SCAP/SREBP pathway is required for the full steroidogenic response to cyclic AMP

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Luteinizing hormone (LH) stimulates steroidogenesis largely through a surge in cyclic AMP (cAMP). Steroidogenic rates are also critically dependent on the availability of cholesterol at mitochondrial sites of synthesis. This cholesterol is provided by cellular uptake of lipoproteins, mobilization of intracellular lipid, and de novo synthesis. Whether and how these pathways are coordinated by cAMP are poorly understood. Recent phosphoproteomic analyses of cAMP-dependent phosphorylation sites in MA10 Leydig cells suggested that cAMP regulates multiple steps in these processes, including activation of the SCAP/SREBP pathway. SCAP [sterol-regulatory element-binding protein (SREBP) cleavage-activating protein] acts as a cholesterol sensor responsible for regulating intracellular cholesterol balance. Its role in cAMP-mediated control of steroidogenesis has not been explored. We used two CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 (CRISPR associated protein 9) knockout approaches to test the role of SCAP in steroidogenesis. Our results demonstrate that SCAP is required for progesterone production induced by concurrent inhibition of the cAMP phosphodiesterases PDE4 and PDE8. These inhibitors increased SCAP phosphorylation, SREBP2 activation, and subsequent expression of cholesterol biosynthetic genes, whereas SCAP deficiency largely prevented these effects. Reexpression of SCAP in SCAP-deficient cells restored SREBP2 protein expression and partially restored steroidogenic responses, confirming the requirement of SCAP–SREBP2 in steroidogenesis. Inhibitors of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase and isoprenylation attenuated, whereas exogenously provided cholesterol augmented, PDE inhibitor-induced steroidogenesis, suggesting that the cholesterol substrate needed for steroidogenesis is provided by both de novo synthesis and isoprenylation-dependent mechanisms. Overall, these results demonstrate a novel role for LH/cAMP in SCAP/SREBP activation and subsequent regulation of steroidogenesis.

steroidogenesis | cholesterol | cAMP | phosphodiesterase | SCAP/SREBP

Luteinizing hormone (LH) binding to its lutropin-choriogonadotropic hormone receptor on Leydig cells initiates a cascade of signaling events, including a surge in levels of the second messenger 3′,5′-cyclic adenosine monophosphate (cAMP) and subsequent activation of protein kinase A (PKA). A large number of cyclic nucleotide phosphodiesterases (PDEs) serve to limit the intracellular temporal and spatial effects of cAMP by hydrolyzing cAMP to 5′-AMP (1, 2). Among the different PDE enzymes, inhibition of PDE4 and PDE8 is known to stimulate steroidogenesis (3–6). Within these gene families, PDE8A and PDE8B both regulate steroidogenesis (5), whereas the role for specific PDE4 family gene products is less well understood. PDE4A, PDE4B, and PDE4C, but not PDE4D, have been shown to be expressed in Leydig cells (5, 7, 8), but the role for specific PDE4s in regulating steroidogenesis is unknown. We have recently demonstrated that simultaneous inhibition of PDE4 and PDE8 in MA10 Leydig cells results in an increase of a large number of phosphorylated PKA consensus sites in multiple proteins (8) concurrent with a very large increase in steroid production (5).

Cellular cholesterol levels are controlled in part by several transcription factors, including sterol-regulatory element-binding proteins (SREBPs) 2 and 1a, that promote cholesterol biosynthetic gene expression when cellular cholesterol levels are too low to meet demand (9, 10). The activities of the SREBPs are precisely controlled by an escort protein, SREBP cleavage-activating protein (SCAP), and the insulin-inducible gene product (Insig) (11–13). When cellular cholesterol levels are high and cholesterol binds to the sterol-binding domain of SCAP, SCAP is tethered by Insig, and the inactive SCAP–SREBP complex is retained in the endoplasmic reticulum (ER) membrane. Once cellular cholesterol levels become lower or demand for cholesterol supply becomes higher, the cholesterol-free form of SCAP is released from Insig, and the SCAP–SREBP complex is transported from the ER to the Golgi in coatomer protein II (COPII) vesicles. In the Golgi, SREBPs are sequentially cleaved by two proteases, site-1 protease and site-2 protease, which allows release of the active N-terminal mature forms of SREBPs and translocation into the nucleus, where they bind to sterol-regulatory elements (SREs) in target genes (14–16). This process has been studied largely in the context of regulation of cholesterol biosynthesis by the liver. A possible role(s) for cAMP in SCAP/SREBP regulation of steroid hormone production has not been extensively explored and is not well-understood.

