Differential Signaling of Cyclic AMP

OPPOSING EFFECTS OF EXCHANGE PROTEIN DIRECTLY ACTIVATED BY CYCLIC AMP AND cAMP-DEPENDENT PROTEIN KINASE ON PROTEIN KINASE B ACTIVATION*

Received for publication, November 13, 2001, and in revised form, January 8, 2002
Published, JBC Papers in Press, January 18, 2002, DOI 10.1074/jbc.M110856200

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The recent discovery of Epac, a novel cAMP receptor protein, opens up a new dimension in studying cAMP-mediated cell signaling. It is conceivable that many of the cAMP functions previously attributed to cAMP-dependent protein kinase (PKA) are in fact also Epac-dependent. The finding of an additional intracellular cAMP receptor provides an opportunity to further dissect the divergent roles that cAMP exerts in different cell types. In this study, we probed cross-talk between cAMP signaling and the phosphatidylinositol 3-kinase (PI3K)/PKB pathways. Specifically, we examined the modulatory effects of cAMP on PKB activity by monitoring the specific roles that Epac and PKA play individually in regulating PKB activity. Our study suggests a complex regulatory scheme in which Epac and PKA mediate the opposing effects of cAMP on PKB regulation. Activation of Epac leads to a phosphatidylinositol 3-kinase-dependent PKB activation, while stimulation of PKA inhibits PKB activity. Furthermore, activation of PKB by Epac requires the proper subcellular targeting of Epac. The opposing effects of Epac and PKA on PKB activation provide a potential mechanism for the cell type-specific differential effects of cAMP. It is proposed that the net outcome of cAMP signaling is dependent upon the dynamic abundance and distribution of intracellular Epac and PKA.

Cyclic adenosine 3′,5′-monophosphate (cAMP) is produced as an intracellular second messenger in response to a variety of extracellular signals, including hormones, growth factors, and neurotransmitters. cAMP regulates a wide range of important biological processes, which, alongside cell metabolism, include cell division, growth, differentiation, secretion, memory, and neoplastic transformation. For many years, major intracellular effects of cAMP in mammalian cells were believed to be mediated by cAMP-dependent protein kinase (PKA). The regulation of PKA is achieved via a unique three-component signaling system in which PKA is composed of two separate subunits, the catalytic (C) and regulatory (R) subunits that interact to form an inactive holoenzyme complex (1). Although phosphorylation of Thr^197 in the activation loop of the C subunit is necessary for the maturation and optimal catalytic activity of PKA (2, 3), unlike most other kinases whose activity is regulated by dynamic phosphorylation/dephosphorylation of the activation loop this phosphorylation step does not seem to be a regulatory mechanism for PKA in vivo. Once phosphorylated, PKA is fully active in its catalytic potential and the Thr^197 phosphate does not turn over readily (4). The activation of PKA is achieved by binding of the second messenger cAMP to the R subunit, which consequently induces a conformational change in the R subunit and leads to the dissociation of the holoenzyme into its constituent subunits (1). The free active C subunit can then affect a range of diverse cellular events by phosphorylating an array of cytoplasmic and nuclear protein substrates, including enzymes and transcription factors (5).

The effect of cAMP on certain cellular functions has been shown to be dependent on cell-type and biological responses (6). For example, in PC12 cells, Swiss 3T3 cells, and thyrocytes, cAMP activates MAP kinases, potentiates the effects of growth factors on differentiation and gene expression, and/or stimulates cell growth and promotes the G1 to S phase transition in the cell cycle (7–10). In contrast, cAMP inhibits the proliferation of many cells, including fibroblasts (Rat1 and NIH 3T3), smooth muscle cells, and cells transformed by oncogenes such as ras (11–14). Despite extensive studies, the molecular mechanism underlying the cell type-specific effects of cAMP remains elusive. The growth inhibitory effect of cAMP is believed to be mediated partly through activation of PKA, which interferes with Ras/MAPK signaling pathways (15). Recent studies suggested that PI3K activity may be required for cAMP-stimulated cell proliferation in thyroid cells (16, 17). Interestingly, the effects of cAMP on PI3K/PKB pathways are also cell type-specific and correlate well with the mitogenic effects of cAMP.

