in the action of forskolin on phospho-Akt (47). Newly identified specific Epac and PKA-activating cAMP analogs (59, 60) were used to directly assess their involvement on cAMP inhibition of Akt. Forskolin, in a PKA-dependent manner, inhibited Akt phosphorylation and its kinase activity, stimulation of either Epac or PKA alone was not sufficient to mimic cAMP action on Akt; however, co-stimulation with both analogs recapitulated the response of cAMP (Fig. 6A). To confirm the role of Epac, dominant negative EpacR279E was co-transfected with Akt (Fig. 6B). Similar to the effects of

FIGURE 5. cAMP increases the rate of Akt dephosphorylation. Top, a scheme of the protocol used (see “Results” for details). Briefly, starved WT HA-Akt cells were labeled with [32P]orthophosphoric acid, followed by a 15-min insulin (1 μg/ml) stimulation. After a washing step, cells were permeabilized with digitonin (40 μg/ml) in the presence of 5 mM ATP, 20 μM cAMP, and calyculin A (calA; 300 nm), as indicated. Upon lysis and HA-immunoprecipitation, HA-AKT phosphorylation level was assessed by SDS-PAGE/autoradiography (bottom). IP, immunoprecipitation.

FIGURE 6. PKA and Epac pathways are required for cAMP-mediated Akt inhibition. Specific PKA (300 μM N6-benzoyladenosine-3',5'-cyclic monophosphate; cA) and Epac (300 μM 8-(4-methoxyphenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate; cE) analogs were used alone or in combination to assess their relative contribution to cAMP-mediated Akt inhibition. Bottom, quantitation (phospho-Akt/total Akt) from three independent experiments (A). EpacR279E was co-transfected with HA-Akt. Upon stimulation, HA-Akt was immunoprecipitated, and its phosphorylation state was analyzed with the indicated antibody (B). Quantitation (phospho-Ser473-Akt/HA-Akt) from two independent experiments is shown. FK, forskolin.

~5 min, consistent with the time frame of physiological inhibition by TSH or forskolin (47). No Akt dephosphorylation was observed in the absence of cAMP, and the addition
PKA inhibition by H89, co-transfection of dominant negative Epac R279E also blocked the ability of forskolin to inhibit Akt.

Taken together, these results confirm the involvement of both effectors, Epac and PKA, on cAMP-dependent Akt dephosphorylation.

Association of Epac1 and Rap1b with a cAMP-stimulated Akt Phosphatase Activity—Genetic (N17-Rap and RapGAP) (47) and pharmacological (cAMP analogs specific for Epac or PKA) (Fig. 6) intervention clearly demonstrated a role for Rap-GTP in the phosphatase-mediated cAMP inhibition of Akt. As a first attempt at understanding some mechanistic aspects of the cAMP-Rap-phosphatase pathway, we asked whether a pool of Epac-Rap1 is present in the cell in association with a phosphatase. For this, we used microcystin-Sepharose, an affinity reagent used to enrich PP1- and PP2A-containing complexes from cell extracts. Epac, Rap, and Akt (Fig. 7A) were observed upon a single microcystin affinity step, and this steady-state association was not affected by forskolin stimulation (not shown). Binding was specific as assessed by co-incubation in the presence of soluble microcystin (Fig. 7A), and no association was observed with the okadaic-insensitive HA-PHLPP Akt phosphatase (not shown). Moreover, Epac and Akt co-localized in a cortical region of the cell when assessed by confocal microscopy, and this compartmentalization was not modified by forskolin stimulation (not shown). Next, we addressed whether a phosphatase activity could be found in association with Epac/Rap1. GST control and GST-Epac were pulled down from transfected cell extracts, and the washed beads were assessed for phosphatase activity using the fluorescent DiFMUP substrate. Results from this experiment are presented in Fig. 7B; a phosphatase activity was detected specifically in GST-Epac-containing beads, and its activity was positively modulated in a forskolin-dependent manner. The effect of forskolin on the phosphatase activity could be blocked by H89, indicating the involvement of PKA. Interestingly, a similar behavior was observed when N17-Rap was co-transfected along with GST-Epac; dominant negative N17-Rap did not significantly affect basal Epac-associated phosphatase activity but blocked its forskolin-mediated stimulation (Fig. 7C). In order to assess the role of the different cAMP effectors on Epac-bound phosphatase activity, specific Epac/PKA analogs were utilized (Fig. 7D). Consistent with the effects on Akt inhibition (Fig. 6A), stimulation of each effector pathway independently showed only a small effect; however, co-stimulation rendered a synergistic activation comparable with forskolin action. These results indicate the presence of a cAMP-stimulated Epac-associated phosphatase, whose activity could be modulated synergistically by PKA, Epac, and Rap1 action.