In this study, we demonstrate using two different CRISPR-Cas9 gene-ablation approaches that cAMP elevation by inhibition of

Significance

Luteinizing hormone stimulates production of testosterone and other steroids largely through a surge in the second messenger cAMP and subsequent activation of protein kinase A (PKA) in target cells. Rates of steroidogenesis are also dependent on the availability of cholesterol, a steroid building block. We propose, based on our results, that cAMP/PKA coordinates the functions of multiple pathways to regulate cellular cholesterol handling and synthesis and downstream steroid output. Activation of the cholesterol-sensing SCAP–SREBP2 pathway plays an important role in cAMP/PKA coordination of steroidogenesis. These cAMP/PKA-induced pathways are likely to be major regulators of sterol biosynthesis and cholesterol recharging in steroid hormone synthetic and other tissues. Cyclic nucleotide phosphodiesterases can be targeted to promote steroidogenesis and cholesterol metabolism.

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Table 1. Phosphorylation of PKA consensus sites of proteins implicated in cholesterol/steroid synthesis or handling that are increased in response to PDE4 plus PDE8 inhibition

Fold PO_4 increase	Protein name	Site	Gene name	Processes regulated
Cholesterol synthesis and delivery				
5.2	Oxysterol-binding protein-like 11	S194	*Osbpl11*	Sterol binding and delivery
2.1	**SREBP cleavage-activating protein**	**S821**	***Scap***	**SREBP pathway**
COPII vesicle trafficking (ER to Golgi)				
7.7	Sec22 homolog B, vesicle-trafficking protein	S137*	*Sec22b*	COPII/SREBP pathways
2.9	Sec23-interacting protein	S748	*Sec23ip*	COPII/SREBP pathways
COPI vesicle trafficking (Golgi to ER)				
11.7	Ral GEF with pleckstrin homology and SH3-binding motif 2	S315	*Ralgps2*	Ral GEF activity
5.7	Golgi autoantigen, golgin subfamily a, 5	S116	*Golga5*	COPI pathways
4.8	ARF GAP1	S360	*Arfgap1*	COPI pathways
4.0	Autophagy 16 like 1	S269	*Atg16l1*	Rab33b interaction? Autophagy
3.7	ARF GEF/Tyr kinase adapter protein 1	S85	*Nck1*	Arf activation
2.1	ARF GEF2	S621	*Arfgef2*	COPI pathways
Cholesteryl ester/lipid droplet hydrolysis				
7.1	**Hormone-sensitive lipase**	**S651**[†]	***Lipe***	**Cholesteryl ester hydrolysis**
4.9	Perilipin 1	S81[‡]	*Plin1*	Lipid droplet hydrolysis
LDL receptor and endosome vesicle trafficking				
9.5	G protein-coupled receptor 107	S537	*Gpr107*	Golgi-to-ER retrograde transport
4.7	Pleckstrin homology domain-containing, family F (with FYVE domain) member 2	S16[‡]	*Plekhf2*	Rab-dependent vesicle trafficking?
4.4	TBC1 domain family, member 10B	S673	*Tbc1d10b*	GAP for Rab family proteins?
4.3	Ras and Rab interactor 2	S510	*Rin2*	Rab-dependent vesicle trafficking
3.0	Amyotrophic lateral sclerosis 2 (juvenile)	S477	*Als2*	Rab-dependent vesicle trafficking
2.3	DENN/MADD domain-containing 1A	S520	*Dennd1a*	Clathrin-mediated endocytosis
Microtubule formation, vesicle trafficking				
13.1	Regulator of microtubular dynamics 2 (FAM82A1)	S51	*Rmdn2*	Microtubular dynamics
12.1	CAP-GLY domain linker protein 1 or 2	S347/353	*Clip1/Clip2*	Microtubule elongation/stability
11.7	Cytoskeleton-associated protein 5	S1861	*Ckap5*	Microtubule elongation/stability; binding of clathrin
8.7	Neuron navigator 1	S651[‡]	*Nav1*	Microtubule elongation/stability
3.7	SLAIN motif family, member 2	S392	*Slain2*	Microtubule elongation/stability
Rho family GTPase activation/inactivation, vesicle trafficking				
2.9	Rho GAP21	S917	*Arhgap21*	Rho inhibition/vesicle trafficking
2.6	Rho GAP17	S698[‡]	*Arhgap17*	Rho inhibition/vesicle trafficking
2.4	Pleckstrin homology domain-containing, family G (with RhoGef domain) member 3	S502	*Plekhg3*	Rho activation/vesicle trafficking
2.4	Rho GEF 17	S1324	*Arhgef17*	Rho activation/vesicle trafficking
2.1	Rho GEF 2	S885	*Arhgef2*	Rho activation/vesicle trafficking
1.9	Rho GAP23	S607	*Arhgap23*	Rho inhibition/Golgi trafficking
1.6	Rho GEF 11	S1353	*Arhgef11*	Rho activation/vesicle trafficking
Others				
13.6	PI 4-kinase, catalytic, beta polypeptide	S511	*Pi4kb*	Phosphatidylinositol/vesicle trafficking?
8.9	A kinase anchoring protein 1	S55	*Akap1*	Steroidogenesis (48, 49)
5.8	Carbohydrate response element-binding protein	S626	*Mlxipl (ChREBP)*	Glucose to acyl CoA synthesis
5.5	DDHD domain-containing 2	S447	*Ddhd2*	Vesicle trafficking