* This work was supported in part by startup funds from the Department of Pharmacology and Toxicology, The University of Texas Medical Branch, a recruitment grant from the John Sealy Memorial Endowment Fund, American Cancer Society Research Scholar Grant RSG-01-033-01-EHR, and Center Grant ES06876 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PKA, cAMP-dependent protein kinase; C, cAMP-dependent protein kinase catalytic subunit; B, cAMP-dependent protein kinase regulatory subunit; Epac, exchange protein directly activated by cAMP; GEF, guanine nucleotide exchange factor; PI3K, phosphatidylinositol 3-kinase; dibutyryl cAMP, N^6,N^6-dibutyryl adenosine-3′,5′-cyclic monophosphate; PBS, phosphate-buffered saline; PKD, phosphoinositide-dependent kinase; HEK, human embryonic kidney; EGFP, epidermal growth factor protein; WRT, Wistar rat thyroid; TSH, thyrotropin.
cAMP effects on PKB and membrane ruffling are PKA independent (16). These findings indicate that multiple cAMP-mediated pathways exist and only some are PKA dependent. Therefore, the recently discovered cAMP receptor Epac (exchange protein directly activated by cAMP) or cAMP-GEF (cAMP-regulated guanine nucleotide exchange factor) may represent an important piece of the puzzle that is critical to our understanding of cAMP-mediated cell signaling. Epac contains a cAMP-binding domain that is homologous to the R subunit of PKA and a guanine exchange factor (GEF) domain (18, 19). Epac proteins bind cAMP with high affinity and activate their downstream target Rap1, a Ras superfamily guanine nucleotide-binding protein. Rap1, initially identified as an antagonist downstream target Rap1, a Ras superfamily guanine nucleotide-binding protein, can be activated by Epac in response to forskolin (18). Moreover, mutation of the PKA phosphorylation site can be activated by Epac in response to forskolin (18). Opposite effects of Epac and PKA on the PI3K/PKB pathway can be observed directly activated by cAMP) or cAMP-GEF (cAMP-regulated guanine nucleotide exchange factor) may represent an important piece of the puzzle that is critical to our understanding of cAMP-mediated cell signaling. Epac contains a cAMP-binding domain that is homologous to the R subunit of PKA and a guanine exchange factor (GEF) domain (18, 19). Epac proteins bind cAMP with high affinity and activate their downstream target Rap1, a Ras superfamily guanine nucleotide-binding protein. Rap1, initially identified as an antagonist downstream target Rap1, a Ras superfamily guanine nucleotide-binding protein, can be activated by Epac in response to forskolin (18). Moreover, mutation of the PKA phosphorylation site can be activated by Epac in response to forskolin (18). Cell Culture and Transfection—HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The cultures were maintained at 37 °C in a humidified chamber supplemented with 5% CO2. The day before the transfection, the cells were cultured into 6-well tissue culture plates, grown to 80% confluence, and further analyzed with Western blot using Rap1 specific antibodies. Rap1 Activation Assay—The GTP loading status of Rap1 was assessed using a gluthatione-S-transferase fusion of the Rap1-binding domain of RalGDS as described earlier (27). Briefly, cells were grown to 75% confluence in 100-mm Corning culture dishes, starved in serum-free medium for 48 h, and treated with 10 μM forskolin for 5 min. Following three washes in PBS, the cells were lysed in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 20 mM MgCl2, 5 mM EGTA, 1% Triton X-100, and Roche EDTA-free protease inhibitors. The cell lysate was mixed with 40 μg of glutathione-Sepharose beads with 30 μg of glutatione S-transferase-RalGDS-Rap1-binding domain and incubated at 4 °C for 2 h with gentle agitation. Following three washes in lysis buffer, the beads were suspended in 40 μl of SDS sample buffer. 15 μl of protein samples were loaded onto a 15% SDS-polyacrylamide gel and further analyzed with Western blot using Rap1 specific antibodies. Western Blot Analysis—Protein concentration of cell lysates was measured with the Bio-Rad Protein Assay reagent, and equal amounts of protein were denatured by heating at 95 °C for 5 min prior to resolution by SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad) using a Trans-Blot SD transfer cell (Bio-Rad) and a transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, 0.0375% SDS (pH 8.3). Membranes were placed in 5% nonfat milk in Tris-buffered saline/Tween buffer (TTBS) at 4 °C for 1 h. Membranes were washed with various antibodies for 1 h at room temperature: anti-PKB antibodies (1:1000), anti-phosphate T308 PKB antibodies (1:1000), anti-phosphate S473 PKB antibodies (1:1000), and affinity purified anti-Epac polyclonal antibody (1 μg/ml). Membranes were then washed with TTBS and incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody for 1 h at room temperature. Following three 10-min washes in TTBS, membranes were treated with horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgGs were from Bio-Rad. The SuperSignal West Pico Chemiluminescent Substrate Reagents kit was obtained from Pierce. Culture media were from Gibco. Fetal bovine serum was from BioWhittaker. N2O4-dibutylryl adenosine-3’,5’-cyclic monophosphate (dibutyryl cAMP) were from Calbiochem. Human embryonic kidney (HEK) 293 cells were from American Type Culture Collection. All chemicals were reagent grade.

