Coordinate Regulation of Forskolin-induced Cellular Proliferation in Macrophages by Protein Kinase A/cAMP-response Element-binding Protein (CREB) and Epac1-Rap1 Signaling

EFFECTS OF SILENCING CREB GENE EXPRESSION ON Akt ACTIVATION*

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Uma K. Misra and Salvatore V. Pizzo

From the Department of Pathology, Duke University, Medical Center, Durham, North Carolina 27710

In this study, we have examined the role of two cAMP downstream effectors protein kinase A (PKA) and Epac, in forskolin-induced macrophage proliferation. Treatment of macrophages with forskolin enhanced [3H]thymidine uptake and increased cell number, and both were profoundly reduced by prior treatment of cells with H-89, a specific PKA inhibitor. Incubation of macrophages with forskolin triggered the activation of Akt, predominantly by phosphorylation of Ser-473, as measured by Western blotting and assay of its kinase activity. Akt activation was significantly inhibited by LY294002 and wortmannin, specific inhibitors of phosphatidylinositol 3-kinase, but not by H-89. Incubation of macrophages with forskolin also increased Epac1 and Rap1-GTP. Immunoprecipitation of Epac1 in forskolin-stimulated cells co-immunoprecipitated Rap1, p-AktThr-308, and p-AktSer-473. Silencing of CREB gene expression by RNA interference prior to forskolin treatment not only decreased CREB protein and its phosphorylation at Ser-133, but also phosphorylation of Akt at Ser-473, and Thr-308. Concomitantly, this treatment inhibited [3H]thymidine uptake and reduced forskolin-induced proliferation of macrophages. Forskolin treatment also inhibited activation of the apoptotic mechanism while promoting up-regulation of the anti-apoptotic pathway. We conclude that forskolin mediates cellular proliferation via cAMP-dependent activation of both PKA and Epac.

The binding of many hormones and growth factors to cells induces activation of adenyl cyclase, which catalyzes synthesis of cAMP from ATP (1–2). CAMP regulates a wide range of processes through its downstream effectors PKA, cyclic nucleotide-gated cation channels, and a small family of guanine nucleotide exchange factors (GEFs) involved in the regulation of Ras-related proteins (Refs. 3–5 and references therein). PKA-dependent pathways regulate cell proliferation and differentiation, microtubules dynamics, chromatin condensation and decondensation, nuclear envelop disassembly and reassembly, and exocytosis (3). The intracellular targeting and compartmentalization of PKA is controlled through association with A kinase-anchoring proteins (3). By binding to cyclic nucleotide-gated channels, cAMP mediates the transduction of olfactory and visual signals (3). Depending on the cell type, cAMP can either inhibit or stimulate cell growth and proliferation in a PKA-dependent and/or PKA-independent manner (4–6). For example, intracellular cAMP-elevating agents promote the G1 to S phase cell cycle transition in cells which include Swiss 3T3, hepatocytes, rat thyroid cells, bone cells, human prostate cancer cells, cardiac myocyte, and PC12 cells (4–6) while inhibiting the proliferation of cells, which include Rat1 and NIH3T3 adipocytes and endothelial cells (4–6). cAMP binds to the regulatory subunit of PKA, which causes dissociation of the catalytic subunit and its translocation to the nucleus, where it affects diverse cellular processes by reversible phosphorylation of enzymes and transcription factors (1, 2). The PKA-dependent effects of cAMP on cell growth inhibition are mediated via activation of Rap1, which binds Raf1 and prevents its activation by Ras and thus inhibits ERK activation. In cells, where cAMP stimulates cell proliferation it activates Rap1, which in turn activates B-Raf (5). This action of cAMP is independent of Ras and provides a pathway for ERK activation. Rap1 is activated rapidly in response to a variety of extracellular and intracellular second messengers, which in certain cases directly bind and activate specific Rap1/GEFs, which include C3G, cAMPGEFs, Cal-DAGGEF, and PDZGEF (3, 5). In certain cell types, stimulation of cell growth and proliferative effects of cAMP in a PKA-independent manner involve activation of Rap1 via Epac. In these cases, the cell-proliferative effects of cAMP mediated by activation of PI 3-kinase/Akt signaling (6, 8, 9). Many cytokines and growth factors promote integrin-dependent cell adhesion through activation of Rap1, and this activity of Rap1 is independent of Ras (5).

Epac directly mediates the effects of cAMP intracellularly (10, 11). This protein contains a catalytic domain, a domain that is responsible for its membrane association, and a cAMP binding domain (10, 11). cAMP is needed for activation of full-length Epac in vitro and deletion of the NH2-terminal region containing cAMP binding domain results in the constitutive activation of Epac (12, 13). cAMP binding to Epac leads to a conformational change that exposes the catalytic domain, and activated Epac in turn activates the downstream target Rap1 (14). Rap proteins are small GTPases of the Ras superfamily, which function as molecular “switches” cycling between an inactive GDP and active GTP forms (15). Rap1 functions as an antagonist of Ras signaling by trapping the Ras effector Raf1 in an inactive complex (14). Rap1 also regulates several important cellular processes independent of Ras such as inte-
grin-mediated cell adhesion (14–16). Mice overexpressing active Rap1 demonstrate increased integrin-mediated cell adhesion to fibronectin through Epac and Rap1 (17), which was abolished by Rap1-GTPase-activating proteins (18). Rap1 mutations disrupt morphogenesis in the eye, ovary, and wings of *Drosophila* embryos (19, 20).

The CREB family regulates responses to growth factors, hormones, and agents, which directly elevate cAMP such as forskolin by binding to cAMP response elements (CRE) (1–2). This DNA sequence is recognized by a diverse family of DNA binding proteins of which the basic leucine zipper transcription factor CREB is best characterized. The activation of CREB is crucially dependent on phosphorylation of Ser-133 by kinases, including PKA, Akt, and PDK1 (1, 2). cAMP agonists protect normal and transformed cells from apoptosis by regulating components of the pro- and anti-apoptotic pathway. Bcl-2 and related cytoplasmic proteins are key regulators of apoptosis (21–23). Bad is a pro-apoptotic protein that neutralizes the anti-apoptotic effects of Bcl-2/Bcl-xl (24,25). Bad has three phosphorylation sites Ser-112, Ser-136, and Ser-15. PKA phosphorylates Bad at Ser-155, whereas Akt phosphorylates Bad at Ser-136 and PAK-2 phosphorylates Bad at Ser-112 (26).

