Functional Roles of Protein Kinase A (PKA) and Exchange Protein Directly Activated by 3′,5′-Cyclic Adenosine 5′-Monophosphate (cAMP) 2 (EPAC2) in cAMP-Mediated Actions in Adrenocortical Cells

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In the adrenal cortex, the biosynthesis of steroid hormones is controlled by the pituitary-derived hormone ACTH. The functions of ACTH are principally relayed by activating cAMP-dependent signaling pathways leading to the induction of genes encoding enzymes involved in the conversion of cholesterol to steroid hormones. Previously, protein kinase A (PKA) was thought to be the only direct effector of cAMP. However, the discovery of the cAMP sensors, exchange proteins directly activated by cAMP (EPAC1 and 2), has led to a reevaluation of this assumption. In the present study, we demonstrate the occurrence of the EPAC2 splicing variant EPAC2B in adrenocortical cancer cells. Immunocytochemistry demonstrated that EPAC2B is localized predominantly in the nucleus. EPAC2B is functional because it activates Rap1 in these cells. Using the cAMP analogs 8-*p*-chlorophenylthio-2′-*O*-methyl-cAMP and *N*6-benzoyl-cAMP, which specifically activate EPAC1/2 and PKA, respectively, we evaluated the contribution of these factors in steroid hormone production, cell morphology, actin reorganization, and migration. We demonstrate that the expression of cAMP-inducible factors involved in steroidogenesis (steroidogenic acute regulatory protein, cytochrome P450 11A1 and 17, and nerve growth factor-induced clone B) and the cAMP-induced biosynthesis of steroid hormones (cortisol and aldosterone) are mediated by PKA and not by EPAC2B. In contrast, both PKA- and EPAC-specific cAMP analogs induced cell rounding, loss of stress fibers, and blocked migration. Taken together, the presented data confirm PKA as the central cAMP mediator in steroid hormone production and reveal the involvement of EPAC2B in cAMP-induced effects on cytoskeleton integrity and cell migration. **(*Endocrinology* 151: 2151–2161, 2010)**

It has long been recognized that cAMP is a ubiquitous second messenger regulating many key cellular processes and that its actions are mediated by the cAMP effector protein kinase A (PKA). However, about a decade ago, two additional cAMP sensors, exchange proteins directly activated by cAMP (EPAC)-1 and -2 (also termed cAMP-binding guanine exchange factors I and II) were discovered (1, 2). Since then, numerous studies have demonstrated that EPAC1/2 mediate a multitude of cellular functions induced by cAMP, *i.e.* proliferation, differentiation, adhesion, apoptosis, and transcription (3, 4). In the endocrine system, the discovery of EPAC1/2 was critical to explain some of the effects mediated by cAMP in hormone signaling that were not mediated by PKA (reviewed in Ref. 4), for example in granulosa cells (5) and thyrocytes (6–8). Moreover, EPAC2 has been implicated in insulin secretion in pancreatic β-cells (9, 10), in the release of α-MSH in the pituitary gland (11), and in the regulation of proglucagon expression in intestinal L cells (12). Both EPAC1 and -2 contain a cAMP-binding domain (CBD) similar to the one

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Abbreviations: CBD, cAMP-binding domain; 8CPT-2M, 8-*p*-chlorophenylthio-2′-*O*-methyl-cAMP; CYP, cytochrome P450; CYP11A1, CYP cholesterol side chain cleavage enzyme; EPAC, exchange proteins directly activated by cAMP; F-actin, filamentous actin; IBMX, isobutyl 3-methylxanthine; N6-Bnz, *N*6-benzoyl-cAMP; NGFI-B, nerve growth factor-inducible clone-B; PDE, phosphodiesterase; PKA, protein kinase A; SDS, sodium dodecyl sulfate; StAR, steroidogenic acute regulatory protein.

found in the PKA regulatory subunit, but the two isoforms differ by the presence of one extra CBD in the N terminus of EPAC2. Binding of cAMP activates EPAC1/2, which then act as guanine exchange factors for the small G proteins Rap1 and Rap2 by catalyzing the exchange of bound GDP for GTP (reviewed in Ref. 13). The extra N-terminal CBD in EPAC2 has low affinity for cAMP (14), and instead, this domain may be involved in plasma membrane localization (15). EPAC1 and -2 are differentially expressed (1). EPAC1 is found ubiquitously, whereas the expression pattern of EPAC2 is restricted to certain regions of the brain, neuroendocrine tissues, and endocrine glands including the adrenal and testis (1, 2, 9). Recently, EPAC2 was described to exist as three different splicing variants, which differ only at their N termini: the full-length protein EPAC2A; EPAC2B, which lacks the N terminus CBD; and EPAC2C, which lacks the N terminus CBD and the Dishevelled-Egl-10-Plekstrin (DEP) (15, 16).

In the adrenal cortex, the biosynthesis of steroid hormones is regulated primarily by the pituitary-derived hormone ACTH. ACTH induces the transcription of steroidogenic enzymes involved in the stepwise conversion of cholesterol to corticosteroids (17). Binding of ACTH to its receptor leads to the activation of multiple signaling pathways (18, 19). The pathway by far the best characterized involves the activation of adenylyl cyclases followed by an elevation of cAMP production. Although cAMP has been recognized as the main second messenger recapitulating the effects of ACTH on steroid hormone output, the downstream signaling events are only partially understood (17–19). cAMP induces the activation of PKA and subsequent phosphorylation of target substrates, such as the cholesterol ester hydrolase and the steroidogenic acute regulatory protein (StAR) (20). These posttranslational modifications activate cholesterol ester hydrolase and StAR and lead to an increased availability of free cholesterol and its delivery to the inner mitochondrial membrane. This allows for the first enzymatic reaction by the cytochrome P450 (CYP) cholesterol side chain cleavage enzyme (CYP11A1), which converts cholesterol to pregnenolone. Pregnenolone is further converted to aldosterone, cortisol, or androstenedione in the different zones of the adrenal gland, depending upon the expression of the different CYP steroidogenic enzymes (CYP17, CYP21, CYP11B1, and CYP11B2) and of the 3β-hydroxysteroid dehydrogenase (21).

Although PKA clearly plays a key role in steroidogenesis, several studies have pointed to cAMP signals mediated independently of PKA in the adrenal cortex (22–24). Tools that allow the specific activation of PKA and EPAC1/2 now exist [*i.e.* *N6*-benzoyl-cAMP (N6-Bnz) and 8-*p*-chlorophenylthio-2′-*O*-methyl-cAMP (8CPT-2M), respectively] (25) but have not previously been used to dissect cAMP-induced signaling cascades in adrenocortical cells. In this study, we establish that the EPAC pathway is functional in these cells and exploit the PKA- and EPAC1/2-specific analogs to discriminate between the involvement of these two pathways in corticosteroid production, changes in cell morphology, microfilament organization, and migration. We found that several cAMP-dependent processes in steroidogenic cells, such as the expression of StAR and steroid hydroxylases, the induction of the transcription factor nerve growth factor-inducible clone-B (NGFI-B), and the production of steroid hormones, are stimulated by cAMP in a PKA-dependent fashion. In contrast, the activation of both pathways leads to the loss of stress fibers, change of cell shape, and a marked decrease in migration.

Materials and Methods

Reagents

Anti-Rap1 (sc-65), anti-EPAC2 (sc-9384 and sc-25633, epitope amino acids 1-220; sc-9383, internal epitope; all three antibodies can detect both EPAC2A and -2B), anti-NGFI-B (sc-5569), anti-StAR (sc-25806), anti-phospho-Tyr 204-ERK1/2 (sc-7383), anti-ERK1 (sc-94), and anti-ERK2 (sc-153) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CYP17 was a generous gift from Dr. M. R. Waterman (Vanderbilt University, Nashville, TN). Anti-CYP11B1 was kindly provided by Dr. Hiroshi Takemori, (University of Osaka, Osaka, Japan). Anti-CYP11A1 (ab-67355) and anti-β-actin (ab-6276) were from Abcam (Cambridge, UK). Antimouse CYP11A1 was from Millipore (Billerica, MA) (AB1294). The EPAC- and PKA-specific cAMP analogs, 8CPT-2M and N6-Bnz, respectively, were from Biolog Life Science Institute (Bremen, Germany). Forskolin, ACTH (amino acids 1-39) and isobutyl 3-methylxanthine (IBMX) were from Sigma-Aldrich Norway AS (Oslo, Norway). Flag-tagged pCMV2-EPAC2A and pCMV2-EPAC2B expression vectors were kind gifts from Dr. S. Seino (Kobe University Graduate School of Medicine, Kobe, Japan).