Protein functions are based on data provided by PhosphoSitePlus (50) and GeneCards (version 4.1, build 29; www.genecards.org), unless otherwise specified. Proteins investigated in the present study are highlighted in bold. Original proteomics data are available at the public MS data repositories MassIVE (ID MSV000079412; massive.ucsd.edu) and ProteomeXchange (accession no. PXD003280; www.proteomexchange.org). The fold increases shown have been calculated from primary data reported in ref. 8. All values are the average fold increases seen from multiple identifications and quantification at the 1-h time point (usually in three to six of six runs) with the exception of the following.

*The Sec22b site (S137) is not a classic PKA consensus site (RRNLGS).

[†]For *Lipe*, no quantification was seen at the 1-h time point but an average increase of 7.1-fold was seen at earlier time points, and the phosphorylation increase was verified by a phospho-specific antibody.

[‡]In these cases, the values reported are the average increase seen in one or two runs at the 1-h time point but are verified by data from other time points.

PDE4 and PDE8 can regulate SCAP/SREBP function in a manner consistent with activation of this pathway being required for maximal steroid hormone biosynthesis. More generally, the data suggest that cAMP/PKA/PDE4+8 coordinate the functions of multiple pathways to regulate steroid output and may also act as a major regulator of cholesterol biosynthesis in Leydig cells and possibly many other tissues.

Results

Activation of PKA by PDE4 and PDE8 Inhibitors Causes Increased Phosphorylation of Many Proteins Likely to Be Regulators of Cholesterol Handling and Steroidogenesis. Based on our previous global screen of phosphorylation events in MA10 Leydig cells in response to PDE inhibition (8), we curated a list of several sites that were the

most highly phosphorylated PKA consensus sites that also might directly influence steroid hormone production, cholesterol synthesis, and/or trafficking (Table 1). Nearly all identified phosphorylation sites showed more than a twofold increase after 1 h of treatment with specific PDE4 and PDE8 inhibitors (rolipram and PF-04957325, respectively) (increases were noted in at least three of five experiments). Many phosphorylation sites were increased more than fourfold. PKA consensus site phosphorylation in response to cAMP elevation occurred in a large number of proteins involved in cholesterol synthesis and delivery pathways, cholesterol and fatty acid liberation from lipid stores, and LDL receptor-, endosome-, and vesicle-trafficking proteins (Table 1). Among the identified phosphorylation sites was S821 of SCAP. Two other proteins also known to be important to the SCAP/COPII-dependent vesicle-trafficking pathway were even more highly phosphorylated (Sec22 homolog B vesicle-trafficking protein and Sec23-interacting protein) (Table 1). COPII vesicles transport a large number of cargo proteins from the ER to other membrane compartments in the cell and regulate a plethora of cellular functions in addition to cholesterol homeostasis. Therefore, the primary goal of this study was to begin to test the role for this pathway as a regulator of cAMP-stimulated steroid hormone production by examining the possible role of SCAP/SREBP in this process.