PP2A Is the Epac-associated Phosphatase Activity—GST-Epac-associated phosphatase activity could be blocked by foscinecin (not shown) and okadaic acid (Fig. 8A) at concentrations
that inhibit PP2A activity (Fig. 8B), suggesting that the phosphatase activity in the Epac complex is a member of the PP2A family. To directly assess this possibility, GST-Epac pull-down material was blotted with antibodies specific for the catalytic PP2A subunit. As can be observed in Fig. 8C, catalytic PP2A is present in a complex with Epac. Consistent with the microcystin-Sepharose affinity results, forskolin treatment did not significantly alter the amount of catalytic PP2A present in the complex, indicating that forskolin action on PP2A represents an activating event rather than an association/recruitment step. Similar results were observed with Akt-associated PP2A (Fig. 8C). Thus, these results indicate that a pool of Epac in the cell is present in a complex with a phosphatase, identified pharmacologically and immunologically as PP2A, whose activity could be modulated by cAMP in a PKA- and Rap1-dependent manner.

**PP2A Is the cAMP-sensitive Akt Phosphatase**—If PP2A is the phosphatase responsible for Akt dephosphorylation downstream of Epac-Rap1b and PKA, its specific inhibition should make the system resistant to cAMP inhibition. In order to specifically block PP2A activity, a dominant negative PP2A construct was utilized (cPP2A-L309A) (61), and its effect on forskolin-mediated Akt inhibition was determined. As can be seen in Fig. 9, forskolin treatment inhibited insulin-stimulated Akt phosphorylation as previously described (47). Co-transfection with dominant negative PP2A-L309A increased steady-state Akt phosphorylation and significantly blocked the inhibitory effect of forskolin. These results confirmed the previous observations based on pharmacological inhibitors (Fig. 4D); moreover, they demonstrate that PP2A is the Akt phosphatase responsible for the Epac-, Rap1b- and PKA-dependent cAMP inhibition of Akt.

**DISCUSSION**

Thyroid cell proliferation is triggered by the synergistic action of TSH and growth factors of the insulin/IGF1 family (62). We have recently demonstrated an active role for both cAMP effectors, Epac and PKA (2), in TSH-mediated mitogenesis; however, the molecular players downstream of cAMP responsible for this synergism are for the moment unknown. We have previously identified Akt activity as a downstream target of TSH-cAMP signaling in thyroid cells (47). TSH/cAMP induced a rapid and transient Akt inhibition, a process that required both PKA and active Rap1 (47). In the present study, we have demonstrated that cAMP, acting via a synergistic action of Epac, PKA, and Rap1b, activates PP2A, increasing the

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**FIGURE 8. Epac-associated phosphatase is PP2A.** A, GST-Epac-transfected cells were incubated with forskolin (FK) as described before in the presence of different amounts of okadaic acid (OA). Samples were processed as described before for phosphatase activity. B, HA-cPP2A-transfected cells were incubated with okadaic acid, as indicated. After immunoprecipitation with HA11 antibody, phosphatase-associated activity was assayed as described. C, GST-, GST-Epac-, and GST-Akt-transfected cells were stimulated with forskolin as indicated before. Upon a glutathione-Sepharose affinity step, associated proteins were resolved on SDS-PAGE and visualized by Western blot with anti-GST and PP2A antibodies.