Epac Antibody Production and Affinity Purification—Specific polyclonal antibodies against Epac were generated by Alpha Diagnostic International Inc. (San Antonio, TX) using synthetic Epac peptide spanning residues 41–148. These observations suggest cAMP-mediated activation of Rap1 may be independent of phosphorylation by PKA. It is most likely that the cAMP-mediated signaling mechanism is much more complex than was believed earlier, and many cAMP-mediated effects that were previously thought to act through PKA are in fact also transduced by Epac. Therefore, it is imperative to reformatte concepts of cAMP-mediated signaling to include the contribution of Epac. The existence of Epac immediately raises many questions regarding the mechanism of cAMP-mediated signaling. Since both PKA and Epac are broadly expressed in many tissues (18, 19), an increase in intracellular cAMP levels will lead to the activation of both enzymes, and possibly other potential cAMP target(s) as well. It is conceivable that while PKA acts through a discrete set of signaling pathways, Epac may enlist distinct signaling pathways, and the net cellular effects of cAMP are dictated by the sum of these events. Therefore, the apparent cellular effect of cAMP can vary depending upon the relative cellular abundance and distribution of Epac and PKA. Our hypothesis is that there is a dynamic control of cellular expression and targeting of Epac and PKA which, coupled with the dynamic changes in the concentration of cAMP, are the controlling mechanisms that determine the observed cell-type-specific cAMP effects. To test this hypothesis, we examined the specific effects of Epac and PKA on the PKB/PKB pathway that has been reported to exhibit cell type-specific responses to cAMP. Our study shows for the first time that although Epac and PKA are activated by a common upstream second messenger cAMP, they can exert opposing effects in regulating downstream targets such as PKB. Therefore, the net outcome of cAMP signaling on PKB activation may be dependent upon the dynamic abundance and distribution of intracellular Epac and PKA.
substrates for 5 min and exposed to x-ray film at room temperature. At least three independent experiments were performed for each Western blot.

**Protein Kinase B Assay**—PKB activity was measured using an Akt Kinase Assay kit from Cell Signaling following manufacturer’s protocol.

**Site-directed Mutagenesis**—Mutagenesis was performed using a QuikChange™ Site-directed Mutagenesis Kit (Stratagene). All DNA constructs were sequenced to confirm the mutation as well as to verify the accuracy of the full-length cDNA sequence.

