cAMP-stimulated transcription of DGKθ requires steroidogenic factor 1 and sterol regulatory element binding protein 1

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Abstract  Diacylglycerol kinase (DGK)θ is a lipid kinase that phosphorylates diacylglycerol to form phosphatidic acid (PA). We have previously shown that PA is a ligand for the nuclear receptor steroidogenic factor 1 (SF1) and that cAMP-stimulated expression of SF1 target genes requires DGKθ. In this study, we sought to investigate the role of cAMP signaling in regulating DGKθ gene expression. Real time RT-PCR and Western blot analysis revealed that dibutyryl cAMP (Bt2cAMP) increased the mRNA and protein expression, respectively, of DGKθ in H295R human adrenocortical cells. SF1 and sterol regulatory element binding protein 1 (SREBP1) increased the transcriptional activity of a reporter plasmid containing 1.5 kb of the DGKθ promoter fused to the luciferase gene. Mutation of putative cAMP responsive sequences abolished SF1- and SREBP-dependent DGKθ reporter gene activation. Consistent with this finding, chromatin immunoprecipitation assay demonstrated that Bt2cAMP signaling increased the recruitment of SF1 and SREBP1 to the DGKθ promoter. Coimmunoprecipitation assay revealed that SF1 and SREBP1 interact, suggesting that the two transcription factors form a complex on the DGKθ promoter. Finally, silencing SF1 and SREBP1 abolished cAMP-stimulated DGKθ expression. Taken together, we demonstrate that SF1 and SREBP1 activate DGKθ transcription in a cAMP-dependent manner in human adrenocortical cells.

Supplementary key words  diacylglycerol kinase θ • adrenal cortex • cAMP

Diacylglycerol kinases (DGKs) are intracellular lipid kinases that phosphorylate diacylglycerol (DAG) to form phosphatidic acid (PA), which is linked to lipid metabolism and signaling (1–3). For example, targeted disruption of DGKθ in mice impairs epidermal growth factor receptor expression and increases protein kinase C (PKC) activity (4). DGKθ-null mice exhibit several neural abnormalities, including a higher resistance of electroconvulsive shock (5) and increased cyclooxygenase 2 and tyrosine hydroxylase expression (6), suggesting a role for DGKθ in regulating synaptic activity. Mice lacking DGKα (7) or DGKζ (8) exhibit enhanced T cell function and demonstrate a role for these kinases in controlling DAG metabolism during the immune response. DGK isoforms have been implicated in various other cellular processes including inhibition of Rap1 signaling (9) and retinoblastoma-mediated cell cycle control (10). DGKθ is activated by nerve growth factor in PC12 cells (11) and thrombin in IIC9 fibroblasts (12, 13), whereas DGKζ promotes myogenesis in C2C12 cells (14) and DGKθ plays a role in regulating the cell cycle in CHO-K1 cells (15).

To date, 10 mammalian DGKs have been identified that are divided into five groups based on functional domains (16, 17). However, all isoforms contain cysteine-rich zinc finger-like structures, a conserved catalytic region (18–21). DGKθ, the sole member of group V, is comprised of three cysteine-rich domains (CRDs), a proline/glycine-rich domain at its N terminus, and a pleckstrin homology (PH) domain with an overlapping Ras-binding domain (22). While the functions of many of the other domains in DGKθ are unclear, the catalytic activity requires all domains of the enzyme (23). It has been postulated that the CRDs of the enzyme are required both for correct folding of the protein and for substrate presentation (23). Mutation of the CRD of DGKθ diminishes DAG-induced translocation of the enzyme to the plasma membrane (24); whereas the interaction between DGKθ and the nuclear receptor steroidogenic factor 1 (SF1) requires the PH domain (25).