Macrophages are present throughout the body, functioning in innate immune surveillance and host defense mechanisms directed against pathogens. In response to stimuli, macrophages undergo a series of processes, including chemotaxis, phagocytosis, intracellular microbial killing, and release of inflammatory cytokines. The CAMP-Epac-1-Rap1 pathway regulates reorganization of the actin cytoskeleton and the resulting morphological changes are important for many cellular processes, including cell migration and adhesion (14, 16–18). Cell-cell interactions are often accompanied by cell spreading that increases the surface contact area between the cells. cAMP induces integrin-mediated cell adhesion through Epac1-Rap1 signaling pathway. In this study, we have examined the effect of elevating intracellular cAMP levels on primary macrophage proliferation by both Epac-1-Rap1 and PKA-CREB signaling pathways. In these studies, we have used forskolin, an adenylyl cyclase activator and a prototype for elevating cAMP levels, for raising intracellular cAMP levels. We show here that in murine peritoneal macrophages, both these pathways regulate forskolin-induced DNA synthesis and cellular proliferation. Furthermore, studies employing the silencing of CREB gene expression indicate that both these pathways exhibit cross-talk at the level of Akt activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Culture media were purchased from Invitrogen. Forskolin, H-89, wortmannin, and LY294002 were purchased from Biomol (Plymouth, PA). Antibodies against CREB, CREB phosphorylated at Ser-133, Akt, Akt phosphorylated at Thr-308 or Ser-473, FOXO1, FOXO1 phosphorylated at Ser-256, GSK3β, GSK3β phosphorylated at Ser-9, p27Kip1, Bcl-2, Bad, Bad phosphorylated at Ser-155 or Ser-136, XIAP, procaspase-3, cleaved caspase-3, procaspase-9, cyclin D1, XIAP, and integrin-linked kinase (ILK) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against the NH2 terminus SH2 domain of the dissociated p85 regulatory subunit of PI 3-kinase, Rap1, and RalGDS-RBD-agarose were purchased from Upstate, Cell Signaling Solutions. (Charlottesville, VA). [3H]Thymidine (specific activity, 71.5 Ci/mmol) was from American Radiochemicals Inc. (St. Louis, MO). [γ-32P]ATP (specific activity, 3000 Ci/mmol) was from PerkinElmer Life Sciences. Peptide substrates for AktSer-473 kinase, NH2–RPPHF-PQFSSYA-COOH, and for AktThr-308 kinase, NH2–KTCGTPPEYLAPE-VRR-COOH (27), were synthesized by Genemed, San Francisco, CA. The control substrate peptide, Zak3tide, was purchased from Upstate Cell Signaling Solutions. Epac1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other reagents of the highest available grade were procured locally.

**Cell Culture**—The use of mice for these studies was approved by the Institutional Animal Use Committee in accordance with relevant federal regulations. Thioglycollate-elicited peritoneal macrophages were obtained from pathogen-free 6 weeks old C57BL/6 mice (NIC, National Institutes of Health) in Hanks’ balanced salt solution containing 10 mM HEPES (pH 7.4) and 3.5 mM NaHCO3 (HHBSS). The cells were washed with HHBSS and suspended in RPMI 1640 medium containing 2 mM glutamine, penicillin (12.5 units/ml), streptomycin (6 μg/ml), and 5% fetal bovine serum; placed in 6-well plates (3 × 105 cells/well); and incubated for 2 h at 37 °C in a humidified CO2 (5%) incubator. The monolayers were washed with HHBSS three times to remove nonadherent cells, which were then incubated overnight at 37 °C in the above RPMI medium before study.

**Measurement of [3H]Thymidine Uptake by Macrophages Exposed to Varying Concentrations of Forskolin**—To assess DNA synthesis in macrophages exposed to forskolin, [3H]thymidine uptake was determined as previously described (28, 29). Briefly, murine peritoneal macrophages (4 × 105 well/cells) in 48-well plates, harvested as above, were allowed to adhere for 2 h in RPMI 1640 medium containing 0.2% fatty acid-free bovine serum albumin, penicillin, streptomycin, and glutamine at 37 °C in a humidified CO2 (5%) incubator. The monolayers were washed twice with HHBSS and a volume of RPMI medium added, followed by the addition of [3H]thymidine (2 μCi/ml), and varying concentrations of forskolin (0–40 μM) to respective wells. The cells were incubated overnight as above. Where the effects of inhibiting PKA activity with H-89 on [3H]thymidine uptake was studied, the cells were pre-incubated with H-89 (10 μM/90 min) before adding forskolin (10 μM), and cells were incubated as above. The incubations were terminated by aspirating the medium and washing the monolayers twice first with chilled 5% trichloroacetic acid (15 min each) and then three times with chilled phosphate-buffered saline. The monolayers were lysed with 1 N NaOH, and an aliquot was used for liquid scintillation counting and protein estimation.

**Determination of Macrophage Cell Number after Stimulation with Varying Concentrations of Forskolin**—Macrophage proliferation was determined by counting the numbers of macrophages 24 h after exposing them to varying concentrations of forskolin (0–40 μM) essentially as described (28, 30). Briefly, peritoneal macrophages were harvested and allowed to adhere in 6-well plates for 2 h as described above. The adhered cells were carefully scraped off the wells, centrifuged at 1200 rpm for 5 min, and suspended in RPMI 1640 medium containing 0.2% fatty acid-free bovine serum albumin. Aliquots containing 2 × 105 cells were pipetted in duplicate into 1.5-ml siliconized polypropylene tubes followed by the addition of varying concentrations of forskolin (0–40 μM) to the respective tubes. After overnight incubation, 10 μl of trypsin blue solution was added to each tube, the tubes were gently shaken during incubation for 2 min, and a 10-μl aliquot was used for counting the number of cells in a hemocytometer. In experiments where the effect of the PKA inhibitor H-89 was studied on forskolin-induced cellular proliferation, it was added to cells (10 μM/90 min) prior to the addition of forskolin.

**Measurement of PKA Activation by Phosphorylation of CREB at Ser133 in Cells Exposed to Forskolin by Western Blotting**—Macrophages incubated overnight in 6-well plates (3 × 105 cells/well) were stimulated with either buffer or forskolin (10 μM) and incubated for varying periods of time as above. The reactions were terminated by aspirating the medium. The lysis of cells, their electrophoresis, and Western blotting were performed according the manufacturer’s instructions. In each
case, an equal amount of protein was used for electrophoresis (31). The detection and quantification of CREB, phosphorylated at Ser-133 was performed by ECF and phosphorimaging employing a Storm 860 PhosphorImager™ (Amersham Biosciences). The membranes were reprobed for CREB and actin as a protein-loading control, according to the manufacturer’s instructions.

Western Blotting for Epac1 and ILK in Cells Exposed to Forskolin—Macrophages incubated overnight in 6-well plates (3 \( \times 10^6 \) cells/well) were stimulated with either buffer or forskolin (10 \( \mu M \)) and incubated for varying periods of time as above. The reactions were terminated by aspirating the medium. The lysis of cells, their electrophoresis, and Western blotting were performed according the manufacturer’s instructions. In each case, an equal amount of protein was used for electrophoresis. The detection and quantification of Epac1 and ILK was performed by ECF and phosphorimaging. The membranes were reprobed for actin as a protein-loading control.

Determination of Rap1 Activation—Macrophages incubated overnight in 6-well plates (3 \( \times 10^6 \) cells/well) were washed with HHBSS and a volume of RPMI 1640 medium containing 5% fetal bovine serum, glutamine, and antibiotics added (13). To duplicate monolayers was a volume of RPMI 1640 medium containing 5% fetal bovine serum, 10 mM Tris-HCl (pH 7.4) supplemented with 1 mM dithiothreitol, 1 mM PMSF, and 1 mM benzamidine by centrifugation at 8000 \( \times g \) for 5 min at 4 °C. To each immunoprecipitate, 40 \( \mu l \) of cold kinase buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl\(_2\), 1 mM dithiothreitol, 1 mM PMSF, 1 mM benzamidine, and 10 \( \mu g/ml \) leupeptin was added followed by the addition of 30 \( \mu M \) p-Akt\(_{Thr-308}^\) kinase substrate peptide (NH\(_2\)-KTF-GGTPEYLPVRR-COOH) or p-Akt\(_{Ser-473}^\) kinase substrate peptide (NH\(_2\)-RRPHFPQSYSYA-COOH) in the respective tubes (27). The peptide NH\(_2\)-GGGEYYGELVKKK-COOH (Zak3tide) was used as the control peptide. The reaction was initiated by adding 50 \( \mu M \) ATP and 2 \( \mu Ci \) of \([\gamma-32P]ATP\) in each tube, and the tubes were incubated for 30 min at 30°C with shaking. The reaction was stopped by adding 5 \( \mu l \) of 0.5 M EDTA to each tube, the tubes centrifuged at 3000 rpm for 3 min, 40 \( \mu l \) of each supernatant applied on p81 phosphocellulose paper (Whatman, NJ), allowed to dry, and the papers were washed four times each time by immersing them in a liter of 1 N phosphoric acid for 3 min. The papers were rinsed with acetone, and their radioactivity was counted in a liquid scintillation counter.