Cell culture

Murine adrenocortical tumor cells (Y1) (26), Sertoli cells (MSC-1) (27), prepubertal Sertoli cells (SMAT-1) (28), and monkey kidney COS-1 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Human adrenocortical carcinoma NCI-H295R (H295R) cells (29) were obtained from American Type Culture Collection and cultured as described previously (30). Mouse Leydig tumor cells (MA-10) were cultured in a 1:1 mixture of DMEM (high glucose) and HAM F12, 15% horse serum, 20 mm HEPES, and 40 μg/ml gentamicin (31). SMAT-1 cells were kindly provided by N. di Clemente (Université Paris-Sud, Paris, France). COS-1 cells were transfected with Flag-EPAC2A or Flag-EPAC2B with SuperFect (QIAGEN, Valencia, CA) at 1:4 (micrograms DNA to microliters SuperFect).

RT-PCR

mRNA was isolated from Y1 and H295R cells using the FastTrack 2.0 kit (Invitrogen, Carlsbad, CA). mRNA (50 ng) was reverse transcribed (30 min at 42 C) using a reverse transcription system (Promega, Madison, WI; A3500). The resulting cDNA was amplified using *Taq* DNA polymerase (New England Biolabs, Beverly, MA). The primers for EPAC1 were as described (2). The following primers were used for EPAC2: forward 5′-ATTAATGGACGCCTGTTTGC-3′ (nucleotides 2393-2412 in EPAC2A) and reverse 5′-CCTCCTCAGGAACAAATCCA-3′ (nucleotides 2643-2625 in EPAC2A). PCR products were separated on 3% agarose gels stained with ethidium bromide.

Western immunoblotting

Whole-cell extracts were prepared by lysis in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, and 2 μg/ml aprotinin and leupeptin]. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk and incubated overnight in primary antibodies and subsequently with anti-mouse/rabbit antibodies conjugated to horseradish peroxidase (1:10,000) for 1 h. Antibody detection was performed using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) according to the manufacturer's instructions and scanned with an LAS-3000 imaging system (Fuji film, Tokyo, Japan). Densitometry measurements were done for each protein examined using ImageJ 1.42q (rsb.info.nih.gov/ij/). Quantifications of the ratios between each protein and β-actin were performed and shown as fold values compared with control. Means ± SD are presented as histograms.

Rap1 activation assay

Y1 and H295R cells were plated on 10-cm plates in serum-supplemented medium and grown for 2–3 d. Cells were rinsed in PBS and incubated in serum-free medium for 24 h and subsequently treated as indicated in the figure legend (see Fig. 3). Cells were washed twice in PBS, lysed in Rap buffer [50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, and 2 μg/ml aprotinin and leupeptin] and incubated with glutathione S-transferase (GST) fused ral guanine nucleotide dissociation stimulator (RalGDS) Rap1-binding domain (RBD) coupled to glutathione beads (300 μl) (Pharmacia, Piscataway, NJ) for 1 h at 4 C. GST-RalGDS-RBD was expressed and purified as described previously (32). After three washes with Rap lysis buffer, bound proteins were eluted with SDS sample buffer for 5 min at 95 C, separated by SDS-PAGE on a 12% gel and subjected to Western blotting using a Rap1-specific antibody. Quantifications of the ratios between GTP-Rap1 and total Rap1 were performed and shown as fold values compared with control. Means ± SD were presented as histograms.

Immunostaining

H295R cells, seeded on coverslips, were fixed in 3.7% formaldehyde in PBS for 15 min, washed twice with PBS, permeabilized with 0.1% Triton X-100 in PBS for 15 min (EPAC2 staining) or 4 min (actin staining), and incubated with blocking buffer (1% BSA in PBS) for 1 h. Cells were incubated with phalloidin conjugated to Alexa 488 (1:40) in PBS for 20 min at room temperature or with anti-EPAC2 (sc-9383) diluted in 0.1% BSA in PBS (1:25) overnight at 4 C, followed by incubation with Cy5-conjugated antigoat antibody (Molecular Probes, Eugene, OR) diluted in 0.1% BSA in PBS (1:200) and mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) after extensive washing in PBS. Control staining with secondary antibody alone under the same staining and exposure conditions showed no unspecific staining. Confocal images of EPAC2-stained cells were acquired with a Zeiss LSM 510 Meta confocal laser scanning microscope equipped with a ×63, numerical aperture 1.4 oil immersion objective and using 633- and 405-nm laser lines. Images of actin-stained cells were obtained with a Nikon TE 2000 fluorescence microscope using a ×63 oil immersion objective.

Migration

Migration was determined after scratch wounding of H295R cell monolayers. Cells were treated with forskolin or the PKA- and EPAC-specific analogs in the presence and absence of IBMX, immediately after creating the wound. Images from three fields per experiment (n = 2) were taken after 48 h, and the wound areas were quantified with the ImageJ software.

Measurements of cortisol and aldosterone

H295R cells plated on six-well plates were grown for 2 d, incubated in DMEM/F12 serum-free and phenol red-free medium for 24 h, and subsequently treated as indicated in Table 1, and conditioned media were collected for cortisol and aldosterone measurements. Cortisol was analyzed by a solid-phase, competitive chemiluminescence enzyme immunoassay on Immulite 2000 from Diagnostics Products Corp. (Los Angeles, CA). Aldosterone was analyzed by the Coat-A-Count procedure, a solid-phase RIA from Diagnostics Products. Cross-reactivity with the antialdosterone antiserum was 0.0004% for 11-deoxycortisol, 0.0005% for DHEA, 0.006% for 11-deoxycorticosterone, and 0.002% for corticosterone, as defined by the manufacturer. Cross-reactivity was not detectable for cortisol and androstenedione.

Statistics

The data are presented as the mean ± SD. Statistics were performed using the GraphPad INSTAT 3 software (GraphPad Software, Inc., San Diego, CA) and one-way ANOVA and Bonferroni as post test, or Student's *t* test.

Results

Adrenocortical cells express EPAC2B

Northern blot analyses have previously demonstrated the presence of EPAC2 but not of EPAC1 in adrenal glands (1, 9). To determine the putative presence of EPAC1/2 in adrenocortical-derived tumor cell lines (murine Y1 cells and human H295R cells), we performed RT-PCR analyses. As shown in Fig. 1A, we found that these cell lines expressed exclusively EPAC2. RNA prepared from NIH-3T3 cells was used as a positive control for EPAC1 (Fig. 1A) (2). In a recent study, Seino *et al.* (15) demonstrated

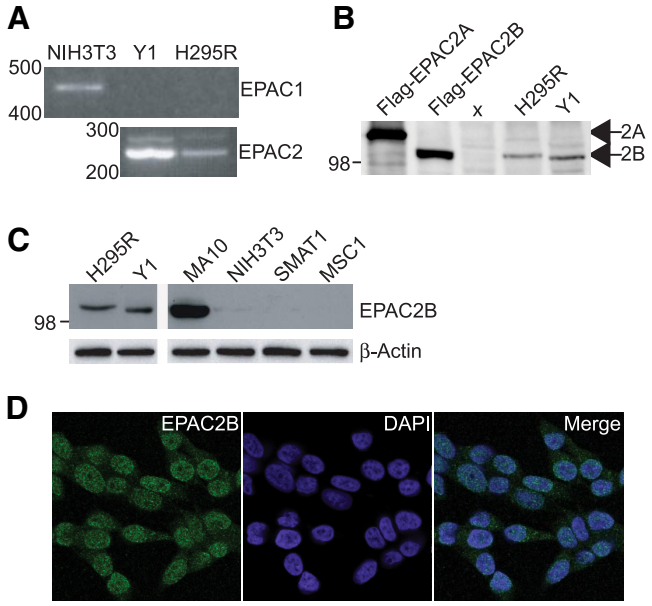

FIG. 1. EPAC2B is expressed in steroidogenic cells. A, RT-PCR was performed on mRNA prepared from NIH-3T3 cells, Y1 cells, or H295R cells using primers specifically amplifying EPAC1 or EPAC2. B and C, Total cell extracts (100 μg) were prepared from H295R, COS-1, and Y1 (B) and H295R, Y1, SMAT1 and MSC-1 (Sertoli-derived cells), and NIH-3T3 (fibroblasts) cells (C) and subjected to Western blotting using an anti-EPAC2 antibody (1:500). In C, COS-1 cells were transfected with Flag-EPAC2A or Flag-EPAC2B and used as positive controls. β-Actin, used as a loading control, was detected from the same cell extracts (10 μg). D, H295R cells were stained with anti-EPAC2 antibody and immunofluorescent secondary antibodies. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Staining was visualized by confocal laser scanning microscopy. These images are representative of at least three independent experiments.