Abbreviations: ACTH, adrenocorticotrophic hormone; bHLH/ZIP, basic helix-loop-helix leucine zipper; Bt2cAMP, dibutyryl cAMP; ChIP, chromatin immunoprecipitation; CRD, cysteine-rich domain; DAG, diacylglycerol; DGK, diacylglycerol kinase; LRH, liver receptor homolog; PA, phosphatidic acid; PH, pleckstrin homology; PK, protein kinase; RIP, radioimmunoprecipitation; SF1, steroidogenic factor 1; shRNA, short hairpin RNA; SREBP, sterol regulatory element binding protein.

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The ability of distinct isoforms to exert regulatory control occurs through unique interactions with protein partners, and differential subcellular localization of DGK isoforms is thought to enable local regulation of DAG and PA concentrations for spatial and temporally separated cellular processes. Many studies have demonstrated roles for compartmentalized DGK activity in nuclear processes. Both DGKα (26) and DGKζ (14) are localized in punctate structures that are enriched in pre-mRNA splicing factors called nuclear speckles. DGKζ is colocalized with hyperphosphorylated RNA polymerase II and the splicing factor SC-35 in the nuclear speckles of various cell types, including PC12, HeLa, and MCF-7 (26). Interestingly, nuclear speckles have been shown to sequester posttranslationally modified SF1 (27, 28).

SF1 induces the transcription of genes involved in steroid hormone biosynthesis and endocrine development and function (29–31). We have previously shown that cAMP signaling increases the transcription of CYP17A1 by stimulating the binding of SF1 to the CYP17A1 promoter (32, 33). We have also shown that DGKα regulates the production of PA, a ligand for SF1 that is produced in response to cAMP signaling (25). DGKα acts as a coregulatory protein by binding to SF1 when the receptor is bound to chromatin. The PA produced in response to DGKα activation stimulates SF1-dependent gene transcription by promoting coactivator recruitment to SF1 target genes, thereby inducing the mRNA expression of CYP17A1 and several other steroidogenic genes. In contrast, inhibition of DGK activity attenuates the binding of SF1-dependent gene expression, and silencing the expression of DGKα expression inhibits cAMP-dependent CYP17A1 transcription. Finally, we have also shown that LXXLL motifs in DGKα mediate a direct interaction of SF1 with the kinase and may facilitate ligand delivery (25). To date, studies have demonstrated that DGKα is regulated by intracellular targeting (24), membrane lipids (12), protein-protein interactions (34), and intrinsic activity (12). However, the factors that control DGKα gene expression in the adrenal cortex are poorly understood. In this study, we defined the role of cAMP signaling in regulating the expression of DGKα.

MATERIALS AND METHODS

Materials

Dibutyryl cAMP (Bt2-CAMP) and tetracycline were obtained from Sigma (St. Louis, MO) and H89 from EMD Biosciences (La Jolla, CA).

Cell culture

H295R adrenocortical cells (35, 36) were generously donated by Dr. William E. Rainey (University of Michigan, Ann Arbor, MI) and cultured in Dulbecco’s modified Eagle’s medium/F-12 (DMEM/F-12) (Invitrogen, Carlsbad, CA) supplemented with 10% Nu-Serum I (BD Bioscience, Palo Alto, CA), 1% ITS Plus (BD Bioscience), antibiotics, and antimitotics. CV-1 monkey kidney cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and cultured in Eagle’s minimum essential medium (MEM) (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Mediatech, Inc.), antibiotics, and antimitotics. SF1 and sterol regulatory element binding protein 1 (SREBP1) knockdown cell lines were generated by transfecting H295R cells with short hairpin RNA (shRNA) plasmids (in the pGFP-V-RS HusH vector; OriGene, Rockville, MD) containing the following oligonucleotides: SF1 5’-TCC TGG CGG TCG CAT CAA GTC TGA CC and SREBP1 5’-ATC TAT GTG CGG GCT GTA TTG AGA GTG AA. Stable clones were selected using 10 μg/ml puromycin (Mediatech, Inc.). H295R cells expressing tetracycline-inducible DGKα shRNA were generated using the BLOCK-iT Inducible H1 RNAi Entry Vector Kit (Invitrogen) as previously described (37). To construct an inducible vector for DGKα shRNA, the following sequences were cloned into pENTR/H1/T0 5’-ACC GCC CAG CAG TGA AGG CCT CAT CTT CAC GAA TGA AGA TGG GCC CCTCAA TAC TGG G-3’ and 5’-AAA CCC AGT ATT GAA GGC GCG C TTC ATC TTG CGT CAA GAT GAG GCC CCC TTC AAT ACT GGG C-3’. H295R-TetR cells were stably transfected with the constructed pENTR/H1/T0-DGKα shRNA expression vector or the control vector using GeneJuice (EMD Biosciences), and cell clones were selected using 5 μg/ml tetracycline for 96 h and suppression of DGKα protein levels in each clone was confirmed by Western blotting using an anti-DGKα antibody (Sigma).

