Cyclic AMP Inhibits Akt Activity by Blocking the Membrane Localization of PDK1*

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Akt is a protein serine/threonine kinase that plays an important role in the mitogenic responses of cells to variable stimuli. Akt contains a pleckstrin homology (PH) domain and is activated by phosphorylation at threonine 308 and serine 473. Binding of 3'-OH phosphorylated phosphoinositides to the PH domain results in the translocation of Akt to the plasma membrane where it is activated by upstream kinases such as (phosphoinositide-dependent kinase-1 (PDK1). Over-expression of constitutively active forms of Akt promotes cell proliferation and survival, and also stimulates p70 S6 kinase (p70S6K). In many cells, an increase in levels of intracellular cyclic AMP (cAMP) diminishes cell growth and promotes differentiation, and in certain conditions cAMP is even antagonistic to the effect of growth factors. Here, we show that cAMP has inhibitory effects on the phosphatidylinositol 3-kinase/PDK/Akt signaling pathway. cAMP potently inhibits phosphorylation at threonine 308 and serine 473 of Akt, which is required for the protein kinase activities of Akt. cAMP also negatively regulates PDK1 by inhibiting its translocation to the plasma membrane, despite not affecting its protein kinase activities. Furthermore, when we co-expressed myristoylated Akt and PDK1 mutants which constitutively co-localize in the plasma membrane, Akt activity was no longer sensitive to raised intracellular cAMP concentrations. Finally, cAMP was also found to inhibit the lipid kinase activity of PI3K and to decrease the levels of phosphatidylinositol 3,4,5-trisphosphate in vivo, which are required for the membrane localization of PI3K. Collectively, these data strongly support the theory that the cAMP-dependent signaling pathway inhibits Akt activity by blocking the coupling between Akt and its upstream regulators, PDK, in the plasma membrane.

The phosphatidylinositol 3-kinase (PI3K)1-dependent cell signaling pathway has emerged as a key regulatory pathway involved in a number of cellular events (1). Upon activation of growth factor tyrosine kinase receptors, the p85 regulatory subunit of PI3K recruits the p110 catalytic subunit to the plasma membrane (2). The p110 catalytic subunit increases the level of PtdIns-3,4,5-P3 or phosphatidylinositol 3,4-bisphosphate (PtdIns-3,4-P2), which induce the membrane translocation of PDK1 and Akt (also called PKB or RAC-PK) by binding to the pleckstrin homology domain (3). In the membrane, PDK1 phosphorylates and activates Akt in a PtdIns-3,4,5-P3- or PtdIns-3,4-P2-dependent manner (4, 5). By a mechanism that involves phospholipase C (6), activated Akt is released from the membrane and phosphorylates various targets.

This complex and unique signaling pathway has been implicated in a variety of cellular events such as cell proliferation and survival (1, 7). Previously, it has been shown that various survival factors, such as nerve growth factor, require the activation of PI3K to prevent various cell types from undergoing apoptosis (8, 9). The mechanism by which the PI3K pathway protects cells from programmed cell death has been studied intensively. Recently, it was shown that Akt can phosphorylate serine 136 of BAD, a member of the pro-apoptotic Bcl-2 family, forming a binding site for 14-3-3 (10, 11). As BAD binds 14-3-3, it can no longer bind to Bcl-2 and Bcl-XL to inhibit their prosurvival activity. Akt also phosphorylates other important cellular factors involved in apoptosis, such as caspase-9 (12) and fork-head transcription factors (13), which results in the inhibition of apoptosis.

Besides blocking apoptosis, the PI3K signaling pathway is involved in glycogen synthesis (14, 15), glucose transport (16), and protein synthesis (17). The activities of Akt especially are closely correlated with these important biological activities. For example, GSK-3β (glycogen synthase kinase-3β) is phosphorylated at serine 9, and its activities are down-modulated by Akt (14). Meanwhile, Tor (target of rapamycin) and p70S6K, which phosphorylate a translation initiation factor, eIF4E-binding protein (eIF4E-binding protein), and a ribosomal protein, S6, respectively, are positioned as downstream targets of Akt (18–21).

The paper is available online at http://www.jbc.org.