SCAP Deficiency Reduces Leydig Cell Steroidogenesis and Cholesterol Biosynthetic Gene Expression. We initially examined whether SCAP/SREBP is required for hormone-stimulated steroidogenesis by knocking down SCAP in the MA10 Leydig cell line because reduction of SCAP is known to also reduce SREBP levels. We used two distinct CRISPR-Cas9 gene-editing techniques. First, SCAP activity and protein were knocked-down in pools of cells by electroporation of Cas9, guide RNA, and a repair construct containing a puromycin-resistance gene. After selection by puromycin, these cell pools had a dramatic reduction in SCAP as well as SREBP2 protein expression compared with wild-type (WT) cells (Fig. 1*A*). We have previously reported that simultaneous inhibition of PDE4 and PDE8 (rolipram at 10 μM and PF-04957325 at 200 nM, respectively) synergistically stimulates steroidogenesis and induction of the steroidogenic acute regulatory (StAR) protein, a protein that can be a rate-limiting factor for steroidogenesis (5). Further, this study demonstrated that both PDEs cooperatively control levels of cAMP-dependent phosphorylation and steroidogenesis (5). We therefore used coinhibition of PDE4 and PDE8 as well as LH stimulation to investigate the role of SCAP/SREBP in the cAMP-dependent component of hormone-stimulated steroidogenesis.

Steroidogenesis induced by PDE4+8 inhibitors in the presence or absence of LH was significantly reduced in the pools of SCAP-deficient cells compared with WT cells (Fig. 1*B*). A similar fold decrease was observed in both LH-stimulated cells and unstimulated cells, although total progesterone production was markedly lower in the latter condition (Fig. 1*B*). However, SCAP-deficient cell pools exhibited equivalent levels of StAR protein induction in response to the PDE inhibitors, indicating that neither the overall viability of the cells nor the mechanism leading to StAR induction was impaired by SCAP deficiency (Fig. 1*A*). Furthermore, phosphorylation of hormone-sensitive lipase (HSL) on S660 (S651 in mouse HSL) was stimulated by PDE inhibition to the same extent in WT and SCAP-deficient cells (Fig. 1*A*), confirming a lack of nonspecific effect of the CRISPR-Cas9 gene targeting of SCAP. These results indicate that SCAP/SREBP is required for steroidogenesis through mechanisms that are distinct from StAR expression and HSL phosphorylation.

In other cells, SREBP2 is well-known to control the level of several cholesterol biosynthetic genes including 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*), 3-hydroxy-3-methylglutaryl-CoA synthase (*Hmgcs*), farnesyl diphosphate synthetase (*Fdps*), and cytochrome P450, family 51 (*Cyp51*), as well as genes required for cholesterol uptake, such as the low-density lipoprotein receptor (*Ldlr*) (17). Messenger RNA levels for each of these genes and SREBP2 (*Srebf2*) itself were significantly reduced in the SCAP-deficient cell pool (Fig. 1*C*), confirming that SCAP deficiency resulted in reduced SREBP2 activity. This was expected, as it has been reported previously in SCAP knockout mice (18). Interestingly, mRNA levels of *Hmgcr*, *Fdps*, *Cyp51*, and *Ldlr* were