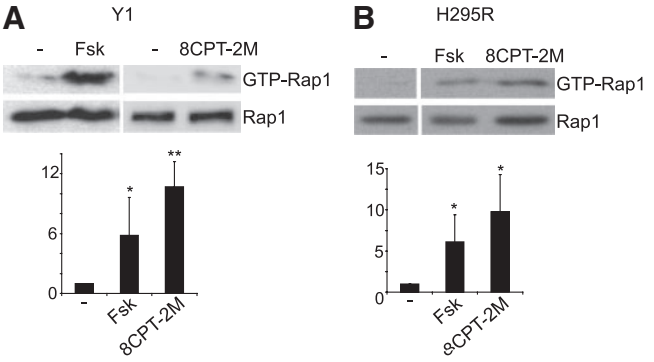

FIG. 2. The EPAC-specific cAMP analog activates Rap1. Serum-starved Y1 cells (A) or H295R cells (B) were incubated in the absence (−) or presence of forskolin (Fsk, 10 μM) or the EPAC-specific cAMP analog (8CPT-2M, 25 μM) for 10 min. Rap1 activation assays were performed as described in *Materials and Methods*. GTP-bound Rap1 (GTP-Rap1) and Rap1 were detected by Western immunoblotting. At least two independent experiments were performed, and the quantification is shown as means ± SD (Student's *t* test: *, $P < 0.05$; **, $P < 0.01$).

that the adrenal cortex expressed a shorter EPAC2 mRNA starting from exon 5 (called isoform EPAC2B). We therefore validated the existence of this shorter variant at the protein level in H295R and Y1 cells by Western immunoblotting, as shown in Fig. 1B. Cell extracts from COS-1 cells transfected with Flag-EPAC2A or Flag-EPAC2B were used as controls. EPAC2B was also detected in testicular Leydig cells (MA-10) but not in nonsteroidogenic testicular Sertoli cells (SMAT-1 and MSC-1) or in fibroblasts (NIH-3T3) (Fig. 1C). The subcellular localization of EPAC2B was evaluated by immunofluorescence staining in H295R cells (Fig. 1D). A punctate staining pattern was detected in the nucleus and to a lesser degree in the cytoplasm. Control labeling with secondary antibodies alone showed no background staining (data not shown). We observed the same pattern of staining in Y1 cells (data not shown).

The EPAC-specific cAMP analog activates Rap1 in adrenocortical cells

To investigate whether the EPAC2B signaling pathway is functional in adrenocortical cells, we determined whether the EPAC-specific cAMP analog (8CPT-2M) led to the activation of Rap1. Y1 and H295R cells were incubated with forskolin (an adenylyl cyclase activator that elevates cAMP levels) or the EPAC-specific cAMP analog, and cell extracts were analyzed for the presence of active Rap1 (GTP-bound Rap1) by Western immunoblotting. As shown in Fig. 2, both agents increased the level of active Rap1 in both cell lines, whereas the levels of total Rap1 remained unchanged. This demonstrates that the EPAC2B signaling cascade is functional in these cells and that EPAC2B activates Rap1 like EPAC2A despite the lack of the N-terminal CBD.

Activation of both PKA and EPAC2B induces cell rounding, in combination with IBMX

In response to cAMP-elevating agents, adrenocortical cells change from a flat and adherent shape to a spherical shape with occasional arborization (33). To evaluate the involvement of PKA and EPAC2B in cAMP-mediated effects on cell morphology, Y1 (Fig. 3A) and H295R (Fig. 3B) cells were incubated with forskolin or with the cAMP analogs specific for PKA or EPAC. Because synthetic cAMP analogs have been reported to be hydrolyzed by phosphodiesterases (PDEs) (34), the PKA- and EPAC-specific analogs were employed both in the presence and absence of the general PDE inhibitor IBMX. In Y1 cells, the effect of forskolin on cell rounding was dramatic and rapid (first observed within 1 h; shown after 6 h in Fig. 3A), as reported previously (35, 36). Cell rounding was reproduced by both the PKA- and EPAC-specific cAMP analogs when combined with IBMX, albeit more potently with the PKA-specific analog (because effects were observed at 100 μM with the PKA analog and required 500 μM for the EPAC analog). IBMX had no effect

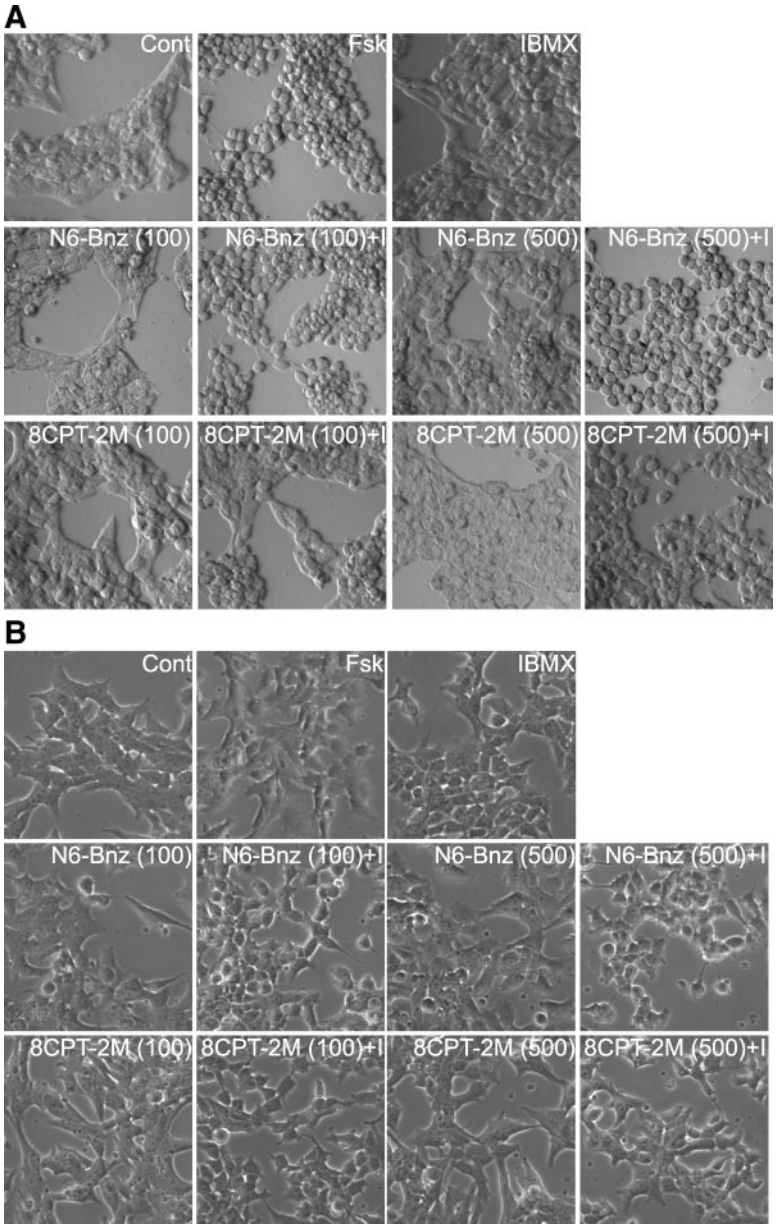

FIG. 3. Both the PKA- and EPAC-specific analogs induce changes in cell morphology. Y1 cells (A) or H295R cells (B) were incubated forskolin (Fsk) alone (10 μM) or with the EPAC-specific cAMP analog (8CPT-2M) and the PKA-specific cAMP analog (N6-Bnz) at 100 and 500 μM for 6 h (Y1 cells) or 24 h (H295R cells) after a 30-min preincubation with IBMX (I) (500 μM) when indicated. Cell morphology was detected by phase-contrast microscopy, and images were taken with a Nikon TE 2000 microscope and a ×20 objective.

alone in Y1 cells. In H295R cells, the effect of forskolin was less dramatic and slower than in Y1 cells (cell rounding was first detected after 16 h; shown after 24 h in Fig. 3B). IBMX had a moderate effect by itself in H295R cells, but the PKA- and EPAC-specific analogs, when combined with IBMX, had a dramatic effect and were equally potent in this cell line. These results indicate that both PKA and EPAC2B are involved in the characteristic changes in cell shape mediated by cAMP and that this effect is dependent upon the concomitant inhibition of PDEs with IBMX.