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1 The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; EGF, epidermal growth factor; PDK, phosphoinositide-dependent kinase; CAMP, cyclic AMP; Br, bromo; PKA, cAMP-dependent protein kinase; myrAkt, myristoylated Akt; p70S6K, p70 S6 kinase; PtdIns-3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns-3,4-P2, phosphatidylinositol 3,4-bisphosphate; HA, hemagglutinin; Jak/STAT, Janus kinase/signaling transducers and activators of transcription.
tor)-dependent activation of the cAMP/PKA pathway (25). There is also evidence that cross-talks between the Ca\(^{2+}\)- and cAMP-dependent pathways exist in the cytoplasm (26) and the nucleus (27). Also, it was demonstrated that cAMP inhibits the Jak/STAT pathway (28), which is important in cytokine signaling. cAMP-dependent protein kinase inhibits G\(_{i}\)-activated PLC\(_{\beta}\) activity by phosphorylating PLC\(_{\beta}\) at a serine residue in vivo (29). In addition, the Ras-mediated mitogen-activated protein kinase pathway is strongly inhibited by cAMP-dependent signaling in various mechanisms (30–33).

Recent findings suggest that there is also cross-talk between the PI3K pathway and the cAMP-dependent pathway. For example, an increased level of cAMP inhibits the interleukin-2-dependent activation of p70S6K (34). In addition, CREB (cAMP response element-binding protein), in which transcriptional activities are induced by phosphorylation at serine 133, is phosphorylated at the same serine residue by both PKA and Akt in vivo (35).

Here, we demonstrate that an increase in the level of intracellular cAMP inhibits the activities of Akt, PI3K, and their downstream target, p70S6K. Interestingly, PDK1 activity was not affected by cAMP treatments, but its plasma membrane localization was dramatically reduced. Taken together, these results support that the cAMP-dependent signaling pathway inhibits Akt through inhibition of PI3K lipid kinase activity and the subsequent inhibition of PDK1 localization at the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—Swiss 3T3, HEK293, COS, and Rat2 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) at 37 °C in a humidified atmosphere with 5% CO\(_2\). Transient transfection in COS cells was performed at 50% confluency by a DEAE-dextran method (Promega) according to the manufacturer’s instructions.

**Preparation of Cell Lysates**—Serum-starved cells were treated with various stimuli: 50 ng/ml EGF (Life Technologies, Inc.), 20 μM forskolin (Sigma or Calbiochem), 20 μM 1,9-dideoxyforskolin (Sigma), 100 ng/ml phorbol 12-myristate 13-acetate (Sigma), 10 μM calyculin A (Calbiochem), 1 μM 8-bromocAMP (8-Br-cAMP, Calbiochem), 1 μM 8-bromo-cGMP (8-Br-cGMP, Calbiochem). Stimulation was terminated by washing cells with ice-cold STE (consisting of 150 mM NaCl, 50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 10 mM MgCl\(_2\), 10 mM MnCl\(_2\), 1 mM dithiothreitol, and 0.2 mM EGTA), and 3 mM \(\text{Ca}^{2+}\). Then, lipids were extracted and separated as described previously by two-dimensional chromatography (TLC). Phosphorylated lipids were visualized by autoradiography.

**Confocal Microscopic Analyses of PDK1**—Rat2 cells were grown on coverslips and transfected with pEGFP-PDK1 by the LipofectAMINE method (Life Technologies, Inc.). Quiescent cells stimulated with the various stimuli were washed three times with cold phosphate-buffered saline and fixed in 3.7% formaldehyde for 40 min. Fixed cells were mounted on slide glasses with phosphate-buffered saline and observed with a laser-scanning confocal microscope (Carl Zeiss LSM 510).

**Immunoblot Analyses**—Cell lysates were boiled in SDS sample buffer for 5 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were incubated for 15 min in blocking solution (Tris-buffered saline containing 0.1% Tween 20, 2% bovine serum albumin, and 0.02% sodium azide) and further incubated with the appropriate primary antibody for 1 h. 12CA5 anti-HA monoclonal antibody was purchased from Roche Molecular Biochemicals. Phosphospecific Akt antibodies were obtained from New England Biolabs. The membranes were then washed with blocking solution and incubated for 30 min with either anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase. Bound antibodies were detected with the enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Detection of in Vivo PtdIns Levels**—Determination of PtdIns-3,4,5-P\(_3\) level in vivo was carried out as described (36, 37). Briefly, 1 × 10\(^6\) COS cells were plated and subjected to serum starvation for 16 h. To metabolically label cells, \(\text{32P}^-\text{PO}_4\) (obtained from New England Nuclear, 50 Ci/ml) was added, and the cells were incubated at 37 °C for 60 min. Excess \(\text{32P}^-\text{PO}_4\) was washed, and the appropriate stimuli were added. Then, lipids were extracted and separated as described previously (36, 37). Phosphorylated lipids were visualized and quantified by autoradiography.