Measurement of the Inhibition of PI 3-Kinase, p-Akt\(_{Thr-308}^\), and p-Akt\(_{Ser-473}^\) Activation by Wortmannin and LY294002 in Cells Stimulated with Forskolin by Western Blotting—To macrophages incubated overnight in 6-well plates (3 \( \times 10^6 \) cells/well) were added varying concentrations of wortmannin (0–60 nM/30 min) or LY294002 (0–40 \( \mu M \)/20 min) prior to stimulation and incubation with forskolin (10 \( \mu M \)/15 min) as above. The reaction was terminated by aspirating the medium. The lysis of cells, their electrophoresis and immunoblotting with antibodies specific for SH2 domain of the dissociated regulatory subunit of PI 3-kinase, and Akt phosphorylated at Thr-308 or Ser-473 were performed according to manufacturer’s instructions. In each case, an equal amount of protein was used for electrophoresis. The detection and quantification of immunoblots was performed by ECF and phosphorimaging. The membranes were reprobed for actin as the protein-loading controls, according to the manufacturer’s instructions.

Determination of Akt\(_{Thr-308}^\) and Akt\(_{Ser-473}^\) Kinase Activities in Cells Stimulated with Forskolin—The p-Akt\(_{Thr-308}^\) and p-Akt\(_{Ser-473}^\) kinase activities in the Akt immunoprecipitates of macrophages stimulated with forskolin was assayed essentially according to Hill and Hemmings (32). Briefly, macrophages incubated overnight in two 6-well plates (4 \( \times 10^6 \) cells/well) were washed in HHBSS twice, and a volume of RPMI 1640 medium containing glutamine, fetal bovine serum, and antibiotics was added to each well. The monolayers were stimulated with buffer or forskolin (10 \( \mu M \)/15 min) in triplicate. The reactions were stopped by aspirating the medium, a volume of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 1% (v/v) Nonidet P-40, 25 mM sodium fluoride, 0.1 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 1 mM benzamidine, and leupeptin (20 \( \mu g/ml \)) was added. The cells were lysed for 10 min over ice, scrapped into tubes, and centrifuged at 8000 \( \times g \) for 10 min at 4 °C, and their protein contents were determined (31). Equal amounts of lysate proteins were immunoprecipitated with Akt antibodies (1:50) at 4 °C overnight with gentle rotation. Akt immunoprecipitates were washed with: 1) lysis buffer supplemented with 0.5 M NaCl, 2) lysis buffer, and 3) 50 mM Tris-HCl (pH 7.4) supplemented with 1 mM dithiothreitol, 1 mM PMSF, and 1 mM benzamidine by centrifugation at 8000 \( \times g \) for 5 min at 4 °C. To each immunoprecipitate, 40 \( \mu l \) of cold kinase buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl\(_2\), 1 mM dithiothreitol, 1 mM PMSF, 1 mM benzamidine, and 20 \( \mu g/ml \) leupeptin was added followed by the addition of 30 \( \mu M \) p-Akt\(_{Thr-308}^\) kinase substrate peptide (NH\(_2\)-KTF-GGTPEYLPVRR-COOH) or p-Akt\(_{Ser-473}^\) kinase substrate peptide (NH\(_2\)-RRPHFPQSYSYA-COOH) in the respective tubes (27). The peptide NH\(_2\)-GGGEYYGELVKKK-COOH was used as the control peptide. The reaction was initiated by adding 50 \( \mu M \) ATP and 2 \( \mu Ci \) of \([\gamma-32P]ATP\) in each tube, and the tubes were incubated for 30 min at 30°C with shaking. The reaction was stopped by adding 5 \( \mu l \) of 0.5 M EDTA to each tube, the tubes centrifuged at 3000 rpm for 3 min, 40 \( \mu l \) of each supernatant applied on p81 phosphocellulose paper (Whatman, NJ), allowed to dry, and the papers were washed four times each time by immersing them in a liter of 1 N phosphoric acid for 3 min. The papers were rinsed with acetone, and their radioactivity was counted in a liquid scintillation counter.

Immunoprecipitation of Epac-1 to Determine Its Association with Rap1, ILK, p-Akt\(_{Thr-308}^\), and p-Akt\(_{Ser-473}^\) in Forskolin-treated Macrophages by Western Blotting—Macrophages incubated overnight, 3 \( \times 10^6 \) cells/well in 6-well plates, were stimulated with buffer or forskolin
(10 μM/15 min) as above. The reaction was stopped by aspirating the medium and adding a volume of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 1% (v/v) Nonidet P-40, 20 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 1 mM PMF, 1 mM benzamidine, and leupeptin (20 μg/ml) and cells lysed over ice for 15 min. The lysates were collected in Eppendorf tubes, DNA strands broken by passing the lysates through 27-gauge needle several times and centrifuged at 1200 rpm at 4 °C. The protein content was determined (31) on supernatants and equal amounts of lysate protein were immunoprecipitated with Epac1 (1:50) as described above. The Epac1 immunoprecipitates were washed with lysis buffer thrice by centrifugation (3000 rpm/5 min each time), a volume of sample buffer was added, and samples heated at 90 °C for 5 min. The samples were centrifuged at 4000 rpm for 2 min, and supernatant was electrophoresed, transferred to membranes, and immunoblotted with antibodies against Epac1, p-AktThr-308, and p-AktSer-473, respectively. The visualization of protein bands on the membrane was performed by ECF and phosphorimaging as described above.

In separate identical experiments, the co-immunoprecipitation of Epac1, Rap1, PKA-CREB, and Epac1-Rap1 with immunoprecipitates of Rap1, ILK, p-AktThr-308, and p-AktSer-473 was also performed, and each immunoprecipitate was probed for Epac1, Rap1, ILK, p-AktThr-308 and p-AktSer-473, respectively, as described above.

**Determination of Pro-caspase-3, Caspase-3, and Pro-caspase-9 in Cells Stimulated with Forskolin by Western Blotting**—Experimental details of cell incubation, forskolin stimulation, cell lysis, electrophoresis, and transfer of gel bands to membranes were as described above. The membranes were immunoblotted with polyclonal antibodies against procaspase-3, cleaved caspase-3, and procaspase-9, respectively. The protein bands on the immunoblots were visualized and quantitated by ECF and phosphorimaging. The membranes were reprobed for actin, Bad, and FoxO1, respectively, according to the manufacturer’s instructions.