**Fluorescence Microscopy**—To detect the subcellular localization of Epac-EGFP and Δ[1-146]Epac-EGFP, cells were subcultured after transfection with an appropriate plasmid in 6-well plates with a poly-2-lysine-coated coverslip in each well and grown for 16–24 h. The cells were fixed in 2% paraformaldehyde in PBS. The samples were rinsed with PBS, mounted on glass slides, and sealed in 70% glycerol. Fluorescent signals were revealed under the fluorescence microscope (Olympus BX51), using an fluorescein isothiocyanate/EGFP filter with maximum excitation at 488 nm and maximum emission at 525 nm.

**RESULTS**

**Epac and PKA Mediate Opposing Effects of cAMP on PKB Activation**—To elucidate the specific roles of Epac and PKA in modulating the PKB signaling pathway, we examined the effects of cAMP-elevating agents on activation of PKB in parental HEK293 and HEK293/Epac cells that have been stably transfected with Epac. As shown in Fig. 1A, HEK293 cells expressed normal levels of PKA but undetectable amounts of Epac when cell lysates from HEK293 and HEK293/Epac cells were probed by affinity purified PKA C antibodies, respectively. A protein with the apparent molecular weight of 100,000 corresponding to the calculated size of Epac was readily detectable in the Epac-
transfected HEK293 cells. Furthermore, overexpression of Epac in HEK293 cells did not affect the protein levels of PKA (Fig. 1A). When treated with forskolin, endogenous PKB activity in HEK293/Epac cells was increased about 3-fold (Fig. 1B), as monitored by phosphorylation of Ser473 and Thr308, whose phosphorylation status is critical for the kinase activity of PKB (33). Mutation at this Arg residue leads to the loss of inhibition in phosphorylation of the endogenous PKB kinase activity directly. Results from these kinase activity assays, and so not expressed in all cells, the actual effect of cyclic nucleotide binding activity and biological activity of Epac (19). Unlike HEK293 cells expressing the wild-type Epac, EpacR279E-expressing HEK293 cells behaved in a manner similar to the parental HEK293 cells in response to forskolin stimulation, resulting in inhibition of PKB activity (Fig. 3). This led us to conclude that the observed cAMP-mediated PKB activation was the direct consequence of Epac activation and required a functional cyclic nucleotide-binding domain.

**Rap1 Activation Is Necessary for Epac-mediated PKB Phosphorylation**—Rap1 is the only known downstream effector for Epac described so far. To test if Rap1 activation contributes to Epac-mediated PKB activation, we compared the levels of GTP-bound Rap1 in serum-starved and forskolin-treated HEK293/Epac and parental HEK293 cells. As shown in Fig. 4A, the amount of GTP-bound Rap1 in parental HEK293 cells was below the detection limit of the pull-down assay and forskolin did not significantly increase the GTP loading of Rap1 in these cells. This fits very well with our observation that the expression of Epac in HEK293 cells is very low, under the detection limit of Western blot using affinity purified Epac antibodies. In contrast, Epac-expressing HEK293 cells exhibited basally elevated levels of GTP-bound Rap1 compared with parental HEK293 cells. This observation is consistent with the original report that Epac overexpression is sufficient to partially activate Rap1 even in the absence of cAMP-elevating agent and Epac is capable of further activating Rap1 specifically in response to cAMP (18, 19). Treatment with forskolin further increased the proportion of GTP-bound Rap1 in HEK293/Epac cells. These results suggest that Rap1 activation is potentially involved in Epac-mediated PKB activation.

To examine if activation of Rap1 is required for Epac-mediated PKB activation, we transiently expressed Rap1A17N, a putative dominant-negative Rap1 mutant in HEK293/Epac cells to block the in vivo activation of endogenous Rap1. Expression of Rap1A17N abolished the ability of Epac to activate PKB in HEK293/Epac cells in response to increased intracellular cAMP. Forskolin actually weakly attenuated rather than activated PKB (Fig. 4B). Considering the fact that Rap1N17 was transiently transfected in HEK293/Epac cells in our assays, and so not expressed in all cells, the actual effect of

### Table 1

|          | Db-cAMP | I89 | PKB-P473 | PKB | 293/Epac |
|----------|---------|-----|----------|-----|----------|
| HEK293   | —       | —   | +        | —   | —        |
| HEK293/Epac | +       | —   | +        | +   | +        |