**RESULTS**

**cAMP Inhibits the Kinase Activity of Akt in Vivo**—To understand how the cAMP-dependent cell signaling pathway modulates Akt, we examined the effects of forskolin, an activator of adenyl cyclase, on the protein kinase activities of Akt induced by a variety of agonists for cell proliferation including EGF, phorbol 12-myristate 13-acetate, calyculin A, and serum. We stimulated pCMV6-HA-Akt-transfected COS cells with various agonists followed by treatment with forskolin as indicated in Fig. 1A. The protein kinase activities of Akt were strongly stimulated by EGF and calyculin A, and weakly by serum as previously reported (19, 38, 39). Interestingly, the Akt activities induced by all of these agonists were strongly inhibited by treatment with forskolin (Fig. 1A). Thus, we decided to investigate further how cAMP-dependent signaling is involved in the regulation of Akt activities as well as the other signaling components downstream of PI3K.

To confirm whether cAMP was indeed responsible for inhibiting Akt activity following forskolin treatment, we treated cells with the cell-permeable cyclic nucleotide analogues 8-Br-cAMP and 8-Br-cGMP. As shown in Fig. 1B, 8-Br-cAMP, but not 8-Br-cGMP, specifically inhibited the EGF-induced Akt activity. Although forskolin or 8-Br-cAMP did not inhibit Akt activity as potently as wortmannin, they decreased it to less than 20% of the EGF-induced activity (Fig. 1, A and B). To
Inhibition of Coupling between Akt and PDK1 by cAMP

Recently it was reported that the activity of Akt is biphasically regulated in a time-dependent manner (40). Rittenhouse and colleagues (40) revealed that in platelets, the generation of PtdIns-3,4,5-P_3 leads to the first phase of activation of Akt, and subsequently the Ca^{2+}-dependent and wortmannin-sensitive accumulation of PtdIns-3,4-P_2 causes the second phase of activation of Akt. Therefore, we examined the effects of cAMP on Akt activities throughout the activation time course of the kinase. We stimulated pCMV6-HA-Akt-transfected COS cells with forskolin for 15 min prior to EGF stimulation. We also completed similar experiments with p70S6K-transfected COS cells. p70S6K is one of the best characterized downstream targets of Akt (19–21) and is negatively regulated by cAMP-dependent cell signaling in immune cells (34). The EGF-induced activities of Akt (Fig. 2A) as well as p70S6K (Fig. 2B) were strongly inhibited by forskolin throughout the time course of the activation. However, one interesting observation is that the early activation peak (2 and 5 min stimulation) of Akt is more resistant to forskolin than that of p70S6K (Fig. 2).

**cAMP Inhibits the Upstream Regulators of Akt—Immunoblot analyses**

Endogenous Akt was biphasically activated by EGF as described in the introduction. Akt must localize in the membrane to be phosphorylated and activated by PDK (3, 41). As described in Fig. 3, the phosphotransferase activities of Akt were strongly inhibited by forskolin for 15 min prior to EGF stimulation. We also completed similar experiments with p70S6K-transfected COS cells. p70S6K is one of the best characterized downstream targets of Akt (19–21) and is negatively regulated by cAMP-dependent cell signaling in immune cells (34). The EGF-induced activities of Akt (Fig. 2A) as well as p70S6K (Fig. 2B) were strongly inhibited by forskolin throughout the time course of the activation. However, one interesting observation is that the early activation peak (2 and 5 min stimulation) of Akt is more resistant to forskolin than that of p70S6K (Fig. 2).

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Next, we examined the possibility that forskolin might inhibit the mechanisms that activate Akt. The molecular mechanism for activation of Akt is well characterized at a molecular level. As described in the introduction, Akt must localize in the membrane to be phosphorylated and activated by PDK (3, 41). Also, several findings support the theory that Akt must be phosphorylated at threonine 308 by PDK1 and at serine 473 by yet unidentified PDK2 to be fully activated (4, 42, 43). To determine whether the mechanism of the inhibition of Akt by cAMP is related to the membrane localization of Akt, we examined the effect of cAMP on myristoylated Akt (myrAkt; kindly provided by Dr. R. A. Roth). Because myrAkt has a myristoylation signal at its N terminus, the protein is not only constitutively localized in the plasma membrane but also maintains a constitutively active status in the cell (compared with the A2 mutant, which has an impaired myristoylation signal) (20). Interestingly, the activity of myrAkt was strongly inhibited by forskolin (−20% activity of the untreated control; Fig. 4A). This implies that cAMP inactivates Akt by some other mechanism besides affecting its localization. Thus, we examined whether cAMP affects the two phosphorylation events required for Akt activation. We found that cAMP severely
impaired the phosphorylation of threonine 308 and serine 473 of Akt (Fig. 4B).