Activation of both PKA and EPAC2B induces loss of stress fibers and inhibits migration in combination with IBMX

Changes in cell morphology are often accompanied by a reorganization of the actin cytoskeleton, which can itself coordinate cell migration (reviewed in Ref. 37). We therefore evaluated the involvement of PKA and EPAC2B in cAMP-induced actin reorganization (Fig. 4A) and cell migration (Fig. 4B). In Fig. 4A, H295R cells were incubated with forskolin or with the cAMP analogs specific for PKA or EPAC in the presence or absence of IBMX and stained with phalloidin to detect filamentous actin (F-actin). Control cells presented characteristic actin stress fibers (as indicated with *arrows*), as previously reported in Y1 and rat glomerulosa cells (38, 39). Treatment with forskolin resulted in loss of stress fibers and the relocation of actin to restricted areas at the cell periphery (as indicated with *arrowheads*). This effect was mimicked when cells were incubated with either of the PKA- and EPAC-specific analogs when combined with IBMX. A modest effect was also observed with IBMX alone. Similar results were obtained in Y1 cells (data not shown).

The effects of the PKA- and EPAC-specific analogs on cell migration were evaluated in H295R cells by scratch assays. Forskolin inhibited cell migration (by 30 ± 5%, Fig. 4B). Interestingly, the EPAC-specific analog also reduced cell invasion into the wound (by 14 ± 0.3%), and in the presence of IBMX, the EPAC-specific analog reduced migration even more than forskolin (by 42 ± 9%). The PKA-specific analog had no effect by itself but inhibited migration severely when IBMX was present (36 ± 7%). IBMX also had an effect on its own (22 ± 0.9%). Taken together, these experiments show that activation of both PKA and EPAC2B induce changes in cell morphology and actin remodeling and that these effects are accompanied by a reduction of cell motility. Agents that elevate cAMP levels are known to inhibit adrenocortical cell proliferation by preventing DNA synthesis (40, 41). We did not observe any effect by the EPAC-specific

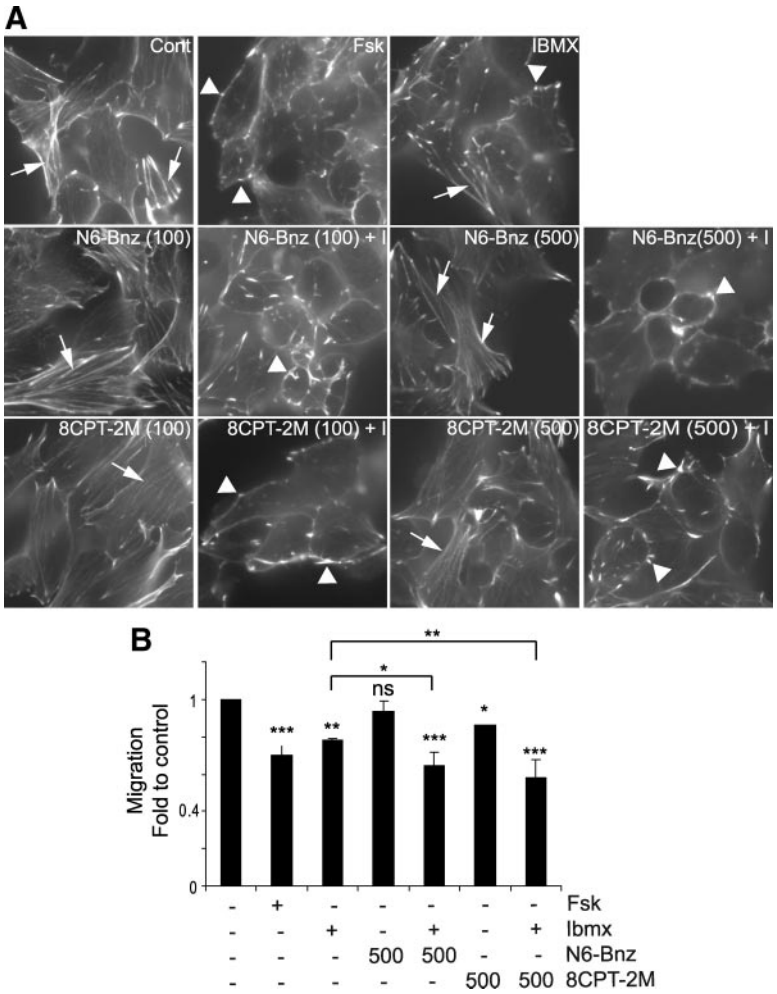

FIG. 4. Both the PKA- and EPAC-specific analogs induce changes in actin organization and decrease in cell migration. A, H295R cells were incubated with forskolin (Fsk) alone (10 μM), the EPAC-specific cAMP analog (8CPT-2M), or the PKA-specific cAMP analog (N6-Bnz) at 100 and 500 μM for 24 h after a 30-min preincubation with IBMX (I) (500 μM), as indicated. Cells were stained with Alexa 488-conjugated phalloidin. *Arrows* point to stress fibers, and *arrowheads* point to punctate actin staining. B, Confluent monolayers of H295R cells were subjected to scratch wounds and subsequently incubated with forskolin alone (10 μM), the EPAC-specific cAMP analog (8CPT-2M), or the PKA-specific cAMP analog (N6-Bnz) at 500 μM for 48 h after a 30-min preincubation with IBMX (500 μM) [one-way ANOVA, Bonferroni post test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control (−); ns, not significant].

analog in either cell line when analyzed by fluorescence-activated cell sorting to examine their cell cycle distribution. In addition, we did not observe any effect on total protein synthesis. This would highlight the fact that our data on migration are not due to variation either on cell division or on hypertrophy.

Activation of PKA, but not of EPAC2B, increases the protein levels of steroidogenic factors

To compare the roles of PKA and EPAC2B in the induction of StAR and steroidogenic enzymes, H295R cells (Fig. 5) and Y1 cells (Fig. 6) were incubated for 24 h with the cAMP analogs specific for PKA or EPAC in the presence or absence of IBMX as well as with forskolin alone. In H295R cells, the protein levels of StAR and the CYP enzymes CYP11A1, CYP17, and CYP11B1 were analyzed by Western immunoblotting (Fig. 5B) and quantified (Fig. 5C). As expected, forskolin increased the levels of StAR, CYP11A1, and CYP17. This effect was reproduced by the PKA-specific cAMP analog (at 500 μM) and was not dependent upon the effect of IBMX. In contrast, the EPAC-specific cAMP analog had no effect alone. Of note, the StAR levels were increased when the EPAC-specific cAMP analog and IBMX were employed together, but this induction was not significantly different from the effect of IBMX alone. The levels of CYP11B1 were not induced by any agent. We observed, however, the presence of a doublet that was apparent only in cells treated with forskolin or the PKA-specific analog. To the best of our knowledge this effect has not been reported before but may warrant further investigation. Mouse adrenocortical cells do not express CYP17 (42), and therefore only the protein levels of StAR and CYP11A1 were determined in Y1 cells (Fig. 6). Again, forskolin increased the levels of StAR and CYP11A1, and this effect was mimicked by the PKA-specific analog (at 500 μM) but not by the EPAC-specific analog. In contrast to H295R cells, the induction by the PKA-specific analog required the presence of IBMX, but there was no effect by IBMX alone (Fig. 6). The levels of β-actin were not affected by any treatment in either cell line.