Cloning and mutagenesis

The human DGKQ promoter was cloned using LA Taq DNA polymerase (Takara, Madison, WI), 500 ng of human genomic DNA (Promega, Madison, WI) and 300 nM of the following primers: forward 5’-CGA GCT CTT AGT ACC GCT CTA GCT CTC TCT CGG CCG CCC CGG C. PCR fragments were cloned into the MluI (5’) and BglII (3’) sites of the pGL3 Firefly luciferase vector (Promega). Putative SF1 and SREBP1 response elements were identified by in silico analysis using MatInspector (Genomatix Software, Ann Arbor, MI) and site-directed mutagenesis performed using the following primers: M1 forward 5’-ACT GGG TGC GAT GGC CCT AAA GCC CTG CCC TCT GC and reverse 5’-GCC CCA CGG GGG CAA AAA CCC...
RNA isolation and quantitative RT-PCR

Cells were sub-cultured onto 12-well plates and 24 h later treated with 0.4 mM Bt2cAMP for 1–24 h. Total RNA was extracted using Iso-RNA Lysis Reagent (5 Prime Inc., Gaithersburg, MD) and amplified using a One-Step SYBR Green RT-PCR Kit (Thermo Fisher Scientific Inc., Waltham, MA) and the primer pairs described in Li et al. (25). DGK expression was normalized to β-actin content and calculated using delta-delta cycle threshold (ΔΔCT) method.

Western blotting

H295R cells were sub-cultured onto 6-well plates and treated with 0.4 mM Bt2cAMP for 24 h, 48 h, or 72 h and harvested into radioimmunoprecipitation assay (RIPA) buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (150 nM aprotinin, 1 mM leupeptin, 1 mM E6-44, 500 mM 4-(2-aminomethyl)benzenesulfonylfluoride; EMD Biosciences). Cells were then lysed by sonication (one 5 s burst) followed by incubation on ice for 30 min. Lysates were centrifuged for 10 min at 4°C and the supernatant collected for analysis by SDS-PAGE. Protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce). Aliquots of each sample (25 μg of protein) were run on 8% SDS-PAGE gels and transferred to Immobilon-FL polyvinylidene difluoride (PVDF) membranes (IPFL00010; Millipore, Billerica, MA). Blots were probed with an anti-DGKα (1:1000, HPA026797; Sigma-Aldrich, St. Louis, MO), anti-SREBP1 (1:1000, sc-8984; Santa Cruz Biotechnology, Santa Cruz, CA), SF1 (1:4000; Millipore, Temecula, CA), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000, sc-25778; Santa Cruz Biotechnology) and an anti-rabbit or mouse secondary antibody (1:5000, ECF Western blotting reagent; GE Healthcare, Piscataway, NJ). Blots were scanned on a VersaDoc 4000 Imager (Bio-Rad, Hercules, CA) and densitometric analysis carried out using Quantity One software (Bio-Rad).