To examine whether the inhibition of Akt activity by cAMP is COS cell-specific, we completed experiments with different cell lines originated from various species, as shown in Fig. 5: Swiss 3T3 mouse fibroblast, COS monkey kidney cell, Rat2 fibroblast, and HEK293 human embryonic kidney cells. cAMP almost completely blocked the EGF-stimulated phosphorylation of endogenous Akt in all of the selected cell lines (Fig. 5). These results strongly suggested that components upstream of Akt must be affected by cAMP.

**cAMP Does Not Inhibit PDK1 Activity but Does Inhibit Its Membrane Localization**—We examined whether the kinase activity of PDK1, the immediate upstream kinase for Akt, is inhibited by cAMP. Myc-tagged PDK1 was transiently expressed in COS cells, and its phosphotransferase activities were measured. As previously reported by others (4, 43), the autophosphorylation and Akt threonine 308 phosphotransferase activities of PDK1 were highly active in quiescent cells and slightly (-10%) increased by EGF stimulation (Fig. 6, inset and upper panels, respectively). Interestingly, forskolin has no effect on the PDK1 activities (Fig. 6, inset and upper panels).

Upon mitogen stimulation, PI3K increases the levels of PtdIns-3,4,5-P3 and PtdIns-3,4-P2 that recruit Akt as well as PDK to the membrane (3). To determine whether cAMP affects the membrane localization of PDK1, we transiently expressed pEGFP-PDK1 in Rat2 cells and examined the changes in subcellular localization of the kinase. Surprisingly, the EGF-induced membrane localization of PDK1 was drastically reduced by treatments with wortmannin and forskolin (Fig. 7). This suggests that cAMP down-modulates Akt activities by blocking the membrane localization of PDK1 and the consequent coupling between PDK1 and Akt in the plasma membrane.

To confirm this inhibitory mechanism of cAMP on PDK1, we...
co-transfected pECE-HA-myrAkt and pBJ5-FLAG-myrPDK1 in COS cells. The transfected cells were treated with forskolin for 20 min, and the Akt activities were measured. As previously shown in Fig. 4A, forskolin treatments reduced myrAkt activity in cells expressing myrAkt alone (Fig. 8, compare lanes 2 and 3). However, forskolin could not inhibit the myrAkt activities from cells co-expressing myrPDK1 (Fig. 8, compare lanes 4 and 5). Consistent with this finding, the faster migrating (inactive and dephosphorylated) form of myrAkt was increased by forskolin treatment only in cells expressing myrAkt alone (Fig. 8, third panel). These results confirm that inhibition of the membrane localization of PDK is the molecular mechanism behind the inhibition of Akt by the cAMP-dependent pathway.

**cAMP Inhibits PI3K Lipid Kinase Activity and in Vivo Production of PtdIns-3,4,5-P3.** As the subcellular localization of PDK1 is regulated by the level of PtdIns-3,4,5-P3, we examined whether cAMP affects the lipid kinase activities of PI3K. We transiently transfected COS cells with pSRα-HA-p110, and treated them with EGF and either forskolin or wortmannin. EGF treatment strongly stimulated the PI3K activity (5.3-fold over unstimulated control) (Fig. 9A, compare lanes 1 and 4). Consistently, both forskolin and wortmannin inhibited the PI3K lipid kinase activities to below the unstimulated control level (Fig. 9A, lanes 2, 3, 5, and 6). To further confirm the effect of cAMP on PI3K, we examined the effect of forskolin on the production of PtdIns-3,4,5-P3 in vivo. Quiescent COS cells were labeled with 32PO4 and treated with forskolin for 15 min prior to EGF stimulation. In Fig. 9B, the levels of phosphatidylinositol monophosphates and PtdIns-3,4,5-P3 were decreased by cAMP and wortmannin. Therefore, we conclude that cAMP inhibits PI3K lipid kinase activity in vivo.
escent COS cells were labeled with $^{32}$PO$_4$ (top panel). The values in the quantified with phosphorimager analyses (middle panel) and quantified with phosphorimager analyses (top panel). Immunoblot analyses showed that similar protein amounts of p110 were used in the assays (bottom panel). The values in the top panel represent the mean of three independent cell preparations $\pm$ S.D. B, quiescent COS cells were labeled with $^{32}$PO$_4$, as described under “Experimental Procedures.” Cells were pretreated with forskolin (F) or wortmannin (wort) for 15 min and treated with EGF for 30 s, and then lipids were extracted and separated by TLC with phosphoinositide standards. Phosphorylated lipids were visualized by autoradiography (middle panel) and quantified with phosphorimager analyses (top panel). The values in the top panel represent the mean of three independent cell preparations $\pm$ S.D.  