**Effect of the PKA Inhibitor H-89 and the PI 3-Kinase Inhibitor LY294002 on Phosphorylation of Akt in Macrophages**—The effects of inhibiting Akt and CREB activation on the anti-apoptotic proteins, Bcl-2, p-BadSer-155, p-BadSer-136, p-FOXO1Ser-256, and XIAP Anti-apoptotic Signaling Molecules in Cells Stimulated with Forskolin by Western Blotting—Experimental details of cell incubation, forskolin stimulation, cell lysis, electrophoresis, and transfer of gel bands to membranes were as described above. The membranes were immunoblotted with polyclonal antibodies against Bcl-2, p-BadSer155, p-BadSer-136, p-FOXO1Ser-256, and XIAP, respectively. The protein bands on the immunoblots were visualized and quantified by ECF and phosphorimaging. The membranes were reprobed for actin, Bad, and FOXO1, respectively, according to the manufacturer’s instructions.

**Effect of the PKA Inhibitor H-89 and the PI 3-Kinase Inhibitor LY294002 on Phosphorylation of Akt in Macrophages**—The effects of inhibiting Akt and CREB activation on the anti-apoptotic proteins, Bcl-2, p-BadSer-155, p-BadSer-136, p-FOXO1, and p-GSK3β was studied by incubating cells with the PKA inhibitor H-89 and the PI 3-kinase inhibitor LY294002. These were added in separate wells before stimulating them with forskolin (10 μM/15 min). The results show that in macrophages the cAMP-elevating agent forskolin functions as a mitogen and enhances [3H]thymidine uptake and cellular proliferation via PKA-dependent mechanisms. However, the contributions of PKA-independent signaling in these processes are not eliminated.

**Forskolin Induces Activation of RAP1**—An increase in intracellular cAMP levels will activate both PKA and Epac and discreet signaling pathways mediated by them. cAMP binding to Epac1 activates Rap1 by converting it from its GDP-associated to a GTP-associated form. In the next series of experiments, we examined the effects of elevating cAMP in macrophages by forskolin on RAP1 activation by assaying RAP1-GTP (Fig. 2). Forskolin treatment of macrophages up-regulated Epac1, which was somewhat dependent upon the concentration (Fig. 2A) and time of incubation (Fig. 2B) with forskolin. The increase in EPAC1 as determined from four separate studies was 3.9 ± 0.2-fold. Forskolin (10 μM/15 min) time of incubation (Fig. 2C) was optimal at 10–20 min of incubation after which the levels declined (Fig. 1C). To analyze the role of PKA in forskolin-induced cellular proliferation and DNA synthesis, we employed a specific inhibitor of PKA, H-89, and we silenced the expression of the PKA effector CREB by RNAi. Incubation of cells with H-89 before forskolin addition, significantly reduced macrophage number (Fig. 1A) and [3H]thymidine uptake (Fig. 1D). Silencing CREB gene expression by RNAi significantly reduced [3H]thymidine uptake (Fig. 1E). The results show that in macrophages the cAMP-elevating agent forskolin functions as a mitogen and enhances [3H]thymidine uptake and cellular proliferation via PKA-dependent mechanisms. However, the contributions of PKA-independent signaling in these processes are not eliminated.

**RESULTS**

**Forskolin Stimulates Macrophage Proliferation, [3H]Thymidine Uptake, and Phosphorylation of CREB at Ser-133**—Forskolin treatment increased macrophage number in a dose-dependent manner, and this proliferative effect was optimal at ~10–20 μM of forskolin (Fig. 1A). Likewise, forskolin increased [3H]thymidine uptake by macrophages, which was optimal at ~20 μM of forskolin (Fig. 1B). Treatment of macrophages with forskolin (10 μM) maximally increased the levels of p-CREBSer-133, p-AktThr-308, and XIAP activation at ~10–20 min of incubation after which the levels declined (Fig. 1C). To analyze the role of PKA in forskolin-induced cellular proliferation and DNA synthesis, we employed a specific inhibitor of PKA, H-89, and we silenced the expression of the PKA effector CREB by RNAi. Incubation of cells with H-89 before forskolin addition, significantly reduced macrophage number (Fig. 1A) and [3H]thymidine uptake (Fig. 1D). Silencing CREB gene expression by RNAi significantly reduced [3H]thymidine uptake (Fig. 1E). The results show that in macrophages the cAMP-elevating agent forskolin functions as a mitogen and enhances [3H]thymidine uptake and cellular proliferation via PKA-dependent mechanisms. However, the contributions of PKA-independent signaling in these processes are not eliminated.

**Forskolin Treatment and Phosphorylation of Akt at Thr-308 and Ser-473 in Macrophages**—The PKA-dependent effects of cAMP on cell growth inhibition are mediated via activation of Rap1, which binds Raf1 and prevents its activation by Ras and thus inhibits ERK activation (7). In cells, where cAMP stimulates cell proliferation, it activates Rap1, which in turn activates B-Raf. This action of cAMP is independent of Ras and thus inhibits ERK activation. In certain cell types, stimulation of cell growth and proliferative effects of cAMP in a PKA-independent manner involves activation of Rap1 via Epac. In these cases, the cell-
proliferative effects of cAMP are mediated by activation of PI 3-kinase/Akt signaling (6, 8, 9). The effects of cAMP on activation of PI 3-kinase signaling correlate with its mitogenic activity (8, 9). In cells where it is mitogenic, it activates PI 3-kinase signaling, and in cells where it inhibits cell growth, it also inhibits PI 3-kinase activation (8, 9). Dysregulation of Akt activity is implicated in the development of a number of diseases, including cancers and diabetes (26, 34, 35). Activation of Akt is dependent upon PI 3-kinase activation, which results upon tyrosine kinase receptor activation consequent to growth factor/mitogen binding (26). Akt is activated by phosphorylation at two residues, Thr-308 in the activation loop and Ser-473 in the hydrophobic motif of the COOH-terminal tail. Although 3-phosphatidylinositol-dependent kinase 1 (PDK1) phosphorylates Akt at Thr-308, identification of the kinase responsible for phosphorylating Akt at Ser-473 is disputed. Mitogen-activated protein kinase-activated kinase-2, integrin linked-kinase (ILK), PKA, PDK1, DNA-dependent protein kinase, and Akt itself have all been implicated (27, 36, 37). Phosphorylation of Akt at both residues is required for its full activation (26, 36, 37). In the present study, we have examined the effect of forskolin treatment of macrophages on the activation of PI 3-kinase and Akt as a result of its phosphorylation at Thr-308 or Ser-473 by Western blotting (Fig. 3, A and B). Forskolin treatment activated PI 3-kinase by 5 min, which was maximal between 10 and 20 min and showed a decline by 60 min of incubation. The kinetics of phosphorylation of Akt at Ser-473 was similar to that of PI 3-kinase activation. The activation of Akt as measured by Western blotting of Akt phosphorylated at Ser-473 was observed at 5 min, and it was maximal at 10–20 min, but rapidly declined at longer periods of incu-
H-89 a specific inhibitor of PKA (Fig. 5). H-89 showed no inhibition of PI 3-kinase, p-Akt\(^{\text{Thr-308}}\), or p-Akt\(^{\text{Ser-473}}\) at any concentration employed. In fact, at higher concentrations of H-89 (20–40 \(\mu\)M) elevated formation of p-Akt\(^{\text{Ser-473}}\) was observed indicating a relief of inhibition on Akt phosphorylation by PKA. Similar observations were reported by Mei et al. (13). In contrast, about a 50% inhibition of the phosphorylation of the transcription factor CREB at Ser-133 occurred at 10 \(\mu\)M of H-89, which was further reduced on increasing the concentration of H-89 (Fig. 5).