Nuclear and cytoplasmic extract isolation

H295R cells were cultured in 100 mm dishes and treated with Bt2cAMP for 48 h. Cytoplasmic and nuclear extracts were harvested from H295R cells and separated using Thermo NE-PER® nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL). Western blotting analysis was carried out as described above. Blots were probed with anti-DGKα (1:1000, HPA026797; Sigma) and anti-lamin A/C (1:5000, sc-376248; Santa Cruz Biotechnology) or anti-β-tubulin (1:2000, sc-23949; Santa Cruz Biotechnology).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as described previously in (32, 39, 40). Briefly, H295R cells were sub-cultured onto 150 mm dishes and treated with Bt2cAMP for 60 min. Cells were treated with 1% formaldehyde for 10 min at room temperature and then incubated for 5 min with 0.125 M glycine. After twice washing with PBS, cells were harvested into RIPA with 0.4 mM Bt2cAMP. Whole cell lysates were harvested and analyzed by SDS-PAGE and Western blotting using anti-DGKα antibody. Data graphed are densitometric analysis of Western blots of DGKα protein expression in cells treated for 24–72 h with 0.4 mM Bt2cAMP. DGKα protein expression normalized to GAPDH expression is graphed and represents the mean ± SEM of four separate experiments, each carried out in triplicate. Asterisks indicate a statistically significant difference compared with the untreated 0 h control group (P < 0.05).
buffer. The purified chromatin solutions were immunoprecipitated using 5 μg of anti-acetyl-K5, K8, K12, K16 histone H4 (60-866; Millipore, Temecula, CA), anti-SF1 (07-618; Millipore), SREBP1 (sc-8984; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-IgG protein A/G plus (sc-2003; Santa Cruz Biotechnology). Real-time PCR was carried out using the following primer sets: forward 5′-CAG AGT CCA CAG CCC CCA GCC CCT TTC AGG and reverse 5′-CTG CCT CGT GCG CGC CAC GGG TCT TGT TCA. Output DNA (immunoprecipitated promoter region) was normalized to input DNA. PCR products were separated on 2% agarose gels and the EtBr-stained bands imaged using a VersaDoc 4000 (Bio-Rad).

**Comminunoprecipitation**

CV1 cells were plated onto 100 mm dishes and transfected with pCMV6-GFP-SF1, pcDNA 3.1-FLAG SREBP1c for 48 h. Five percent of lysates were retained as input and the remaining cell lysates were incubated with an anti-FLAG M2 mouse monoclonal antibody (5 μg; F1804, Sigma) and protein A/G agarose beads (Santa Cruz Biotechnology) overnight at 4°C with rotation. Beads were washed three times with RIPA buffer and twice with PBS and the immobilized proteins separated by SDS-PAGE. Output blots were probed with anti-SF1 (1:5000, 07-618; Millipore) and input blots with anti-FLAG (1:2500, F1804, Sigma). Expression was detected using an ECF Western blotting kit (GE Biosciences) and visualized using a VersaDoc 4000 imager (Bio-Rad).

**PA assay**

H295R cells were grown on 100 mm dishes and then treated with Bt2cAMP from 24 h to 48 h and total lipid extract was harvested. Nuclear were purified using a Nuclei Pure kit (Sigma) and PA content was quantified using a Total PA kit (Cayman, Ann Arbor, MI) on a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA) at an excitation wavelength of 530–540 nM and an emission wavelength of 585–595 nm. Data was quantified using through SoftMax Pro software (Molecular Devices).

**DAG assay**

H295R cells were cultured onto 6-well plates and treated with Bt2cAMP from 72 h and cells were harvested with PBS. PBS was aspirated and the content of DAG in each sample was determined using a Human DAG ELISA kit (MyBioSource, Inc., San Diego, CA).

**Statistical analysis**

One-way ANOVA and Tukey-Kramer multiple comparisons were performed using Prism 5.0 (GraphPad Software, San Diego, CA). Significant difference value was set as $P < 0.05$.