NGFI-B is an immediate-early gene that is rapidly induced by cAMP and ACTH in adrenocortical cells (43, 44). This nuclear receptor is implicated in cAMP-dependent transcription of multiple steroid hydroxylase genes (44–46) and is thus an important factor in cAMP-induced steroidogenesis. As demonstrated in Fig. 7, the levels of NGFI-B were robustly increased by forskolin after 2 h of treatment in both cell lines, in concordance with previous studies (43, 44). The PKA-specific analog also increased the levels of NGFI-B. IBMX had no effect by itself but enhanced greatly the effects of the PKA-specific analog, thereby recapitulating the effects of fors-

TABLE 1. PKA but not EPAC2B increases cortisol and aldosterone secretion

	Cortisol (nM)	Aldosterone (pM)
Control	32.3 ± 5.9	<69
IBMX	78.3 ± 19.0 (NS)	73.7 ± 5.7
Forskolin	521.4 ± 172.2[a]	186.8 ± 67.7
N6-Bnz (100 μM)	67.3 ± 31.2 (NS)	<69
N6-Bnz (500 μM)	311.4 ± 79.7[a]	159.4 ± 67.0
IBMX + N6-Bnz (500 μM)	705.5 ± 142.8[b]	328.5 ± 34.4[c]
8CPT-2M (100 μM)	46.7 ± 32.3 (NS)	<69
8CPT-2M (500 μM)	42 ± 10.2 (NS)	<69
IBMX + 8CPT-2M (500 μM)	107.5 ± 14.7 (NS)	115.2 ± 27.9 (NS)[d]

H295R cells were incubated with forskolin (10 μM), the PKA-specific cAMP analog N6-Bnz at 100 or 500 μM, or the EPAC-specific cAMP analog 8CPT-2M at 100 or 500 μM for 24 h. Preincubation with IBMX (100 μM) was for 45 min. Mean values are presented ± SD; n = 3–7. For aldosterone, <69 indicates values below the detectable threshold of the assay. Statistics were determined by one-way ANOVA, Bonferroni multiple-comparison post test. NS, Not significant compared with control.

[a] $P < 0.01$ compared with control.

[b] $P < 0.01$ for IBMX + N6-Bnz (500 μM) compared with both N6-Bnz (500 μM) and IBMX alone.

[c] $P < 0.01$ for IBMX + N6-Bnz (500 μM) compared with both N6-Bnz (500 μM) and IBMX alone.

[d] NS for IBMX + 8CPT-2M (500 μM) compared with both 8CPT-2M (500 μM) and IBMX alone.

kolin. The EPAC-specific analog had no effect in either the presence or absence of IBMX. These results indicate therefore that the cAMP-induced expression of steroidogenic enzymes as well as that of NGFI-B is mediated by PKA.

Activation of PKA, but not of EPAC2B, induces the synthesis of cortisol and aldosterone

To further characterize the roles of PKA and EPAC pathways in steroidogenesis, H295R cells were treated with forskolin alone or with the cAMP analogs specific for PKA and EPAC in the absence or presence of IBMX. The levels of cortisol and aldosterone were subsequently measured in the media (Table 1). The PKA-specific analog induced the production of both cortisol and aldosterone in a dose-dependent manner, and this was further enhanced by IBMX. As expected, forskolin also induced the production of both hormones. In contrast, as predicted from the lack of effect on the expression of StAR and the steroid hydroxylases, the EPAC-specific analog had no effect on cortisol or aldosterone secretion.

Discussion

Recently, EPAC2 was described to exist as three different splicing variants, which differ only at their N termini: EPAC2A (full length), which is expressed in the cerebral cortex and pancreas; EPAC2B, which lacks the N-terminal CBD and has, until now, been detected only in the adrenal cortex; and EPAC2C, which lacks the N-terminal CBD and the Dishevelled-Egl-10-Plekstrin (DEP) domain and is specifically expressed in the liver (15, 16). In this study, we have shown that the EPAC2B protein is expressed in Y1 and H295R cells (derived from adrenocortical cells) and MA-10 cells (derived from testicular Leydig cells). Neither EPAC1 nor EPAC2A were detected in adrenocortical cells. The finding that treatment with the EPAC-specific analog activates Rap1 in Y1 and H295R cells (Fig. 2) demonstrates that EPAC2B is functionally active in adrenocortical cells despite the lack of the N-terminal CBD. This is in agreement with a previous report asserting that the low-affinity N-terminal CBD is not required for cAMP-induced activation of EPAC2 (14). It is interesting to note that whereas EPAC2A (that contains the N-terminal CBD) was shown to be localized to the cytoplasm, near the plasma membrane (15), EPAC1 and EPAC2B are found in the nucleus (47, 48) (Fig. 1D), indicating that the N-terminal part of EPAC2A may be involved in cellular distribution of the protein. Interestingly, the localization of EPAC1 has recently been linked to the nuclear/cytoplasmic trafficking of the DNA-dependent protein kinase (48).

In several biological systems, EPAC1/2 potentiates the positive effects of PKA on cell differentiation (*e.g.* for neurite extension in pheochromocytoma cells (49) and adipose conversion of preadipocytes (50) and cell division (in thyrocytes; 51). Considering that the EPAC2B variant appears to be specifically expressed in cells with steroidogenic capacity, this prompted us to systematically examine the contribution of the PKA and EPAC pathways in steroidogenesis. We found that the biosynthesis of both cortisol and aldosterone was mediated by PKA and not by EPAC2B in adrenocortical cells (Table 1), as previously indicated by comparative studies in Y1 and Kin-8 cells (cell line derived from Y1 cells that harbor an inactivating mutation in the regulatory subunit of PKA) (52). In addition, EPAC2B was not able to potentiate the effects of PKA on steroid biosynthesis when the cAMP analogs were combined at suboptimal concentrations (data not shown). Although EPAC2B is also expressed in testicular cells, PKA was also the sole inducer of steroid production in MA-10 cells (data not shown). In sum, PKA clearly plays a dominant role in the synthesis of steroid hormones. We cannot exclude, however, that other cAMP-induced pathways may also be involved, particularly leading to aldosterone synthesis because the Ca^{2+}/calmodulin kinases were shown to play a major role in cAMP-induced aldosterone synthesis in zona glomerulosa cells (24).

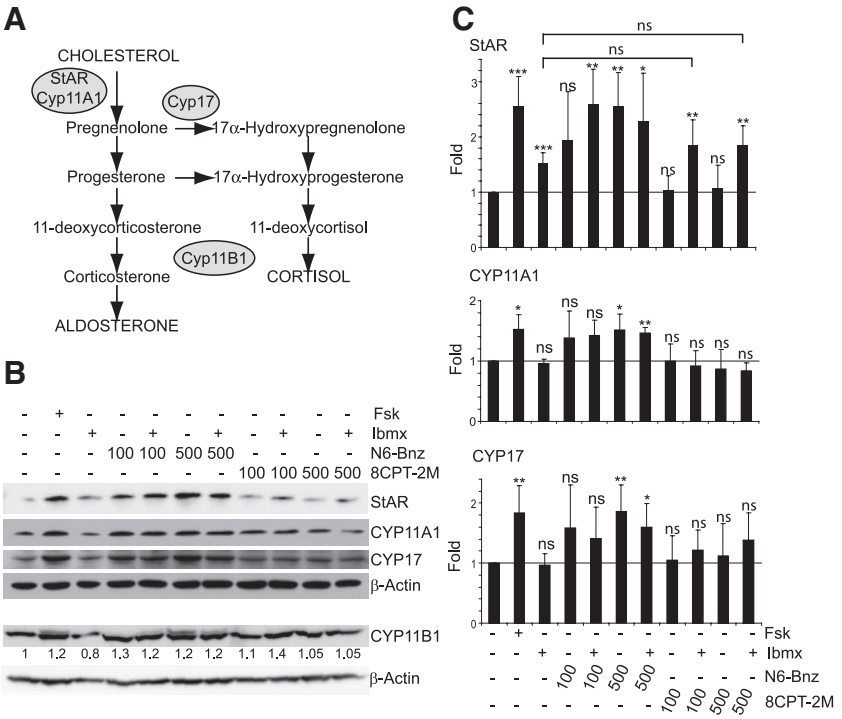

FIG. 5. The PKA-specific cAMP analog induces the expression of StAR and steroid hydroxylases in H295R cells. A, Diagram representing the enzymes and the pathways involved in the synthesis of cortisol and aldosterone in human adrenocortical cells. B, H295R cells were incubated with forskolin (Fsk) alone (10 μM), the EPAC-specific cAMP analog (8CPT-2M), or the PKA-specific cAMP analog (N6-Bnz) at 100 and 500 μM for 24 h after a 30-min preincubation with IBMX (500 μM), as indicated. Cell extracts (25 μg) were subjected to Western immunoblotting using antibodies against StAR, CYP11A1, CYP17, CYP11B1, and β-actin. C, For each factor analyzed, two to four independent experiments were performed, and the densitometric quantifications are shown as means ± SD [Student's *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control (−); ns, not significant].