**Forskolin Activates Akt Kinase Activity in Macrophages**—Next we determined the p-Akt\(^{\text{Thr-308}}\) and p-Akt\(^{\text{Ser-473}}\) activities of cells stimulated with forskolin using specific substrates for p-Akt\(^{\text{Ser-473}}\) and p-Akt\(^{\text{Thr-308}}\) kinases, respectively (Fig. 6). The results corroborate the Western blotting data (Fig. 3) and further show that indeed forskolin is a strong activator of p-Akt\(^{\text{Ser-473}}\) kinase in macrophages. Similar observations in cell types where cAMP is mitogenic have been noted on strong activation of Akt\(^{\text{Ser-473}}\) phosphorylation by cAMP-elevating agents (13).

p-Akt\(^{\text{Thr-308}}\) and p-Akt\(^{\text{Ser-473}}\) Co-immunoprecipitate with Epac1 in Macrophages Stimulated with Forskolin—cAMP can also activate Rap1 in a PKA-independent manner via cAMP-binding proteins that contain Rap1-GEF domains. These proteins, called Epacs (exchange protein directly activated by cAMP), which are widely expressed in brain, pituitary, adrenal, and liver, are targetted to membranes via DEP domains. Epacs are found within perinuclear regions, nuclear membranes, and mitochondria. Epacs may be involved in cAMP-induced Akt activation, which requires its translocation from the cytosol to membranes. The membrane attachment of Akt brings about conformational change as well as proximity to the kinases involved in its phosphorylation at Thr-308 and Ser-473 (26). After phosphorylation, Akt dissociates from the membranes and translocates to nuclei (39). We hypothesized that, if Epac1 plays a membrane targeting role for Akt activation, then the p-Akt\(^{\text{Thr-308}}\) and p-Akt\(^{\text{Ser-473}}\) might be associated with Epac1 and if so then they would co-immunoprecipitate with Epac1 in macrophages stimulated with forskolin. Both p-Akt\(^{\text{Thr-308}}\) and p-Akt\(^{\text{Ser-473}}\) co-immunoprecipitated with Epac1 (Fig. 7, A–E). Because activated Akt species co-immunoprecipitated with Epac1, these results suggest a role of Epac1 in membrane targeting of activated Akt in forskolin-stimulated cells. The DEP domain of Epac is responsible for membrane association, and Epac is mainly localized to the perinuclear region (13). Interestingly, ILK also co-immunoprecipitated with Epac1 (Fig. 7, A–E), and forskolin treatment up-regulated the levels of ILK protein by ~2-fold in macrophages (Fig. 7F). Interestingly, reverse immunoprecipitation experiments demonstrated that Epac1, Rap1, ILK, p-Akt\(^{\text{Thr-308}}\), and p-Akt\(^{\text{Ser-473}}\) co-immunoprecipitate with each other forskolin-stimulated cells (Fig. 7, A–E). These results suggest the possibility that Epac1-Rap1 associates with ILK, p-Akt\(^{\text{Thr-308}}\), and p-Akt\(^{\text{Ser-473}}\) to form signaling complexes and possibly are involved in targeting them to their site of action.

**Silencing of CREB Gene Expression Inhibits Forskolin-induced Enhanced Activation of PI 3-Kinase/Akt Signaling**—In the bas al state, PKA resides in the cytoplasm function as an inactive heterodimer of paired regulatory (R) and catalytic (C) subunits. Induction of cAMP synthesis liberates the C subunit, which diffuses into the nucleus and induces cellular gene expression by phosphorylating the transcription factor CREB at Ser-133 (1, 2). In the next series of experiments, we studied the effect of silencing CREB gene expression by RNAi on forskolin-induced mitogenesis and cellular proliferation (Fig. 1) and activation of PI 3-kinase/Akt signaling in macrophages. In preliminary experiments, we determined the concentration of CREB dsRNA that would give the maximal silencing of CREB gene expression as determined by

![FIGURE 3. Effect of time of incubation on up-regulation of the PI 3-kinase signaling cascade in forskolin-stimulated macrophages. See “Experimental Procedures” section for details. A, bar diagram showing changes in the 85-kDa regulatory subunit of PI 3-kinase (dark gray), p-Akt\(^{\text{Ser-473}}\) (white), p-Akt\(^{\text{Thr-308}}\) (black), and ILK (light gray) in cell stimulated with forskolin (10 \(\mu\)M) for 0–60 min. The changes are expressed in arbitrary units and are mean ± S.E. from four independent experiments. B, corresponding representative immunoblots of the 85-kDa regulatory subunit of PI 3-kinase (designated PI 3-K), p-Akt\(^{\text{Thr-308}}\), and p-Akt\(^{\text{Ser-473}}\). Only one immunoblot of actin, the protein-loading control is shown, although it was performed for all the immunoblots shown.](image-url)
PKA-CREB and Epac1-Rap1-GTP Cross-talk at Akt

measuring the levels of CREB protein by Western blotting. Under our experimental conditions the maximal silencing of CREB gene expression as determined by the analysis of protein levels of CREB by Western blotting was achieved at 25 μg of CREB dsRNA (between 60 and 70%, Fig. 8, A and C), and therefore we have used this concentration of CREB dsRNA in all our experiments. These results were corroborated by the reduced mRNA levels of CREB under these conditions (Fig. 8B). In Fig. 1 we have shown that silencing of CREB gene expression significantly reduced forskolin-induced increased [3H]thymidine uptake and cellular proliferation. Because agonist-induced PI 3-kinase/Akt signaling is crucial for cellular proliferation, and because inhibition of PKA activity with its inhibitor, H-89, profoundly inhibited forskolin-induced cellular proliferation of macrophages, we next determined the effect of silencing CREB gene expression on PI 3-kinase and Akt activation by Western blotting (Fig. 8D). Silencing of the CREB gene significantly decreased PI 3-kinase activation (Fig. 8D), p-AktThr-308 (Fig. 8D), and p-AktSer-473 (Fig. 8D) compared with forskolin-stimulated cells. Treatment of cells with scrambled dsRNA under identical conditions had little or no effects on these signaling components. These results are quite surprising because CREB is downstream of Akt and Akt phosphorylates CREB at Ser-133 (1, 2); however, they are consistent with results shown in Fig. 3.

Forskolin Down-regulates Activation of Caspase-9 and Caspase-3 in Macrophages—Cellular proliferation and cell survival are multicomponent processes that include inhibition of cell death cascades (40, 41). Initiation of an intrinsic apoptotic pathway by a wide variety of stimuli causes the release of cytochrome c from mitochondria, which binds to cytosolic Apaf-1 and induces oligomerization of Apaf-1, leading to recruitment and activation of procaspase-9 in apoptosomes. The apoptotic program then involves activation of a cascade of caspases. Caspase-9 cleaves procaspase-3 and -7 thus inducing biochemical and morphological changes associated with apoptotic cell death (40, 41). Therefore, we next examined the levels of procaspase-9 and cleaved caspase-3 by Western blotting in macrophages stimulated with forskolin (Fig. 9, A and B). Forskolin inhibited the cleavage of procaspase-9 and procaspase-3 (Fig. 9, A and B). The results show that in macrophages forskolin promotes cell survival by inhibiting the activation of initiator and effector caspases. Akt activation inhibits caspase-9 activation downstream of cytochrome c release by phosphorylation at Ser-196. Recently cAMP has also been reported to inhibit the activation of caspase-9 by Apaf-1 in response to cytochrome release in a PKA-dependent manner (42). Because forskolin up-regulates both PKA-Akt and PKA-CREB signaling, these results suggest that “cross-talk” occurs between the PI 3-kinase and PKA signaling cascades (see also Refs. 43–46).