**RESULTS**

**cAMP induces DGKα mRNA expression**

We have previously shown that adrenocorticotropic hormone (ACTH) signaling rapidly increases DGKα activity (25). Therefore, in this study we sought to determine the effect of increased intracellular cAMP on DGKα gene expression. H295R human adrenocortical cells were treated with Bt2cAMP for 24 h and RNA isolated for qRT-PCR. DGKα mRNA expression was increased by 2.9-fold after 24 h treatment with Bt2cAMP (Fig. 1A), but had no effect on the mRNA expression of other DGK isoforms (Fig. 1A). Next, we assessed the kinetics of the DGKα response to Bt2cAMP by treating H295R cells for 1–24 h. The results revealed that Bt2cAMP activation rapidly increased DGKα mRNA expression by 1.5-fold at the 3 h time point with a maximal 3.7-fold increase in DGKα mRNA expression occurring at the 21 h time point (Fig. 1B). Consistent with an increase in mRNA expression, Bt2cAMP treatment led to an increase in DGKα protein expression by 2.2- and 2.7-fold after 48 h and 72 h treatment, respectively (Fig. 1C).

**Effect of kinase on cAMP-stimulated DGKα mRNA expression**

In the human adrenal cortex, the action of cAMP is mediated by the cAMP-dependent protein kinase A (PKA) (41). To determine if Bt2cAMP stimulated DGKα expression required PKA, H295R cells were treated with H89 or the mitogen-activated protein kinase inhibitor U0126. As shown in Fig. 2, H89 treatment attenuated the cAMP activation on DGKα mRNA expression. Conversely, no significant effect was observed with U0126.

**cAMP increases DGKα reporter gene activity**

Next we sought to define the mechanism by which cAMP stimulation induces DGKα expression and cloned 1.5 kb of the DGKα promoter into a reporter gene plasmid fused to the Firefly luciferase gene and transfected the construct into H295R cells. As shown in Fig. 3A, Bt2cAMP treatment significantly increased the transcripational activity of the 1.5 kb reporter gene by 1.9-fold. In silico analysis of the DGKα promoter revealed several putative SF1 binding sites (Fig. 3B). Notably, one of these putative SF1 binding sites overlapped with response elements for the SREBP family. SREBPs are a family of basic helix-loop-helix leucine zipper...
cAMP signaling induces DGK0 gene expression

To determine the effect of SF1 and SREBP1c on DGK0 reporter gene transcription, we transfected expression plasmids for these transcription factors in H295R cells and quantified luciferase activity. Consistent with the Bt2cAMP effect, cotransfection with an SF1 expression and SREBP1c plasmids resulted in a 1.8- and 2.7-fold increase in DGK0 reporter gene activity, respectively. Moreover, overexpression of both transcription factors resulted in a 4.2-fold increase in DGK0 luciferase activity, with Bt2cAMP further stimulating DGK0 reporter gene transcription. Transfection of DGK0 reporter gene plasmids harboring mutations at putative SF1/SREBP1c sites (Fig. 3B) revealed that mutation of region M5 (−775/−776) had no significant effect on basal DGK0 promoter reporter gene activity, whereas...
mutation of regions M1 (997/−986) and M6 (760/−751) increased basal luciferase activity (Fig. 3C). Compared with the wild-type promoter, mutation of M2 (−976/−967), M3 (−910/−904), and M4 (−817/−809) significantly attenuated the SF1 response. As shown in Fig. 3D, mutation of regions M1 and M2 significantly reduced SREBP1c-stimulated DGKα reporter gene activity. In contrast to the requirement of region M3 for SF1-dependent transcription, mutation of M3 had no effect on SREBP1-stimulated transcriptional activity of the DGKα reporter gene. Further, mutation of M4, M5, and M6 were unable to reduce SREBP1-stimulated DGKα luciferase activity. Collectively, these studies indicate that M2 (976/−967), M3 (−910/−904), M4 (−817/−809) contribute to SF1-dependent transactivation, whereas SREBP1 requires region M2.