**Opposite Effects of Epac and PKA on Regulation of PKB/Akt**

**Endogenous PKB activity in HEK293 and HEK293/Epac cells**

Endogenous PKB activities in HEK293 and HEK293/Epac with and without forskolin treatments were measured using Akt Kinase Assay kit from Cell Signaling following the manufacturer’s protocol.

|          | −Forskolin | +Forskolin | −Forskolin + H89 |
|----------|------------|------------|-----------------|
| HEK293   | 1.0±0.4    | 0.3±0.2    | 1.5±0.3         |
| HEK293/Epac | 1.0±0.6   | 3.4±0.9    | 5.9±1.0         |

*a* The basal PKB activity in the absence of forskolin treatment was set to 1.0 and PKB activities in the presence of treatments were normalized to the basal PKB level.

*b* The basal PKB activity in HEK293/Epac cells is about 40% higher than that in HEK293 cells.
Rap1N17 may be more remarkable. Taken together, these results suggest that Rap1 is downstream of Epac and that Rap1 activation is required for Epac-mediated PKB activation in HEK293/Epac cells.

Proper Subcellular Targeting of Epac Is Essential for Rap1 Activation and PKB Activation

To test if proper subcellular targeting of Epac is required for cAMP-mediated PKB activation and to further confirm that the stimulatory effect of cAMP on PKB phosphorylation observed in HEK293/Epac cells was indeed due to the presence of a functional Epac, we introduced into the HEK293 cells a deletion mutant, \( \Delta (1-148) \)Epac, whose first 148 amino acid residues had been removed. This Epac mutant is functionally inactive because it cannot be targeted to the correct subcellular locations where Epac exerts its function. As shown in Fig. 5A, like the control GFP protein, \( \Delta (1-148) \)Epac-GFP fusion protein was diffused across the whole cell volume while the full-length Epac showed a distinct perinuclear localization, indicating the N-terminal of the Epac is responsible for proper cellular targeting of Epac. When HEK293 cells stably expressing the \( \Delta (1-148) \)Epac mutant were treated with forskolin, instead of activating PKB as shown in HEK293/Epac cells, cAMP inhibited the phosphorylation of PKB at residues Ser\(^{473} \) in HEK293/\( \Delta (1-148) \)Epac cells. Pretreatment with H89 completely blocked the inhibitory effect of cAMP on PKB phosphorylation.

Proper Subcellular Targeting of Epac Is Essential for Rap1 Activation and PKB Activation—To test if proper subcellular targeting of Epac is required for cAMP-mediated PKB activation and to further confirm that the stimulatory effect of cAMP on PKB phosphorylation observed in HEK293/Epac cells was indeed due to the presence of a functional Epac, we introduced into the HEK293 cells a deletion mutant, \( \Delta (1-148) \)Epac, whose first 148 amino acid residues had been removed. This Epac mutant is functionally inactive because it cannot be targeted to the correct subcellular locations where Epac exerts its function. As shown in Fig. 5A, like the control GFP protein, \( \Delta (1-148) \)Epac-GFP fusion protein was diffused across the whole cell volume while the full-length Epac showed a distinct perinuclear localization, indicating the N-terminal of the Epac is responsible for proper cellular targeting of Epac. When HEK293 cells stably expressing the \( \Delta (1-148) \)Epac mutant were treated with forskolin, instead of activating PKB as shown in HEK293/Epac cells, cAMP inhibited the phosphorylation of PKB at residues Ser\(^{473} \) in HEK293/\( \Delta (1-148) \)Epac cells. Pretreatment with H89 completely blocked the inhibitory effect of cAMP on PKB phosphorylation.
presence or absence of pretreatment with 10 nM wortmannin (B). Cell lysates were subjected to Western blot analyses as described under "Experimental Procedures" using anti-phospho-Ser^{473}-specific (PKB-P473) PKB antibodies.