Forskolin Up-regulates the Protein Levels of XIAP—Akt induces NFκB transcriptional activity (26, 47), and the genes induced by NFκB include prosurvival Bcl-2 family members and inhibitors such as XIAP, which inhibits apoptosis by binding directly to the initiator and effector caspases (26, 48). Stimulation of macrophages with forskolin also elevated XIAP protein levels, which were maximal by ~60 min of incubation (Fig. 9, A and B). cAMP protects normal and transformed intestinal epithelial cells from apoptosis induced by diverse stimuli (49). This protection was associated with rapid induction of XIAP, which required its transcriptional up-regulation by phosphorylation of CREB (49). Treatment of the cells with the PI 3-kinase inhibitor LY294002 (20 μM/20 min) before addition of forskolin (10 μM) profoundly reduced the levels of XIAP protein (Fig. 9, A and B), which shows the role of Akt, the downstream effector of PI 3-kinase, in promoting cell survival. Under the experimental conditions, the PKA inhibitor H-89 (10 μM/10 min) minimally affected XIAP expression as measured by Western blotting in forskolin-stimulated cells (Fig. 10D).

Forskolin Up-regulates the Expression of the Prosurvival Protein Bcl-2, in Macrophages—Bcl-2 is the prototype for a large family of structurally related proteins that regulate cell death in mammalian cells (26). Some of these such as Bax and Bad promote cell death, whereas Bcl-2 and BclXl promote cell survival. The various Bcl-2 family members are located both in the cytoplasm and at intracellular membranes, including outer mitochondrial membranes. Changes in the balance between prosurvival and prodeath Bcl-2 family members cause translocation of proapoptotic Bcl-2 family members from the cytoplasm to the mitochondrial membrane, which initiates the cell death program (26). BclXl binds...
to Apaf-1 and inhibit caspase-9 activation, and this binding is antagonized by Bax. Bad, a pro-apoptotic protein, binds to Bcl-2/BclXL neutralizing their anti-apoptotic effects. Phosphorylation of Bad results in the release of the anti-apoptotic proteins Bcl-2/BclXL, which interact with Bax to inhibit apoptosis, and Bad interacts with 14-3-3 proteins, which may protect Bad from dephosphorylation or sequester Bad away from its mitochondrial targets (26). Bad has three phosphorylation sites; namely, Ser-112, Ser-136, and Ser-155. Akt phosphorylates Bad at Ser-136 (26), and PKA phosphorylates Bad at Ser-155 (50). The promoter region of Bcl-2 contains a CRE site, and the transcription factor CREB, when activated, is a positive regulator of Bcl-2 (51, 52). We next determined the role of Bcl-2 in forskolin-induced cell proliferation in macrophages by quantifying the levels of Bcl-2 protein, Bad, and Bad phosphorylated at Ser-155, and Bad phosphorylated at Ser-136 by Western blotting (Fig. 9, A and C). Forskolin increased the phosphorylation of Bad at Ser-155 at early periods of incubation (5–20 min), but the p-BadSer-155 levels reached to basal values at longer periods of incubation. Similar pattern in the levels of Bcl-2 was observed in forskolin-treated cells. That the increased expression was mediated by CREB activation in forskolin-treated cells is supported by markedly reduced levels of Bcl-2 in these cells upon silencing CREB gene expression (TABLE ONE and Fig. 9D). Forskolin treatment also up-regulated the phosphorylation of Bad at Ser-136. Because Akt phosphorylates Bad at Ser-136, the results also indicate the role of Epac-activated Akt in the anti-apo-

**FIGURE 5.** Effect of the PKA inhibitor H-89 on PI 3-kinase signaling cascade components and phosphorylation of CREB at Ser-133. A, PI 3-kinase (○), p-AktThr-308 (□), p-AktSer-473 (▲), and p-CREBSer-133 (●). H-89-induced inhibition is shown as % change from cells treated with forskolin (10 μM) alone, and which has been considered as 100%. B, immunoblots representative of two to three independent experiments along with an immunoblot of actin are also shown. See “Experimental Procedures” for details.
The effects of forskolin in macrophages. Cells were incubated with either the PKA inhibitor H-89 (10 μM/90 min) or the PI 3-kinase inhibitor LY294002 (20 μM/20 min) before stimulation with forskolin. To assess the role of PKA and PI 3-kinase/Akt signaling in the up-regulation of Bcl-2, p-BadSer-155, and p-BadSer-136. Forskolin-induced increases in Bcl-2 protein were reduced by ~50% in cells pretreated with H-89 or LY294002 demonstrating the involvement of both PKA and Akt signaling in the up-regulation of Bcl-2. H-89 treatment reduced forskolin-induced increases in p-BadSer-155 and LY294002 reduced forskolin-induced increases in p-BadSer-136 by more than 50% (Fig. 10D). These results suggest an interplay of both PKA and Akt-dependent signaling in promoting cell survival by up-regulating anti-apoptotic protein.

Phosphorylated FOXO1 in Forskolin-treated Macrophages—In the next series of experiments we examined the effect of forskolin treatment of macrophages on phosphorylated FOXO1 (Fig. 10, A and B). Pro-survival signaling cascades regulate apoptosis by modulating the expression of a defined subset of genes such as the FOXO family (26, 53–55). These transcription factors are substrates for Akt, and phosphorylation of FOXO1 promotes its export from the nucleus to the cytosol thus prevents FOXO1 interaction with DNA and up-regulation of transcription factors involved in the apoptotic pathway (26). FOXO1 also interacts with 14-3-3 protein, which serves to localize phosphorylated FOXO1 in the cytosol (56, 57). Treatment of macrophages with forskolin elevated the levels of phosphorylated FOXO1 at ~10 min of incubation, and these levels remained elevated at longer time intervals (Fig. 10, A and B). Akt promotes cell survival by phosphorylating transcription factor FOXO1, which causes the inhibition of the expression of apoptotic genes by its nuclear exclusion and cytoplasmic retention. The treatment of cells with LY294002 reduced forskolin-induced protein levels of p-FOXO1Ser256 (Fig. 10D). The results thus show the involvement of PI 3-kinase/Akt signaling promoting cell survival. Because forskolin treatment activated both PKA-CREB and Epac-Akt signaling cascades, it is likely that both these pathways are involved in the regulation of FOXO1. FOXO1 has been implicated in the regulation of the PKA signal transduction pathway (58). It has been reported that CREB-binding protein (CBP) associates with FOXO factors and causes its acetylation, which attenuates FOXO-mediated transcriptional activity of target genes (59).