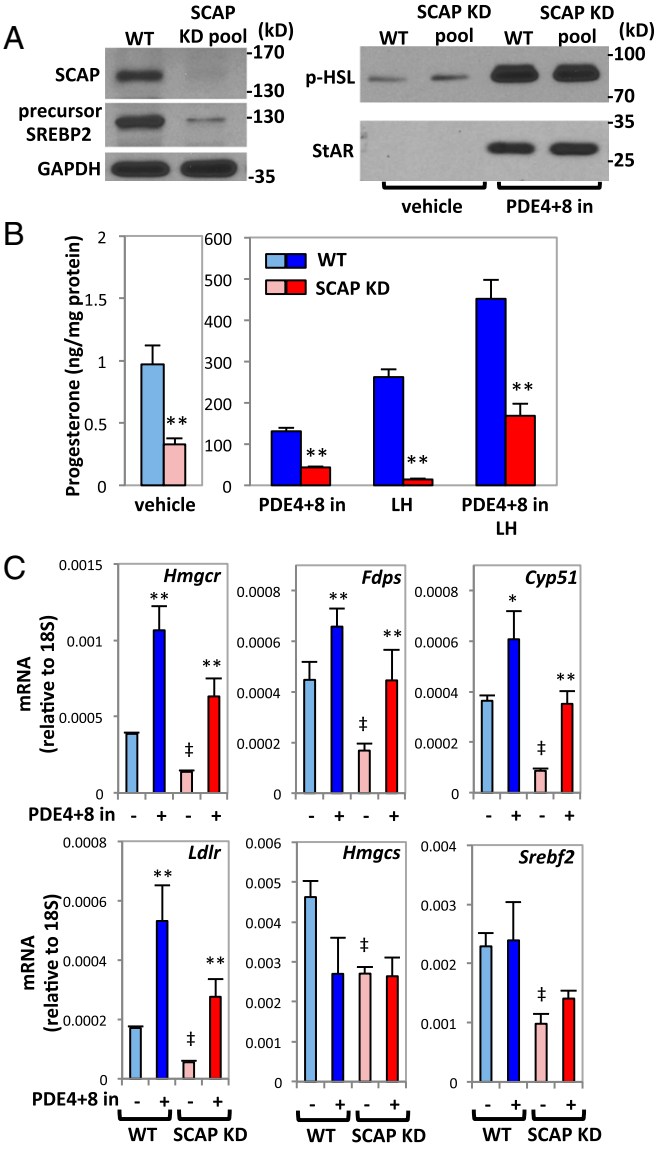

Fig. 1. SCAP deficiency generated by the CRISPR-Cas9 system elicited reductions in SREBP2, steroidogenesis, and cholesterol biosynthetic genes. (*A*) MA10 cell extracts collected from a SCAP knockdown (KD) cell pool and a WT cell pool were used to determine SCAP and SREBP2 protein levels. For determination of p-HSL (Ser660 rat, Ser651 mouse) and StAR, both cell groups were treated with vehicle or PDE4+8 inhibitors (PDE4+8 in; 10 μM rolipram and 200 nM PF-04957325) for 15 min (p-HSL) or 2 h (StAR) before harvest. (*B*) A SCAP KD cell pool (red bars) and WT cell pool (blue bars) were serum-starved for 3 h and then treated with either vehicle, PDE inhibitors, LH (20 ng/mL), or LH plus PDE inhibitors for an additional 2 h. Progesterone released into the medium was quantified by ELISA. Each value represents mean ± SD ($n = 4$). Representative results from four repeated experiments are shown. (*C*) Messenger RNA levels were determined by quantitative real-time PCR using total RNA collected from SCAP KD cell pools and WT cell pools. Both groups were treated with vehicle or PDE inhibitors for 18 h under serum-starved conditions. Each value represents mean ± SD ($n = 4$). Data represent one of two sets of analyses. Statistical significance is shown as $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. (-) inhibitors; $^{‡}P < 0.01$ vs. WT.