**FIGURE 9. PP2A is the Akt phosphatase mediating cAMP inhibition.** PCCL3 cells were co-transfected with Myc-Akt and HA-cPP2A-L309A (dnPP2A) or pcDNA3.1 as control (Vector). Upon a 16-h period of serum starvation, cells were stimulated with forskolin (10 μM) for the indicated times, followed by insulin (1 μg/ml for 5 min). Upon lysis, Myc-Akt was immunoprecipitated from the extracts (9E10 antibody), and proteins were resolved on SDS-PAGE and assayed for Myc (bottom) and Akt phosphorylation (anti-phospho-Akt Ser473) (top). For unknown reasons, opposite to the observation made with calyculin A, HA-cPP2A-L309A consistently increased the steady-state levels of Myc-Akt. Quantitation (phospho-Ser473-Akt/Myc-Akt) from two independent experiments is shown.
rate of phospho-Akt dephosphorylation, leading to inhibition of its activity.

cAMP modulates Akt in different cell types, including fibroblasts (33, 34, 45, 63), hepatocytes (42), adipocytes (41, 64), cardiomyocytes (40), skeletal muscle (43), neurons (48), Schwann (39), endothelial (46), and endocrine cells (38, 65). Specifically, in thyroid cells, the role of Akt in TSH-mediated proliferation of nontransformed cells is still controversial; either no effects (66) or both positive (67–69) and negative (47) effects of TSH on Akt phosphorylation/activity have been reported. The stimulation of Akt activity by cAMP is of small magnitude (1.5–2-fold), and it occurs with relatively slow kinetics (69). In contrast, cAMP elicits a robust (5–10-fold), rapid and transient inhibition of Akt activity with half-maximal effects developing within 2 min (47), returning to basal levels in 30–45 min. Since the transient inhibitory effect of cAMP on basal Akt activity is not always easily observed, as opposed to the effects on insulin-stimulated Akt activity, it is therefore possible that the slow positive action of cAMP on Akt reported by others represents the slow recovery phase upon inhibition.

Both cAMP effector pathways, Epac/Rap1 and PKA, seem to be actively involved in Akt regulation. A dual cAMP action on Akt was recently proposed, where a finely tuned regulation is achieved by a balance between opposite Epac/Rap1-dependent and PKA-dependent actions (63). It has recently been suggested that distinct cAMP pools, shaped by the action of phosphodiesterases, might be responsible for these specific cAMP effector responses (64). Our results in thyroid cells differ markedly with this model; effector-specific cAMP analogs do not show any effect on Akt when added independently, despite the fact that Epac- and PKA-selective analogs activated Rap1b and CREB phosphorylation, respectively (2). Importantly, when added together, Epac- and PKA-selective agents fully mimicked forskolin or TSH action. Moreover, inhibitors of either effector branch completely abrogated cAMP-dependent Akt inhibition (47). Our results clearly indicate that a synergistic action of Epac and PKA is responsible for cAMP inhibition of Akt in a manner that depends on Rap1b.

From a mechanistic point of view, the effects of cAMP on steady-state phospho-Akt could represent a regulatory action in any of the upstream kinase-activating events (i.e. PIK/PDK-1/mTORC2) or, alternatively, an effect on the regulation of its dephosphorylation. Moreover, a series of Akt binding partners were reported to modulate its phosphorylation and activity; whereas JIP-1 (70), Ft-1 (71), and TCI-1 (72) can directly modulate Akt activity, others, like Hsp90, act indirectly by protecting it from the action of inactivating phosphatases (73). Interestingly, Hsp90 was recently identified as a PKA substrate, and its phosphorylation negatively affects its ability to associate with endothelial nitric-oxide synthase (74). Whether an analogous PKA-dependent effect on Hsp90/Akt accounts for the inhibitory action of cAMP is for the moment unknown. Our findings described here are consistent with a model in which cAMP deactivates Akt by increasing an Akt phosphatase activity. Contrary to previous reports (45), cAMP did not affect in our system either PIK or PDK1 activity/translocation; constitutionally active and calycin A-stimulated Akt were both resistant to the inhibitory action of cAMP, and, most directly, cAMP affected the rate of Akt dephosphorylation (Fig. 5), thus indicating an Akt phosphatase as the cAMP target. Most importantly, we identified a new Epac-PP2A signaling module, whose phosphatase activity can be positively modulated by cAMP in a PKA- and Rap1-dependent manner. Consistent with the effects on Akt, a synergistic action of both cAMP effector branches was observed at the level of Epac-associated PP2A activity.