**Effect of Forskolin on Phosphorylation of GSK3β**—GSK3α/β is a serine/threonine kinase that phosphorylates glycogen synthase causing its inactivation (60, 61). Phosphorylation of GSK3α at Ser-21 and GSK3β at Ser-9 by several kinases, including PKA and Akt, results in its inactivation (60, 61). Several transcription factors are directly phosphorylated by p-GSK3βSer-9 (60, 61). The majority of these transcription factors are inhibited after phosphorylation; however, activated CREB is stimulated after phosphorylation by p-GSK3βSer-9 (62, 63). GSK3β has been implicated in the regulation of cell survival (60–62). Treatment of macrophages with forskolin increased p-GSK3βSer-9 by ~1.5-fold at early periods of incubation, and this elevation was sustained upon longer periods of incubation (Fig. 10, A and B). Akt promotes the survival potential of cells by phosphorylating GSK3β. Pretreatment of cells with the PI 3-kinase inhibitor LY294002 before forskolin treatment inhibited the activation of GSK3β by ~40–50% (Fig. 10D), which demonstrates the cell-survival-promoting effect of PI 3-kinase/Akt signaling in forskolin-treated cells. The results indicate that phosphorylation of GSK3β in forskolin-treated cells may regulate both cell survival by inhibiting transcription of pro-apoptotic genes, as well as up-regulating the activation of CREB.

**Effects of Forskolin on p27kip1 and Cyclin D1 Protein Levels in Macrophages**—p27 is a member of the Kip family of CDK inhibitor induced by extracellular antimitogenic signals. Inhibition of proliferation occurs in the G1 phase of the cell cycle and involves cyclin-dependent kinase (CDK) complexes with cyclins as well as the CDK inhibitor
p27kip (64, 65). Mitogen-activate protein kinase regulates cyclin levels transcriptionally, via the ATF/CREB transcription factors (66). The cell cycle inhibitor p27kip acts during the late G1 phase by binding and inhibiting CDK2-cyclin E/A complexes. Cells can only progress through the cell cycle when p27kip is dissociated from CDK2-cyclin E/A complexes, and this is generally achieved by degradation of p27kip. Thus inhibition of cell proliferation by cAMP often correlates with increased levels of p27kip and decreased levels of cyclin D1. Therefore agents inducing cellular proliferation should generally decrease p27kip and elevated expression of cyclin D1, and that is what we observe in macrophages treated with forskolin. Forskolin treatment decreased the levels of p27kip, but caused a 2- to 3-fold increase in the levels of cyclin D1 (Fig. 10, A and C). These results suggest that cAMP promotes cell cycle progression.

DISCUSSION

In this study, we have examined the role of two cAMP effector systems; namely, Epac- and PKA-initiated signaling cascades, on forskolin-induced macrophage proliferation. Our studies indicate a number of points: 1) forskolin treatment elevated p-CREBSer-133 levels and stimulated mitogenesis and cell proliferation, which were inhibited by the PKA inhibitor H-89 or by silencing CREB gene expression by RNAi; 2) forskolin treatment activated PI 3-kinase and elevated the levels of Epac, Rap1-GTP, ILK, and p-AktSer-473 with a lesser effect on p-AktThr-308; 3) in forskolin-stimulated cells p-AktThr-308, p-AktSer-473, Rap1, and ILK co-immunoprecipitated with Epac; 4) the PI 3-kinase inhibitors wortmannin and LY294022 decreased p-AktSer-473 by over 80% while decreasing p-AktThr-308 by nearly 50%. In contrast, the PKA inhibitor H-89 showed almost no effect on these parameters, but it inhibited the phosphorylation of CREB at Ser-133; 5) forskolin treatment modulated anti-apoptotic cascades by inhibiting the activation of caspase-9, and caspase-3, decreasing p27kip, and up-regulating the levels of p-BadSer-136 and p-BadSer-155, Bcl-2, p-FoxO1Ser-256, p-GSK3βSer-9, and cyclin D1; 6) silencing of CREB gene expression reduced by 50–70% the activation of PI 3-kinase, as well as p-AktSer-473 and p-AktThr-308 in forskolin-treated cells.

The results presented show a coordinated regulation of forskolin-induced mitogenesis and cell proliferation by PKA-CREB and Epac1-Rap1 signaling and suggest that these two pathways converge and/or cross-talk at PI 3-kinase/Akt signaling. By activating PKA, cAMP regulates specific steps in cell proliferation and differentiation, an effect requiring control of cell cycle-dependent protein kinase cascades (1, 2). cAMP also affects cell spreading by activating PKA (67), whereas the cAMP-dependent Epac1-Rap1 pathway is involved in regulating cellular adhesion (10, 11, 14–19). The cAMP-dependent signal transduction pathways also modulate apoptosis, and cross-talk with the PI 3-kinase signal transduction pathway is necessary for this effect (8–11, 26). Recruitment and activation of PI 3-kinase at the plasma membrane catalyzes the generation of 3’-phosphorylated phosphoinositides from membrane-bound inositol 1,4,5-bisphosphate. These lipids then function as intermediates that regulate the activation of Akt and downstream signaling (26). Akt promotes cell survival by regulating Bad,
PKA-CREB and Epac1-Rap1·GTP Cross-talk at Akt

FIGURE 9. Effect of forskolin treatment of macrophages on the apoptotic pathway. The data from four separate studies are shown for the effect of forskolin on expression of caspases and anti-apoptotic protein. A, bar diagram showing changes in protein levels of: p-BadSer~136~ (black), p-BadSer~155~ (horizontal lines), Bad (dark gray); pro-caspase-9 (stippled); cleaved caspase-3 (white); and XIAP (light gray) in cells stimulated with forskolin for 0–60 min. The results shown are in arbitrary units (AU × 10^6) and are expressed as mean ± S.E. from three to four independent experiments. Representative immunoblots are: B, pro-caspase-9, procaspase-3, cleaved caspase-3, and XIAP; C, p-BadSer~136~. Bad protein, Bcl-2, and p-BadSer~155~. Not shown is an immunoblot of the protein-loading control actin. D, Bcl-2 protein levels in cells transfected with dsCREB RNA. The lanes are: 1, Lipofectamine plus buffer; 2, Lipofectamine plus forskolin; 3, dsCREB RNA plus forskolin; and 4, scrambled dsRNA plus forskolin.

caspase-9, FOXO1, and nuclear factor NFκB (26). Although Akt is the downstream target of PI 3-kinase products, Akt stimulation in an Epac-dependent manner has also been reported (38, 68). Rap1 is the downstream effectors of Epac as demonstrated by the increase in Rap1-GTP levels. In cells overexpressing Epac, forskolin treatment further augments this effect. In these cells, forskolin increased Akt kinase activity by ∼3-fold compared with wild type (13). Expression of a dominant negative Rap1 mutant in cells overexpressing Epac abolished its ability to activate Akt (13). The Epac NH2-terminal DEP domain directs its subcellular targeting, and cells expressing an Epac deletion mutant, Δ(1–148), were unable to phosphorylate Akt at Ser-473 when treated with forskolin (13). Deletion mutants in the DEP domain primarily distribute in the cytosolic fraction, whereas full-length Epac1 is present only in the particulate fraction (13). The co-immunoprecipitation of p-AktThr~308~ and p-AktSer~473~ with Epac1 in forskolin-treated macrophages, suggests a possible role of Epac1 in membrane targeting of activated Akt. Because Rap1, along with p-AktThr~308~ and p-AktSer~473~, co-immunoprecipitated with Epac1, and because Rap1 is localized in plasma membranes, it is likely that Epac1 in complex with activated Akt and Rap1 may be involved in targeting Akt to plasma and nuclear membranes.