cAMP promotes the recruitment of SF1 and SREBP1 to the DGKα promoter

We next determined the effect of cAMP stimulation on the recruitment of SF1 and SREBP1 to the endogenous DGKα promoter by performing ChIP assays using chromatin isolated from H295R cells that were treated with 0.4 mM Bt2cAMP for 1 h and found that Bt2cAMP increased the acetylation of histone H4 (Fig. 4A). cAMP stimulation promoted the enrichment of SF1 and SREBP1 at the DGKα promoter by 3.8- and 3-fold, respectively. The proximity of the SF1 and SREBP1 binding sites on the DGKα promoter (Fig. 3B) and the effect of mutating the M2 region on the activity, we quantified PA concentrations in cells treated for 24–72 h with Bt2cAMP. As shown in Fig. 6B, the total PA concentration was increased by 34-fold at the 72 h time point, with a concomitant 48% decrease in the cellular amount of DAG (Fig. 6C). Consistent with the increase in total cellular PA, nuclear PA concentrations were also increased with Bt2cAMP treatment in a time-dependent manner. Finally, to assess the relative contribution of DGKα to the cAMP-stimulated increase in PA, we quantified the concentrations of PA in wild-type and DGKα knockdown (Fig. 6B) H295R cell lines. Knockdown of DGKα
cAMP signaling induces DGK0 gene expression

DISCUSSION

DGKs modulate the concentration of DAG and PA, key second messengers in numerous signaling pathways (48–52). Recent studies have revealed that DGKs regulate immunity, inflammation, and the nervous system (53–57), and aberrant DGK activity is implicated in the etiology of type 2 diabetes, cardiovascular disease, and cancer (49, 58, 59). We have previously identified a role for DGK0 in glucocorticoid production. By virtue of its ability to produce PA, a ligand for the nuclear receptor SF1, DGK0 regulates the transcription of multiple genes required for cortisol biosynthesis, including CYP17A1 (25). Our present studies provide further support that phospholipid metabolism plays a key role in cAMP-dependent steroidogenesis. We demonstrate that the expression of DGK0 is induced by cAMP (Fig. 1A). Although DGKα, DGKγ, DGKδ, DGKε, DGKη, DGKθ, and DGKζ are expressed in H295R cells (25), the mRNA expression of these isoforms is not affected by Bt2cAMP. In agreement with our previous findings (25), our data suggest that DGK0 is the main PA source in cAMP-stimulated human adrenocortical cells (Fig. 6B). We previously demonstrated that cAMP rapidly induced nuclear DGK0 catalytic activity within 5 min (25). Herein, we showed that cAMP, in addition to an acute effect on DGK enzymatic activity and activation of the cAMP signaling pathway, also chronically increased the expression (Fig. 1) and activity (Fig. 6A) of DGK0.

Luciferase reporter assays revealed that SF1 and SREBP1c increased the transcriptional activity of a DGK0 reporter gene (Fig. 3). The activation of DGK0 luciferase activity and the recruitment of the receptor to the endogenous DGK0 promoter (Fig. 4A) suggest that cAMP signaling may activate a feed-forward mechanism that enables the sustained activation of SF1 target genes that are required for glucocorticoid production. We envision that optimal steroid hormone production requires not only a rapid increase in nuclear PA production in response to ACTH/cAMP (25), but also a mechanism to facilitate the continued ability of SF1 to activate target gene transcription. One mechanism to achieve SF1 activation is to allow for an increase in DGK0 expression, and subsequently PA production. SF1 plays an essential role in inducing the transcription