Consistent with the effects on steroid hormones synthesis, the PKA-specific cAMP analog, but not the EPAC-specific cAMP analog, stimulated the expression of different factors involved in the metabolism of cholesterol, (*i.e.* StAR, NGFI-B, CYP11A1, and CYP17). The results on the regulation of CYP11A1 and CYP17 expression are in agreement with previous reports either comparing Y1 and Kin-8 cells (CYPT11A1) (53, 54) or using a pharmacological inhibitor of PKA in Leydig cells (CYP17) (55), which show that cAMP-induced transcription of the corresponding genes is dependent upon PKA activation. We have also observed that the CYP17 promoter is activated by the PKA- but not the EPAC-specific cAMP analog in H295R cells (data not shown). ACTH and cAMP both induce transcription from the NGFI-B gene (43, 44) and regulate its phosphorylation state (56). Pharmacological inhibition of PKA showed that cAMP induced the transcription of NGFI-B in corticotropes (57) in a PKA-dependent manner, and our data now clarified the primary involvement of PKA in the induction of NGFI-B at the protein level. StAR levels were also increased only after PKA activation, which is consistent with previous studies (54). The regulation of StAR expression by cAMP is unlike that of steroid hydroxylases. PKA is not involved in the transcriptional expression of StAR but rather functions by regulating its levels posttranslationally, presumably by regulating its stability (54). In support of these data, we have observed that the activation of neither PKA nor EPAC could recapitulate the effects of cAMP-elevating agents on the StAR promoter activity in luciferase reporter assays in Y1 cells (data not shown). Other signaling molecules downstream of cAMP are therefore likely to be required for StAR transcription, and ERK1/2 (58) and Ca^{2+}/calmodulin kinases (24) have been shown to be possible candidates.

cAMP is well known to induce characteristic changes in the cell shape and concomitant reorganization of F-actin microfilaments in adrenocortical cells (reviewed in Ref. 18). These functional responses have previously been attributed partly to PKA (52, 59). For instance, Kin-8 cells demonstrate only a partial resistance to cell rounding after 8-Br-cAMP treatment (52). Although it is not excluded that Kin-8 cells have residual PKA activity, this result supports the data presented in Figs. 3 and 4, namely that the EPAC-specific analog, in addition to the PKA-specific analog, can reproduce the effects of cAMP on cell rounding and the concomitant reorganization of F-actin microfilaments. Our results are also consistent with the effect of the EPAC-specific analog on the breakdown of actin stress fibers in endothelial cells (60–63) and in prostate cancer cells (64). Activation of EPAC2B also significantly inhibited cell migration in H295R cells (Fig. 4B), and this is in line with a recent study by Grandoch *et al.* (64) showing that the EPAC-specific cAMP analog inhibited the migration of prostate cancer cells. As has been extensively reported previously, we found that PKA also inhibits migration (reviewed in Ref. 65). Both the PKA- and EPAC-activated pathways required simultaneous inhibition of PDEs to achieve a similar level of response as with forskolin for the regulation of cell shape and actin remodeling (Figs. 3 and 4A). Synthetic cAMP analogs are hydrolyzed by PDEs (34, 66), which might explain the requirement of IBMX in these experiments. In the scratch assay, IBMX had a significant effect by itself on cell migration (Fig. 4B), and we cannot exclude a direct role for PDEs in actin remodeling

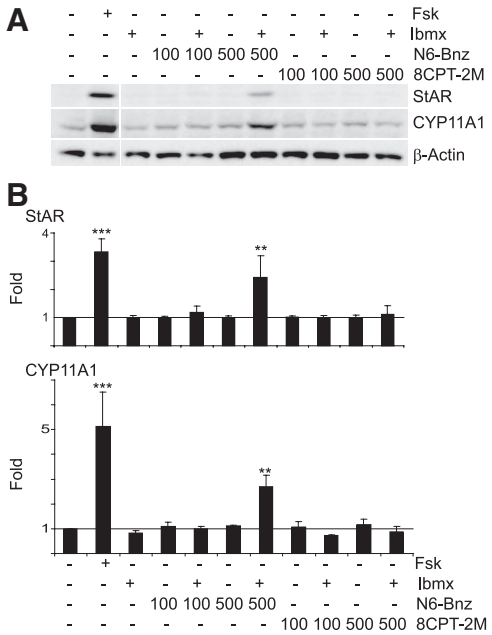

FIG. 6. The PKA-specific cAMP analog induces the expression of StAR and CYP11A1 in Y1 cells. A, Y1 cells were incubated with forskolin (Fsk) alone (10 μM), the EPAC-specific cAMP analog (8CPT-2M), or the PKA-specific cAMP analog (N6-Bnz) at 100 and 500 μM for 24 h after a 30-min preincubation with IBMX (500 μM) as indicated. Cell extracts (25 μg) were subjected to Western immunoblotting using antibodies against StAR, CYP11A1, and β-actin. B, For each factor analyzed, two to three independent experiments were performed, and the densitometric quantifications are shown as means ± SD [one-way ANOVA: **, $P < 0.01$; ***, $P < 0.001$ compared with control (−)].

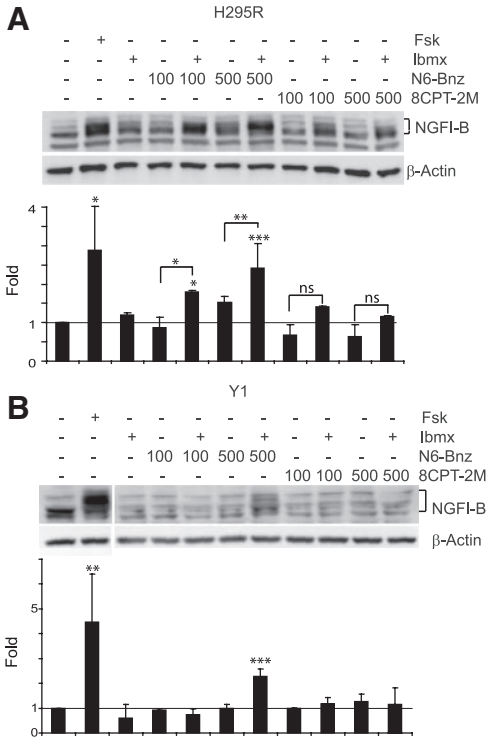

FIG. 7. PKA, but not EPAC2B, induces the expression of NGFI-B in H295R and Y1 cells. H295R (A) and Y1 (B) cells were incubated with forskolin (Fsk) alone (10 μM), the EPAC-specific cAMP analog (8CPT-2M), or the PKA-specific cAMP analog (N6-Bnz) at 100 and 500 μM for 2 h after a 30-min preincubation with IBMX (500 μM) as indicated. Cell extracts (40 μg) were subjected to Western immunoblotting using antibodies against NGFI-B and β-actin. The densitometric measurements of two to three independent experiments were quantified and shown as the mean ratios ± SD [one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control (−); ns, not significant].

and migration. Accordingly, a recent study demonstrates that the inhibition of PDE4 (using the specific inhibitor rolipram) stimulated integrin-induced actin assembly and reduced cell migration, clearly pointing to a more direct role for PDE4 (67). To the best of our knowledge, there are no reports on the expression and the exact function of PDE4 in the adrenal cortex. PDE2, PDE8B, and PDE11A have been shown to be expressed in the adrenal cortex (68–70), and in particular, the activity of PDE2 was demonstrated to be tightly connected to the regulation of cAMP levels in rat glomerulosa cells (71). The aforementioned PDE members may therefore be plausible candidates for the effects observed with IBMX.