ILK, which co-immunoprecipitated with Epac1, may also contribute to the activation of Akt, as reported in other cell types (67–69). Using RNAi, it has recently been shown that ILK gene expression did not affect the phosphorylation of Akt at Thr-308, but almost completely inhibited that of Akt at Ser-473 and this was accompanied by a significant increase in apoptosis (69). ILK is expressed in most mammalian cell types and it interacts with the cytoplasmic domain of the integrin β1 and β3 subunits (19, 21). Integrin ligation and clustering results in the generation of intracellular signals that regulate cell survival, cell differentiation, and cell motility (19, 20). In macrophages, we observed that forskolin treatment elevated the levels of ILK by ∼1.5- to 2-fold. This suggests a role for forskolin-activated Rap1 in regulating macrophage cell shape and spreading.

Rap proteins are small GTPases from the Ras family that function as molecular "switches" cycling between inactive GDP and active GTP forms. Rap1 regulates ERK activation by Ras-dependent and independent pathways that program cell proliferation in a cell-type-specific manner (4, 5). Rap1 also stimulates cellular proliferation in some cell types in a PKA-independent manner by activating PI 3-kinase/Akt signaling (4–6). In a macrophage cell line, complement-mediated phagocytosis was abolished by inhibition of Rap1 signaling (Ref. 18, and references therein). Rap1 also regulated integrin-mediated cell adhesion and cell motility. Rap1 is required for β-integrin-mediated platelet adhesion, and it is rapidly activated by a variety of platelet agonists. In macrophages, β2-integrin activation requires Rap1. Mice overexpressing active Rap1 showed increased integrin-mediated cell adhesion to fibronectin through Epac and Rap1, which was abolished by Rap1-GTPase-activating proteins (17). Rap1 mutations disrupt normal cell shape and morphogenesis in the eye, ovary, and wings of Drosophila embryos (see Refs. 17 and 18, and references therein). Rap1 activation potentiates the response to mitogenic stimuli in thyroid follicular cells and antigen-challenged thymocytes and links cAMP signaling to the regulation of ERK activity and cell proliferation (4–7). In a previous report, we have shown that cell proliferation in macrophages treated with various cAMP-elevating agents was accompanied by ERK activation and up-regulation of B-Raf (30). In this study, we further show that
both PKA-dependent and PKA-independent pathways coordinately regulate cell proliferation of forskolin-treated macrophages.

Forskolin treatment stimulated macrophage [3H]thymidine uptake and increased their numbers. That these effects of forskolin are mediated by PKA-CREB signaling is supported by our data demonstrating profound inhibition by the PKA inhibitor H-89 or silencing of CREB gene expression. CREB is essential for cell survival and co-transcription of a dominant negative CREB mutant decreased cell survival (1, 2).

Expression of a mutant form of CREB, KCREB, in human melanoma cells decreased their tumorigenic and metastatic potential in nude mice (72). Expression of KCREB also blocked adipocyte differentiation and induced apoptosis in these cells (73). KCREB expression increased the expression of several pro-apoptotic genes and decreased the expression of anti-apoptotic signaling as well as down-regulated the expression of Akt, a key mediator of cell survival. Mutation of serine to alanine at position 133 (CREBM1) functions as a dominant negative repressor of CREB-dependent genes expression (74). Transgenic mice with CREBM1 expression have defective T lymphocyte helper function with increased susceptibility of T lymphocytes helper cells to activation induced cell death and had decreased levels of Bcl-2. Overexpression of Bcl-2 in these cells reversed cell death indicating the crucial role of CREB in up-regulating the Bcl-2 gene (74).

Consistent with these data are studies in PC12 cells where pharma-

**TABLE ONE**

Effect of silencing the expression of the CREB gene on CREB and activation of Akt

| Treatments                  | CREB protein | PI 3-kinase | p-Akt<sup>Thr-308</sup> | p-Akt<sup>Ser-473</sup> | Bcl-2 |
|-----------------------------|--------------|-------------|--------------------------|--------------------------|-------|
| Lipofectamine plus the buffer | 1327 ± 140   | 255 ± 23    | 217 ± 19                 | 238 ± 25                 | 102 ± 9 |
| Lipofectamine plus forskolin   | 2738 ± 250   | 575 ± 52    | 427 ± 46                 | 464 ± 40                 | 174 ± 16 |
| Lipofectamine plus dsCREB     | 1624 ± 160   | ND          | ND                       | ND                       | ND    |
| Lipofectamine plus dsCREB plus forskolin | ND     | 261 ± 24    | 289 ± 30                 | 170 ± 15                 | 95 ± 10 |
| Lipofectamine plus scrambled dsRNA | 2765 ± 280 | 474 ± 50    | 435 ± 45                 | 295 ± 31                 | 162 ± 15 |

<sup>a</sup> ND, not done.
colocational inhibition of PI 3-kinase decreased Bcl-2 promoter activity by ~45% (75). By contrast, PC12 cells overexpressing Akt demonstrated a 2-fold increase in Bcl-2 mRNA. Overexpression of the catalytic subunit of PI 3-kinase enhanced Bcl-2 gene promoter activity by ~2-fold, and expression of a dominant negative p85 subunit construct of PI 3-kinase inhibited Bcl-2 gene promoter activity by ~44% (73). In our study, silencing CREB gene expression profoundly decreased activated Akt species as well as that of BCI-2 protein. We suggest, therefore, that transcriptional regulation of Akt by CREB occurs as well as regulation of Akt by the Epac1-Rap1 signaling cascade. These results indicate that Akt coordinates both CREB and Epac1-Rap1 signaling in promoting cellular proliferation and mitogenesis in forskolin-treated peritoneal macrophages.

In this report, we also show that, under our experimental conditions, forskolin is a strong promoter of Akt phosphorylation at Ser-473, but a weak promoter of Akt phosphorylation at Thr-308. Phosphorylation at both Thr-308 and Ser-473 residues is required for full activation of Akt, and similar observations have been made in other cell types (26, 27, 37). PI 3-kinase-dependent phosphorylation of Akt at Thr-308 residue by PDK1 has been widely reported. However, PI 3-independent kinases have also been suggested to phosphorylate this site on Akt (76). We also find that the phosphorylation of both Thr-308 and Ser-473 residues is required for full activation of Akt, and that forskolin is a weak promoter of Akt phosphorylation at Thr-308. Phosphorylation at Ser-473 on Akt is increased by forskolin. However, a forskolin-induced decrease in Bcl-2 promoter activity was observed in murine peritoneal macrophages, which is consistent with the findings that the phosphorylation of Akt at Ser-473 in forskolin-treated macrophages. We do not know whether this complex of Akt silencing and Bcl-2 gene promoter activity is regulated by multiple factors acting on Akt at different sites.

In summary, we show in this report that cAMP elevated by forskolin in murine peritoneal macrophages activates PKA and Epac1-dependent signal transduction. Activated PKA phosphorylated CREB, which up-regulates transcriptional expression of the Akt gene. PKA also inhibits the activation of pro-apoptotic proteins. Binding of cAMP to Epac1 activates Rap1 and subsequently Akt, which promotes cell survival and proliferation by down-regulating the expression of apoptotic proteins and up-regulating anti-apoptotic proteins. We conclude that both PKA-CREB and Epac1-Rap1 signaling converge at the level of Akt and this is important in macrophage functions.

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