Fig. 5. Silencing SF1 and SREBP1 suppresses DGK0 gene expression. A: H295R wild-type and SF1 knockdown (k.d.) cells were treated with 0.4 mM Bt2cAMP for 48 h and cell lysates were harvested and analyzed by SDS-PAGE (8%), followed by Western blotting for DGK0, SF1, SREBP1, and GAPDH. B: Real time RT-PCR was used to assess the mRNA expression of DGK0, SREBP1, and SF1 using total RNA that was isolated from wild-type and SF1 knockdown H295R. Data are graphed as fold change in mRNA expression normalized to the mRNA expression of β-actin and represent the mean ± SEM of three separate experiments, each performed in triplicate. *Statistically different from untreated control group, P < 0.05. C: Wild-type and SREBP1 knockdown cells were treated with 0.4 mM Bt2cAMP for 48 h and cell lysates were harvested and analyzed by SDS-PAGE and Western blotting for DGK0, SF1, SREBP1, and GAPDH. D: RNA isolated from wild-type and SREBP1 knockdown H295R cells was subjected to qRT-PCR analysis. Data are graphed as fold change in DGK0, SREBP1, or SF1 expression mRNA expression normalized to the mRNA expression of β-actin and represent the mean ± SEM of three separate experiments, each performed in triplicate. *Statistically different from untreated control group, P < 0.05.
of multiple steroidogenic genes, including cytochrome CYP17A1 in the adrenal cortex and gonads. The ability of SF1 to activate target genes is regulated by mechanisms including coregulatory proteins (60–63), posttranslational modification (27, 28, 64–68), and ligand binding (25, 69–72).

SREBPs are considered as master regulators of lipid metabolism. In general, SREBP target genes include cholesterol biosynthetic (e.g., HMG-CoA synthase, LDLR receptor) and lipogenic genes (e.g., acetyl-CoA carboxylase, fatty acid synthase). However, we have also previously shown that SREBP1 is recruited to the CYP17A1 promoter in response to stimulation by sphingosine-1-phosphate (47). Our current studies demonstrate that SREBP1 is recruited to the DGKθ promoter (Fig. 4A) and is required for both basal and cAMP-stimulated DGKθ expression (Fig. 5C, D).

Interestingly, we also found that SREBP1 and SF1 interact (Fig. 4B), suggesting coordinated action between these two transcription factors. The ability of SF1 to act cooperatively with other transcription factors is well documented. SF1 synergizes with several transcription factors, including GATA transcription factors (73–76), cAMP regulatory element binding proteins (77–79), AP1 family members (80), and β-catenin (81). Significantly, the likelihood of a physical interaction between SF1 and SREBP1 is supported by studies demonstrating that both SREBP1 and SREBP2 interact with hepatic nuclear factor 4 (82) and with the liver receptor homolog (LRH)1 (83). LRH1 and SF1 belong to the NR5A subfamily of nuclear receptors and share greater than 90% conservation in the DNA binding domain and are >50% conserved in the ligand binding domain (71, 84), so it is not surprising that SF1 also interacts with SREBP1. However, despite similarities in the ability of the two NR5A family members to interact with SREBP1, the functional consequences on target gene expression differ. Whereas we show herein that SREBP1 and SF1 cooperate in the activation of DGKθ reporter gene activity (Fig. 3A), SREBPs inhibit the ability of LRH1 to activate target genes in HepG2 and Huh7 hepatoma cells by preventing the interaction of LRH1 with the coactivator PGC1α (peroxisome proliferator-activated receptor γ coactivator 1α) (83).

Fig. 6. cAMP increases PA production. A: H295R cells were grown on 10 cm dishes and treated with 0.4 mM Bt2cAMP and the cytoplasmic and nuclear fractions isolated for SDS-PAGE and Western blotting for DGKθ, β-tubulin, and lamin. Data graphed represent densitometric analysis of DGKθ cytoplasmic and nuclear expression, normalized to β-tubulin and lamin expression, respectively. B: Wild-type or DGKθ knockdown (kd) cells were treated with Bt2cAMP for 24–72 h and the cellular or nuclear lipids isolated for quantification of PA. The cellular or nuclear amount of PA was normalized to the protein concentration. Data graphed represent the mean ± SEM of three separate experiments, each performed in triplicate. *Statistically different from untreated control group, P < 0.05. Inset: Representative Western blot of controls and tetracycline treated H295R cells demonstrating decreased DGKθ protein levels. C: Wild-type or DGKθ knockdown H295R cells were grown on 6-well plates and treated with 0.4 mM Bt2cAMP for 72 h and the cellular content of DAG quantified by ELISA. The graphed data represent the mean ± SEM of three independent experiments, each performed in triplicate.
We also observed that silencing SF1 in the H295R cell line suppresses the expression of SREBP1 (Fig. 5C, D). Microarray analysis (K. Cai et al., unpublished observations) revealed that silencing SF1 reduced the expression of several genes in the SREBP regulatory pathway, including SREBP2, insulin induced gene 1 (INSIG1), and SREBP cleavage-activating protein, suggesting a role for the nuclear receptor in regulating cholesterol homeostasis in the adrenal cortex. These findings are inconsistent with studies performed in HuH7 human hepatoma cells demonstrating that silencing LRH1 led to an increase in the expression of SREBP target genes when the cells were cultured in cholesterol-free media (83). However, further studies are required to delineate the role of SF1 in regulating the expression of SREBP1.