**FIG. 6. Epac-mediated PKB activation is PI3K dependent.** HEK293 cells stably transfected with Epac were serum-starved and stimulated with 10 μM forskolin or 1 mM sodium dibutyryl cAMP in the presence or absence of pretreatment with 10 μM LY294002 (A) or 100 nM wortmannin (B). Cell lysates were subjected to Western blot analyses as described under "Experimental Procedures" using anti-phospho-Ser^{473}-specific (PKB-P473) PKB antibodies.

cAMP, just as we observed in the parental HEK293 cells (Fig. 5B). Pull-down assays using glutathione S-transferase-Ral-GDS-Rap1-binding domain also showed that Δ(1–148)Epac was incapable of activating Rap1 in the presence and absence of stimulation (Fig. 5B). Since Δ(1–148)Epac possesses normal cAMP binding activity and its GEF activity can be tightly regulated by cAMP as the full-length Epac when tested *in vitro* with purified components (34), these data combined with our observations suggest deletion of the N-terminal targeting domain of the Epac prevents Δ(1–148)Epac from being targeted to its proper subcellular location where it activates the downstream target Rap1 that is necessary for PKB activation in response to increase intracellular cAMP concentration. These observations further demonstrated that Epac is responsible for mediating the stimulating effects of cAMP on PKB in HEK293/Epac cells and proper targeting of Epac is essential for the apparent cAMP-mediated activation of PKB in vivo.

**Epac-mediated PKB Activation Is Dependent on PI3K Activation**—As a downstream target of PI3K, the activation of PKB is dependent upon the presence of PI3K products in many cases (28, 29), although stimulation of PKB sometimes can also be achieved in a PI3K-independent manner (35, 36). To test if Epac-mediated PKB activation is dependent on PI3K activity, we examined the effect of LY294002, a specific PI3K inhibitor, on cAMP-mediated activation of PKB in Epac-transfected HEK293 cells. As can be seen in Fig. 6A, forskolin and dibutyryl cAMP-induced phosphorylation of Ser^{473} of PKB in HEK293 cells expressing full-length Epac was completely abolished by pretreatment of the cells with LY294002. Identical results were obtained with wortmannin (Fig. 6B). These observations indicate that Epac-mediated PKB activation by forskolin occurs by a PI3K-dependent mechanism.

**cAMP Activates PKB in WRT Cells That Express High Levels of Endogenous Epac**—One tissue that potentially expresses high levels of Epac naturally is the thyroid as it has been shown by Northern hybridization analysis that the mRNA level of Epac is highest among more than 20 human tissues tested (19). When total cell extract from Wistar rat thyroid (WRT) cells was analyzed by Western blot using affinity purified Epac antibodies, a single protein band was detected (Fig. 7A), suggesting Epac is expressed in WRT cells. In fact, the expression of Epac in WRT was quite high with a level similar to that of the HEK293/Epac stable cell line. Interestingly, unlike the predominant PKA-expressing HEK293 cells, WRT cells respond to the cAMP-elevating reagents including thyrotropin (TSH) in a manner similar to that of HEK293/Epac cells: cAMP modestly inhibited while Epac/cAMP-GEF may act as a positive modulator of PKB activity; however, elevation of intracellular cAMP concentration can lead to either inhibitory (45–47) or stimulatory (35, 36, 48) effects on PKB activity. At present, the role that Epac plays in this process is unclear.