In summary, we have shown that PKA and EPAC2B have distinct but also overlapping functional roles in adrenocortical cells. PKA is clearly the mediator of cAMP-induced effects on steroidogenesis. However, both PKA and EPAC2B affect the actin cytoskeleton integrity and cell migration. This study is the first report showing the involvement of the splicing variant EPAC2B in these cAMP-mediated responses and further establishes a role for EPAC1/2 in actin reorganization and migration. Furthermore, the role of EPAC2B in cell motility may have wider implications, such as for the organization of the adrenal gland and for adrenal cancer cell invasion.

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References

1. **Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM** 1998 A family of cAMP-binding proteins that directly activate Rap1. Science 282:2275–2279
2. **de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL** 1998 Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 396: 474–477
3. **Borland G, Smith BO, Yarwood SJ** 2009 EPAC proteins transduce diverse cellular actions of cAMP. Br J Pharmacol 158:70–86
4. **Sands WA, Palmer TM** 2008 Regulating gene transcription in response to cyclic AMP elevation. Cell Signal 20:460–466
5. **Gonzalez-Robayna IJ, Falender AE, Ochsner S, Firestone GL, Richards JS** 2000 Follicle-stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-induced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. Mol Endocrinol 14:1283–1300
6. **Dremier S, Pohl V, Poteet-Smith C, Roger PP, Corbin J, Doskeland SO, Dumont JE, Maenhaut C** 1997 Activation of cyclic AMP-dependent kinase is required but may not be sufficient to mimic cyclic AMP-dependent DNA synthesis and thyroglobulin expression in dog thyroid cells. Mol Cell Biol 17:6717–6726
7. **Cass LA, Summers SA, Prendergast GV, Backer JM, Birnbaum MJ, Meinkoth JL** 1999 Protein kinase A-dependent and -independent signaling pathways contribute to cyclic AMP-stimulated proliferation. Mol Cell Biol 19:5882–5891
8. **Mei FC, Qiao J, Tsygankova OM, Meinkoth JL, Quilliam LA, Cheng X** 2002 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. J Biol Chem 277:11497–11504
9. **Ozaki N, Shibasaki T, Kashima Y, Miki T, Takahashi K, Ueno H, Sunaga Y, Yano H, Matsuura Y, Iwanaga T, Takai Y, Seino S** 2000 cAMP-GEFII is a direct target of cAMP in regulated exocytosis. Nat Cell Biol 2:805–811
10. **Kashima Y, Miki T, Shibasaki T, Ozaki N, Miyazaki M, Yano H, Seino S** 2001 Critical role of cAMP-GEFII-Rim2 complex in incretin-potentiated insulin secretion. J Biol Chem 276:46046–46053
11. **Sedej S, Rose T, Rupnik M** 2005 cAMP increases Ca^{2+}-dependent exocytosis through both PKA and Epac2 in mouse melanotrophs from pituitary tissue slices. J Physiol 567:799–813
12. **Islam D, Zhang N, Wang P, Li H, Brubaker PL, Gaisano HY, Wang Q, Jin T** 2009 Epac is involved in cAMP-stimulated proglucagon expression and hormone production but not hormone secretion in pancreatic α- and intestinal L-cell lines. Am J Physiol Endocrinol Metab 296:E174–E181
13. **Bos JL** 2003 Epac: a new cAMP target and new avenues in cAMP research. Nat Rev Mol Cell Biol 4:733–738
14. **Rehmann H, Prakash B, Wolf E, Rueppel A, de Rooij J, Bos JL, Wittinghofer A** 2003 Structure and regulation of the cAMP-binding domains of Epac2. Nat Struct Biol 10:26–32
15. **Niimura M, Miki T, Shibasaki T, Fujimoto W, Iwanaga T, Seino S** 2009 Critical role of the N-terminal cyclic AMP-binding domain of Epac2 in its subcellular localization and function. J Cell Physiol 219:652–658
16. **Ueno H, Shibasaki T, Iwanaga T, Takahashi K, Yokoyama Y, Liu LM, Yokoi N, Ozaki N, Matsukura S, Yano H, Seino S** 2001 Characterization of the gene EPAC2: structure, chromosomal localization, tissue expression, and identification of the liver-specific isoform. Genomics 78:91–98
17. **Sewer MB, Waterman MR** 2003 ACTH modulation of transcription factors responsible for steroid hydroxylase gene expression in the adrenal cortex. Microsc Res Tech 61:300–307
18. **Gallo-Payet N, Payet MD** 2003 Mechanism of action of ACTH: beyond cAMP. Microsc Res Tech 61:275–287
19. **Cooke BA** 1999 Signal transduction involving cyclic AMP-dependent and cyclic AMP-independent mechanisms in the control of steroidogenesis. Mol Cell Endocrinol 151:25–35
20. **Schimmer BP** 1995 The 1994 Upjohn Award Lecture. Molecular and genetic approaches to the study of signal transduction in the adrenal cortex. Can J Physiol Pharmacol 73:1097–1107
21. **Payne AH, Hales DB** 2004 Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 25:947–970
22. **Liu H, Enyeart JA, Enyeart JJ** 2008 ACTH inhibits bTREK-1 K^+ channels through multiple cAMP-dependent signaling pathways. J Gen Physiol 132:279–294
23. **Le T, Schimmer BP** 2001 The regulation of MAPKs in Y1 mouse adrenocortical tumor cells. Endocrinology 142:4282–4287
24. **Gambaryan S, Butt E, Tas P, Smolenski A, Allolio B, Walter U** 2006 Regulation of aldosterone production from zona glomerulosa cells by ANG II and cAMP: evidence for PKA-independent activation of CaMK by cAMP. Am J Physiol Endocrinol Metab 290:E423–E433
25. **de Rooij J, Rehmann H, van Triest M, Cool RH, Wittinghofer A, Bos JL** 2000 Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. J Biol Chem 275:20829–20836
26. **Schimmer BP** 1979 Adrenocortical Y1 cells. Methods Enzymol 58: 570–574
27. **Peschon JJ, Behringer RR, Cate RL, Harwood KA, Idzerda RL, Brinster RL, Palmiter RD** 1992 Directed expression of an oncogene to Sertoli cells in transgenic mice using Mullerian inhibiting substance regulatory sequences. Mol Endocrinol 6:1403–1411
28. **Dutertre M, Rey R, Porteu A, Josso N, Picard JY** 1997 A mouse Sertoli cell line expressing anti-Mullerian hormone and its type II receptor. Mol Cell Endocrinol 136:57–65
29. **Rainey WE, Bird IM, Mason JI** 1994 The NCI-H295 cell line: a pluripotent model for human adrenocortical studies. Mol Cell Endocrinol 100:45–50
30. **Hoivik EA, Aumo L, Aesoy R, Lillefosse H, Lewis AE, Perrett RM, Stallings NR, Hanley NA, Bakke M** 2008 Deoxyribonucleic acid methylation controls cell type-specific expression of steroidogenic factor 1. Endocrinology 149:5599–5609
31. **Ascoli M** 1981 Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses. Endocrinology 108:88–95
32. **van Triest M, de Rooij J, Bos JL** 2001 Measurement of GTP-bound Ras-like GTPases by activation-specific probes. Methods Enzymol 333:343–348
33. **Rainey WE, Saner K, Schimmer BP** 2004 Adrenocortical cell lines. Mol Cell Endocrinol 228:23–38
34. **Holz GG, Chepurny OG, Schwede F** 2008 Epac-selective cAMP analogs: new tools with which to evaluate the signal transduction properties of cAMP-regulated guanine nucleotide exchange factors. Cell Signal 20:10–20
35. **Cuprak LJ, Lammi CJ, Bayer RC** 1977 Scanning electron microscopy of induced cell rounding of mouse adrenal cortex tumor cells in culture. Tissue Cell 9:667–680
36. **Voorhees HL, Aschenbrenner J, Carnes J, Mrotek JJ** 1984 Rounding and steroidogenesis of enzyme- and ACTH-treated Y-l mouse adrenal tumor cells. Cell Biol Int Rep 8:483–497
37. **Le Clainche C, Carlier MF** 2008 Regulation of actin assembly associated with protrusion and adhesion in cell migration. Physiol Rev 88:489–513
38. **Han JD, Rubin CS** 1996 Regulation of cytoskeleton organization and paxillin dephosphorylation by cAMP. Studies on murine Y1 adrenal cells. J Biol Chem 271:29211–29215
39. **Côté M, Payet MD, Gallo-Payet N** 1997 Association of α_S-subunit of the G_S protein with microfilaments and microtubules: implication during adrenocorticotropin stimulation in rat adrenal glomerulosa cells. Endocrinology 138:69–78
40. **Masui H, Garren LD** 1971 Inhibition of replication in functional mouse adrenal tumor cells by adrenocorticotropic hormone mediated by adenosine 3′:5′-cyclic monophosphate. Proc Natl Acad Sci USA 68:3206–3210