Our data demonstrate that the cellular content of PA increases in response to Bt2cAMP treatment, concomitant with a decrease in DAG (Fig. 6). The time course of this increase supports a role for cAMP-stimulated DGKθ transcription in mediating PA production. However, given our previous studies demonstrating that Bt2cAMP rapidly increases nuclear PA (25), it is likely that activation of the cAMP signaling pathway acutely regulates DGKθ activity and chronically regulates DGKθ expression. Indeed, we have preliminary mass spectrometric evidence that DGKθ is phosphorylated at multiple sites (D. Li et al., unpublished observations). Published findings from other laboratories have demonstrated that phosphorylation plays a key role in regulating DGK activity, thus it is plausible that posttranslational modification also modulates DGKθ function. PKA and PKC have been shown to phosphorylate DGK in COS7 cells (85). Phosphorylation of DGKα by the tyrosine kinase Src confers hepatocyte growth factor-induced cell motility (86, 87), whereas PKC-catalyzed phosphorylation of DGKζ promotes the dissociation of the lipid kinase from PKC (88, 89). Given that DAG stimulates PKC activity, the association with DGKζ provides a mechanism to limit the ability of PKC to phosphorylate target proteins. Studies are ongoing to investigate the role of phosphorylation in regulating DGKθ function in response to activation of the cAMP signaling cascade.

Consistent with our previous studies (25), and the work of others, DGKθ is expressed in the nucleus of H295R cells. As shown in Fig. 6B, most of the increase in PA production in response to cAMP at the 24 h time point is due to an increase in nuclear PA biosynthesis, demonstrating the importance of spatially regulated phospholipid metabolism in cell signaling. Because other DGK isoforms also exhibit nuclear localization (90, 91), DGKθ for example (25, 92–95), it was important to determine the relative contribution of DGKθ to the increased PA production observed in response to Bt2cAMP. Though DGKθ was the sole isoform whose mRNA expression increased after cAMP stimulation (Fig. 1A), it is possible that other isoforms may be positively regulated by cAMP at the posttranscriptional level. H295R cells that were stably expressing a shRNA targeted against DGKθ exhibited a 50% decrease in basal and Bt2cAMP-stimulated concentrations of PA, indicating that DGKθ plays a prominent role in the capacity of adrenocortical cells to produce PA in response to cAMP signaling. However, these findings also suggest that other DGK isoforms or members of the phospholipase D family may also contribute to the increased biosynthesis of PA. In an elegant study recently reported by Mitra et al. (96), targeted disruption of the PA phosphatase lipin1 in adipocytes revealed a novel role for the transcriptional coactivator and lipid phosphatase in the regulation of cAMP/PKA signaling. These findings provide support for the role of lipid-metabolizing enzymes as key regulators, not only of lipid homeostasis, but also of signal transduction and cellular processes.

In summary, we found that the expression of DGKθ is induced by cAMP. Both SF1 and SREBP1 are required for constitutive and cAMP-stimulated DGKθ expression. Additionally, SF1 is a novel regulator of SREBP1 expression. Given the role of DGKθ in synthesizing the agonist for SF1 (25), our studies identify a feed-forward mechanism by which the capacity of adrenocortical cells to produce PA in response to cAMP is regulated by SF1.

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