Since cAMP can either inhibit or stimulate PKB activity, it is conceivable that Epac and PKA, acting as downstream receptors, may differentially mediate these effects, e.g. PKA inhibits PKB activation while Epac/cAMP-GEF may act as a positive modulator of PKB in response to cAMP. To test this hypothesis, we introduced Epac into HEK293 cells that showed minimal effects of cAMP-elevating agents on activation of PKB in parental HEK293 and Epac-expressing cells. When treated with forskolin, endogenous PKB activity in HEK293 cells was incapable of activating Rap1 in the presence and absence of stimulation (Fig. 5B). These observations further demonstrated that Epac is responsible for mediating the stimulating effects of cAMP on PKB in HEK293 cells and proper targeting of Epac is essential for the apparent cAMP-mediated activation of PKB in vivo. In addition to directly regulating many important cellular processes, cAMP influences an array of intracellular signaling pathways such as, the MAP kinase pathways (7, 11–14), cyclin-dependent kinase (37), Ca^{2+}-dependent signaling pathways (38, 39), and the Jak/STAT pathway (40). Recent studies indicate that cAMP-dependent signaling is also closely interwoven with the PI3K/PKB pathway (41, 42). The PI3K/PKB signaling pathway is a key component in control of cell survival and proliferation (43, 44). cAMP has been implicated in modulating PKB activity; however, elevation of intracellular cAMP concentration can lead to either inhibitory (45–47) or stimulatory (35, 36, 48) effects on PKB activity. At present, the role that Epac plays in this process is unclear.
PKB activation.

response to the common activator cAMP, mediate opposing effects on PKB regulation by cAMP in which Epac and PKA, in

implies that only a specific pool of Rap1 (cAMP-dependent) is

is only slightly elevated in 293/Epac cells (Fig. 1)

observed PKB activation since the basal levels of PKB activity

higher than normal Epac expression and slow GTPase activity

Epac cells. This basal activation of Rap1, probably due to

forskolin-independent, basal activation of Rap1 in HEK293/

induced PKB activation as transient expression of a dominant

Epac activates PKB through a Rap1-independent pathway, our

mediated either by a Rap1-independent or Rap1-dependent

On the other hand, our study establishes that cAMP can also

Rap1 is potentially an important mechanism for

in vivo Rap1 regulation. It has been previously reported that Ra-GEF2, an

other Rap-specific guanine nucleotide exchange factor that shares a homology GEF domain with Epac, can increase the plasma membrane fraction of Rap1GTP while the Rap1GTP level from the total cellular extract decreases (50).

Based on our observations, a model for cAMP-mediated PKB regulation is proposed. As shown in Fig. 8, the two intracellular cAMP receptors, Epac and PKA, mediate the opposing effects of cAMP on PKB activation. On the one hand, cAMP, acting through PKA, can inhibit PKB activation. A recent study by Kim et al. (47) showed that inhibition of PKB by PKA is serine/threonine phosphatase independent. It was further suggested that PKA inhibits PKB activity by blocking PI3K lipid kinase activity and consequently decoupling PKB from its upstream regulator, PDK, at the plasma membrane (47). The exact mechanism of PKA-mediated PI3K inhibition is not clear and currently under investigation. It is possible that PKA directly phosphorylates PI3K and consequently down-regulates its lipid kinase activity. It has also been proposed that PKA negatively regulates Rap1 activation through direct phosphorylation (51).

On the other hand, our study establishes that cAMP can also activate PKB through an Epac-mediated signaling pathway in a PI3K-dependent manner. Results of Epac-mediated activation of PKB expand our understanding of the cAMP-mediated signaling pathways. Our observations of opposing effects of Epac and PKA on PKB activation provide a potential mechanism for the apparent cell type-specific differential effects of cAMP. It is conceivable that the net outcomes of the cellular effects of cAMP may be dependent upon the dynamic expression of Epac and PKA and their specific subcellular distribution in a particular cell/tissue. Our results that forskolin activates PKB in WRT cells that naturally express high levels of Epac strongly support our conclusion that Epac activates while PKA inhibits PKB in response to cAMP and the final cellular effects of cAMP are integration of the Epac and PKA signaling pathways. Furthermore, our data suggest that the temporal effects of Epac and PKA activation on a specific pathway may provide an additional regulatory mechanism for intracellular cAMP signaling. Given the complex and important role that cAMP plays in regulating cell proliferation, differentiation, and survival, it is possible that other cAMP-mediated signaling pathways may also exist.

Acknowledgments—We thank Dr. Johannes L. Bos (University Medical Center Utrecht, The Netherlands) for providing the Epac cDNA and Mardelle Susman for help in manuscript preparation.

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