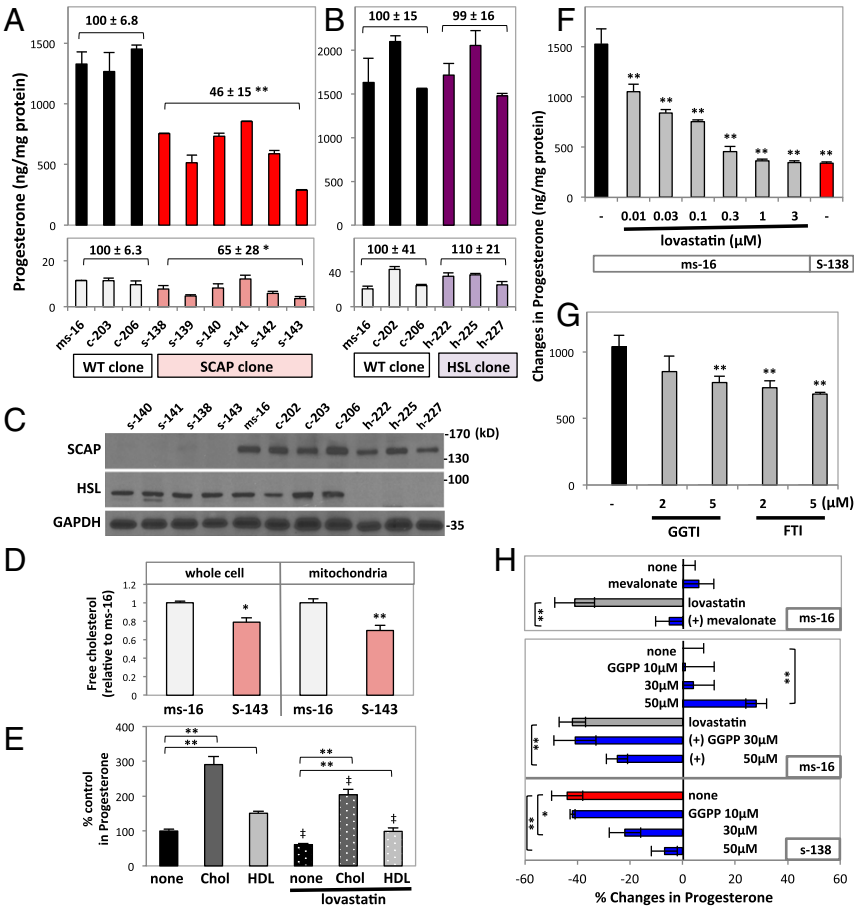

Fig. 2. Steroidogenesis and cholesterol/isoprenoid biosynthetic pathway in SCAP-deficient cell clones generated by the CRISPR-FokI/dCas9 system. (*A* and *B*) SCAP-deficient clonal cells (s-138, s-139, s-140, s-141, s-142, and s-143) (*A*), HSL-deficient clonal cells (h-222, h-225, and h-227) (*B*), and WT clonal cells (ms-16, c-202, c-203, and c-206) were serum-starved for 3 h and treated with vehicle (*Lower*) or PDE4+8 inhibitors (10 μM rolipram and 200 nM PF-04957325; *Upper*) for 2 h under serum-starved conditions. Each value represents mean ± SD ($n = 4$). The numbers are represented relative to WT, mean ± SD ($n = 3$–6). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. WT. (*C*) SCAP and HSL levels were determined by Western blot analysis using whole-cell extracts of each clonal cell line. GAPDH was used as a loading control. (*D*) Whole-cell extracts and mitochondrial fractions were isolated from ms-16 and s-143 cells. Free cholesterol was measured in each fraction. Results are presented as relative to ms-16 (WT). Each value represents mean ± SD ($n = 3$) obtained from three repeated experiments. Free cholesterol levels were 11.4 ± 1.1 ($n = 4$) and 120.0 ± 8.5 ($n = 4$; pmol/μg protein) in whole-cell extract and mitochondrial fraction, respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$ denote statistical significance. (*E*) ms-16 cells were pretreated with methyl-β-cyclodextrin cholesterol (Chol; 1 mM) or mouse HDL (25 μg/mL) with or without lovastatin (0.5 μM) for 30 min. Cells were then stimulated with PDE4+8 inhibitors for 2 h under serum-starved conditions. Each value represents the relative changes to PDE inhibitor-induced steroidogenesis without lovastatin as 100%; mean ± SD ($n = 3$). Progesterone levels in cells exposed to PDE inhibitors but not to exogenous cholesterol (indicated as "none" in the figure) were 791 ± 60 ($n = 3$; ng/mg protein). $^{**}P < 0.01$ vs. none; $^{‡}P < 0.01$ vs. (-) lovastatin. (*F*) ms-16 cells were pretreated with the indicated concentrations of lovastatin for 30 min and then treated with vehicle or PDE4+8 inhibitors for an additional 2 h under serum-starved conditions. Progesterone production in the absence of lovastatin is indicated by the black bar. The SCAP-deficient s-138 cell clone (red bar) was treated with PDE4+8 inhibitors in the absence of lovastatin. Each value represents mean ± SD ($n = 4$) of PDE inhibitor-stimulated progesterone levels after subtraction of basal levels. Representative results from three repeated experiments are shown. $^{**}P < 0.01$ vs. cells in the absence of lovastatin (-). (*G*) ms-16 cells were pretreated with GGTI (2 and 5 μM) or FTI (2 and 5 μM) for 30 min and then treated with vehicle or PDE4+8 inhibitors for an additional 2 h under serum-starved conditions. Each value represents mean ± SD ($n = 4$) of PDE inhibitor-stimulated progesterone levels after subtraction of basal levels. Representative results from three repeated experiments are shown. $^{**}P < 0.01$ vs. (-). (*H*) ms-16 cells were pretreated with mevalonate (10 mM) or GGPP (10, 30, and 50 μM) with or without lovastatin (0.5 μM) for 30 min. s-138 cells were pretreated with GGPP (10, 30, and 50 μM) for 30 min, and then both cell clones were stimulated with PDE4+8 inhibitors under serum-starved conditions. Each value represents the relative change to the level of PDE inhibitor-induced steroidogenesis in ms-16 cells; mean ± SD ($n = 4$). Representative results from three repeated experiments are shown. $^{*}P < 0.05$ and $^{**}P < 0.01$ denote statistical significance.