41. **Armelin MC, Gambarini AG, Armelin HA** 1977 On the regulation of DNA synthesis in a line of adrenocortical tumor cells: effect of serum, adrenocorticotropin and pituitary factors. J Cell Physiol 93:1–9
42. **Perkins LM, Payne AH** 1988 Quantification of P450scc, P450(17) alpha, and iron sulfur protein reductase in Leydig cells and adrenals of inbred strains of mice. Endocrinology 123:2675–2682
43. **Davis IJ, Lau LF** 1994 Endocrine and neurogenic regulation of the orphan nuclear receptors Nur77 and Nurr-1 in the adrenal glands. Mol Cell Biol 14:3469–3483
44. **Wilson TE, Mouw AR, Weaver CA, Milbrandt J, Parker KL** 1993 The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase. Mol Cell Biol 13:861–868
45. **Zhang P, Mellon SH** 1997 Multiple orphan nuclear receptors converge to regulate rat P450c17 gene transcription: novel mechanisms for orphan nuclear receptor action. Mol Endocrinol 11:891–904
46. **Bassett MH, Suzuki T, Sasano H, De Vries CJ, Jimenez PT, Carr BR, Rainey WE** 2004 The orphan nuclear receptor NGFIB regulates transcription of 3β-hydroxysteroid dehydrogenase: implications for the control of adrenal functional zonation. J Biol Chem 279:37622–37630
47. **Qiao J, Mei FC, Popov VL, Vergara LA, Cheng X** 2002 Cell cycle-dependent subcellular localization of exchange factor directly activated by cAMP. J Biol Chem 277:26581–26586
48. **Huston E, Lynch MJ, Mohamed A, Collins DM, Hill EV, MacLeod R, Krause E, Baillie GS, Houslay MD** 2008 EPAC and PKA allow cAMP dual control over DNA-PK nuclear translocation. Proc Natl Acad Sci USA 105:12791–12796
49. **Christensen AE, Selheim F, de Rooij J, Dremier S, Schwede F, Dao KK, Martinez A, Maenhaut C, Bos JL, Genieser HG, Døskeland SO** 2003 cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. J Biol Chem 278:35394–35402
50. **Petersen RK, Madsen L, Pedersen LM, Hallenborg P, Hagland H, Viste K, Døskeland SO, Kristiansen K** 2008 Cyclic AMP (cAMP)-mediated stimulation of adipocyte differentiation requires the synergistic action of Epac- and cAMP-dependent protein kinase-dependent processes. Mol Cell Biol 28:3804–3816
51. **Hochbaum D, Hong K, Barila G, Ribeiro-Neto F, Altschuler DL** 2008 Epac, in synergy with cAMP-dependent protein kinase (PKA), is required for cAMP-mediated mitogenesis. J Biol Chem 283:4464–4468
52. **Olson MF, Krolczyk AJ, Gorman KB, Steinberg RA, Schimmer BP** 1993 Molecular basis for the 3′,5′-cyclic adenosine monophosphate resistance of Kin mutant Y1 adrenocortical tumor cells. Mol Endocrinol 7:477–487
53. **Wong M, Rice DA, Parker KL, Schimmer BP** 1989 The roles of cAMP and cAMP-dependent protein kinase in the expression of cholesterol side chain cleavage and steroid 11β-hydroxylase genes in mouse adrenocortical tumor cells. J Biol Chem 264:12867–12871
54. **Clark BJ, Ranganathan V, Combs R** 2001 Steroidogenic acute regulatory protein expression is dependent upon post-translational effects of cAMP-dependent protein kinase A. Mol Cell Endocrinol 173:183–192
55. **Laurich VM, Trbovich AM, O'Neill FH, Houk CP, Sluss PM, Payne AH, Donahoe PK, Teixeira J** 2002 Mullerian inhibiting substance blocks the protein kinase A-induced expression of cytochrome p450 17α-hydroxylase/C(17–20) lyase mRNA in a mouse Leydig cell line independent of cAMP responsive element binding protein phosphorylation. Endocrinology 143:3351–3360
56. **Li Y, Lau LF** 1997 Adrenocorticotropic hormone regulates the activities of the orphan nuclear receptor Nur77 through modulation of phosphorylation. Endocrinology 138:4138–4146
57. **Kovalovsky D, Refojo D, Liberman AC, Hochbaum D, Pereda MP, Coso OA, Stalla GK, Holsboer F, Arzt E** 2002 Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. Mol Endocrinol 16:1638–1651
58. **Gyles SL, Burns CJ, Whitehouse BJ, Sugden D, Marsh PJ, Persaud SJ, Jones PM** 2001 ERKs regulate cyclic AMP-induced steroid synthesis through transcription of the steroidogenic acute regulatory (StAR) gene. J Biol Chem 276:34888–34895
59. **Colonna C, Podestá EJ** 2005 ACTH-induced caveolin-1 tyrosine phosphorylation is related to podosome assembly in Y1 adrenal cells. Exp Cell Res 304:432–442
60. **Kooistra MR, Corada M, Dejana E, Bos JL** 2005 Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. FEBS Lett 579:4966–4972
61. **Cullere X, Shaw SK, Andersson L, Hirahashi J, Luscinskas FW, Mayadas TN** 2005 Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. Blood 105:1950–1955
62. **Fukuhara S, Sakurai A, Sano H, Yamagishi A, Somekawa S, Takakura N, Saito Y, Kangawa K, Mochizuki N** 2005 Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway. Mol Cell Biol 25:136–146
63. **Wittchen ES, Worthylake RA, Kelly P, Casey PJ, Quilliam LA, Burridge K** 2005 Rap1 GTPase inhibits leukocyte transmigration by promoting endothelial barrier function. J Biol Chem 280:11675–11682
64. **Grandoch M, Rose A, ter Braak M, Jendrossek V, Rübben H, Fischer JW, Schmidt M, Weber AA** 2009 Epac inhibits migration and proliferation of human prostate carcinoma cells. Br J Cancer 101:2038–2042
65. **Howe AK** 2004 Regulation of actin-based cell migration by cAMP/PKA. Biochim Biophys Acta 1692:159–174
66. **Poppe H, Rybalkin SD, Rehmann H, Hinds TR, Tang XB, Christensen AE, Schwede F, Genieser HG, Bos JL, Doskeland SO, Beavo JA, Butt E** 2008 Cyclic nucleotide analogs as probes of signaling pathways. Nat Methods 5:277–278
67. **Fleming YM, Frame MC, Houslay MD** 2004 PDE4-regulated cAMP degradation controls the assembly of integrin-dependent actin adhesion structures and REF52 cell migration. J Cell Sci 117:2377–2388
68. **MacFarland RT, Zelus BD, Beavo JA** 1991 High concentrations of a cGMP-stimulated phosphodiesterase mediate ANP-induced decreases in cAMP and steroidogenesis in adrenal glomerulosa cells. J Biol Chem 266:136–142
69. **Horvath A, Giatzakis C, Tsang K, Greene E, Osorio P, Boikos S, Libè R, Patronas Y, Robinson-White A, Remmers E, Bertherat J, Nesterova M, Stratakis CA** 2008 A cAMP-specific phosphodiesterase (PDE8B) that is mutated in adrenal hyperplasia is expressed widely in human and mouse tissues: a novel PDE8B isoform in human adrenal cortex. Eur J Hum Genet 16:1245–1253
70. **Boikos SA, Horvath A, Heyerdahl S, Stein E, Robinson-White A, Bossis I, Bertherat J, Carney JA, Stratakis CA** 2008 Phosphodiesterase 11A expression in the adrenal cortex, primary pigmented nodular adrenocortical disease, and other corticotropin-independent lesions. Horm Metab Res 40:347–353
71. **Côté M, Payet MD, Rousseau E, Guillon G, Gallo-Payet N** 1999 Comparative involvement of cyclic nucleotide phosphodiesterases and adenylyl cyclase on adrenocorticotropin-induced increase of cyclic adenosine monophosphate in rat and human glomerulosa cells. Endocrinology 140:3594–3601