Unlike PIK, PP2A is not a classical target for cAMP action, although reports exist describing cAMP-dependent PP2A activation (75, 76); however, the mechanistic details for cAMP stimulation of PP2A are still unknown. PP2A is a major serine/threonine phosphatase involved in cell signaling. It forms a heterotrimeric complex composed of A, B, and C subunits (77, 78). The 37-kDa catalytic subunit (C) and 65-kDa scaffold A subunit/PR65, form the PP2A core structure. This A/C core associates with a member of a large family of nonrelated regulatory/targeting B subunits (B/PR55, B'/B56/PR61, B'/PR72, B'/PR93/PR110), which modulate subcellular localization, substrate specificity, and catalytic activity (79). Recent studies by independent groups suggest that members of the B family (20, 31, 80, 81) and PP2A-B'/B56 heterotrimers might be specifically involved in Akt regulation (27, 29, 82). More specifically, it has been recently shown that cAMP could activate PP2A via PKA-dependent phosphorylation of B56 (83); however, dominant negative B56 did not block the effect of cAMP on Akt. The identity of the PP2A regulatory subunit involved in Epac-PP2A-Akt is under investigation.

PP2A activity is modulated by post-translational modifications. CAMP-dependent regulation of any of the PP2A subunits and/or the enzyme machinery responsible for their post-translational modifications therefore represent potential targets. For instance, it has been reported that PKA phosphorylates a B regulatory subunit, altering substrate specificity (84). Catalytic C subunit possesses a unique C-terminal sequence that binds to a groove in the interface between A and B subunits (77, 78). This C-tail is subjected to tyrosine phosphorylation (85, 86) and carbamoylation (87, 88). Tyrosine phosphorylation of Tyr307 at its C terminus inactivates PP2A (85), potentially by disrupting its interaction with B subunit (77); however, preliminary data from our laboratory suggest that cAMP does not modify the levels of Tyr307 phosphorylation. Carbamoylation of Leu309 is considered necessary for the recruitment of the B regulatory subunit (89–93). However, new in vitro studies using a C subunit deficient in the last 15 residues did not interfere with the ability to form the holoenzyme (78), indicating that methylation might facilitate in vivo heterotrimer assembly, but it is certainly not required in vitro. Since cAMP increased carbamoylation of PP2A in Xenopus eggs (94), the possibility that cAMP might affect the phosphatase methyltransferase or methyltransferase activity deserved further investigation. In any case, the mechanisms involved should take into account the synergistic behavior of both cAMP effector branches, PKA and Epac.

Compartmentalization via formation of macromolecular complexes is an efficient way to attain signaling specificity (95),

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4 K. Hong and D. L. Altshuler, unpublished data.
and particularly the association of Akt and phosphatases in such complexes was reported in several models. Integrin activation induces the formation of a complex leading to PP2A activation and Akt dephosphorylation (22, 96); dopaminergic responses in striatum are mediated by an Akt-β-arrestin 2-PP2A complex (20); p38α-dependent caveolin-1/PP2A association in cardiomyocytes leads to Akt dephosphorylation (31); TPA-mediated PKCζ and PKCe activation in keratinocytes induces PP2A association and Akt dephosphorylation (97). All of these studies suggest that local recruitment and/or activation of subpopulations of PP2A in specific complexes confers specificity to Akt regulation by upstream signals. Our studies provide a framework for cAMP regulation of Akt in the context of a new Epac-mediated complex, integrating Rap- and PKA-dependent events. Interestingly, in cardiomyocytes, an AKAP-tethered complex containing Epac, PKA, phosphodiesterases, PP2A, and members of the Erk family coordinates the integration of Campbell-dependent events (51). Whether an analogous AKAP-mediated complex is involved in the Epac-PP2A-Akt regulation is currently under investigation.

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