DISCUSSION

We have demonstrated that cAMP down-modulates Akt activity by interfering with the membrane localization of PDK1 and inhibiting the lipid kinase activity of PI3K. When we observed that the kinase activity of myristoylated Akt was strongly inhibited by forskolin, we first suspected that PKA might directly phosphorylate Akt and consequently inhibit the phosphotransferase activity of Akt, as Akt has a putative PKA phosphorylation site in its catalytic domain. However, the catalytic subunit of PKA was unable to phosphorylate Akt in vitro, and an Akt mutant that lacks the putative PKA phosphorylation site was still strongly inhibited by forskolin in vivo (data not shown). Furthermore, the phosphorylations required for the activity of Akt were greatly diminished by forskolin treatment in vivo (Figs. 4B and 5). These results led us to study the effects of cAMP on PDK1 and PI3K, two known upstream regulators of Akt.

Previous studies have suggested that PDK1 is constitutively active and that its activity is regulated mainly by membrane localization via binding to PtdIns-3,4,5-P$_3$ (3, 4, 40). In support of this hypothesis, a PDK1 mutant containing an N-terminal myristoylation signal constitutively activates co-expressed Akt protein in vivo (3). These results suggest that the pleckstrin homology domain-mediated localization of PDK1 and Akt in the plasma membrane is critical for the functional coupling between the two protein kinases. Interestingly, our results strongly support the theory that cAMP signaling interferes with this process by inhibiting the lipid kinase activity of PI3K and the in vivo production of PtdIns-3,4,5-P$_3$ (Fig. 9, A and B, respectively). However, we do not currently understand how cAMP inhibits PI3K in the cell. Our preliminary data suggest at least that a direct phosphorylation of the p110 and p85 subunits of PI3K by PKA is not a mechanism behind the inhibition of the PI3K activity. We also examined the changes in tyrosine phosphorylation status of the p85 subunit of PI3K by cAMP but found that basal and EGF-stimulated tyrosine phosphorylation of the p85 protein was not significantly changed by cAMP under our experimental conditions. In addition, we found that cAMP does not affect the expression of PTEN. Thus, although we have excluded several possibilities for the mechanism of PI3K inhibition by cAMP, further studies are needed to elucidate this matter fully.

Although we consistently observed an inhibitory role of cAMP for Akt in various cultured cells (Fig. 5), Van Obberghen and colleagues (44) reported on the activation of Akt by PKA through a PI3K-independent pathway. However, not only was the activation of Akt by cAMP and PKA only minor, cAMP rather inhibited phosphorylation at serine 473 of Akt (44). This finding does not agree with studies by other groups demonstrating that phosphorylation at serine 473 of Akt is necessary for its activity (4, 42, 43). On the other hand, we demonstrated that the EGF-stimulated phosphorylation of endogenous Akt at both threonine 308 and serine 473 was inhibited to basal levels by forskolin treatments (Fig. 5). We also demonstrated the inhibition of PI3K activity (Fig. 9A), the in vivo production of PtdIns-3,4,5-P$_3$ (Fig. 9B), translocation of PDK1 to the plasma membrane (Fig. 7), and p70S6K activity (Fig. 2B) by cAMP. We believe these findings reflect the general nature of the inhibitory effect of cAMP on the PI3K pathway, as it would be illogical for cAMP to activate Akt while inhibiting its upstream regulators. Furthermore, our findings were consistent in a variety of cell lines. While we were revising this manuscript, others also revealed, in agreement with our data, that cAMP...
cannot induce the phosphorylations of Akt at threonine 308 and serine 473 and cannot activate Akt activity (45, 46). They also mentioned that cAMP decreases the activity of Akt (45). Therefore, we believe that we have demonstrated without a doubt the inhibition of Akt and other components of the PI3K pathway by cAMP under more relevant and natural experimental conditions. This conclusion is further supported by other groups’ results that cAMP indirectly inhibits p70S6K, a downstream target of Akt and PI3K, in vivo (34) and that cell-permeable cAMP fails to activate Akt in vivo (47).

Akt plays important roles in protecting cells from various apoptotic pressures. Recent studies have shown that Akt exerts its anti-apoptotic activities by phosphorylating important regulators for apoptosis such as BAD (10, 11), caspase-9 (12), and other groups’ results that cAMP indirectly inhibits p70S6K, a downstream target of Akt and PI3K, in vivo (34) and that cell-permeable cAMP fails to activate Akt in vivo (47).

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