up-regulated by PDE4+8 inhibition in both WT and SCAP-deficient cell pools, although the absolute levels were lower in SCAP-deficient cells. PDE inhibitor-inducible up-regulation in mRNA levels was also seen for steroidogenic acute regulatory protein (*Star*), cytochrome P450, family 11, subfamily a, polypeptide 1 (*Cyp11a1*), and acyl-CoA synthetase long-chain family member 1 (*Acsl1*) in both WT and SCAP-deficient cells (Fig. S1). HSL (*Lipe*) and acyl-CoA synthetase long-chain family member 4 (*Acsl4*) were significantly induced by PDE4+8 inhibition in WT cells (Fig. S1). ACSL enzymes act to convert free fatty acids into acyl-CoAs for further processing, including cholesteryl ester formation and inhibition of cholesterol efflux (19, 20). ACSL4 has been reported to contribute to steroidogenesis through increasing arachidonic acid delivery to mitochondria, where this fatty acid could enhance cholesterol transport (21, 22). Thus, PDE4+8 inhibition is likely to increase cholesterol biosynthetic gene products through multiple mechanisms, including both SCAP-dependent and SCAP-independent mechanisms.

To confirm that the effects of SCAP deficiency were not due to off-target effects of Cas9, we took advantage of a more specific

CRISPR approach to generate individual SCAP-deficient cell clones. We used the FokI/dCas9 CRISPR gene-editing system (23) to delete SCAP. The FokI/dCas9 dimeric system requires two guide RNAs for FokI to cleave the target DNA, and therefore is likely to have much higher specificity than what can be achieved with the canonical Cas9 system (23). We used the clonal daughter cell line ms-16, derived from the original MA10 cells, for these experiments because we found that individual clonal cells derived from the original MA10 cell WT population varied substantially in their ability to produce progesterone. For example, in the ms-16 cell clone line, PDE4+8 inhibition resulted in at least sixfold higher induction of steroidogenesis, compared with the mixed-parent MA10 cells (e.g., compare the response in MA10 cells in Fig. 1 with the response in ms-16 cells in Fig. 2). Some other clonal cell lines showed much lower responses than the ms-16 cells. Following electroporation with the FokI/dCas9 and multiplex guide RNA plasmids, individual SCAP-deficient clonal cell lines were first screened by PCR amplification of the CRISPR target region (SCAP intron/exon 16) (Fig. S2). In selected clones showing multiple PCR products, SCAP protein levels were determined by Western blot (Fig. 2*C*) and genomic mutagenesis was confirmed by sequencing (Fig. S2). All clones with successful SCAP deletions exhibited significantly reduced progesterone production, compared with either the parent ms-16 cells or two other WT clonal lines, c-203 and c-206 (Fig. 2*A*). The average reduction in progesterone production in SCAP-deficient cells reached 54% under serum-starved conditions (Fig. 2*A*) and 60% in the presence of serum (Fig. S3*A*). These results suggested that SCAP contributes significantly to steroidogenesis even when cells have access to cholesterol from extracellular sources. Free cholesterol levels were determined in whole-cell extracts and mitochondrial fractions both in WT and SCAP-deficient cells. Mitochondrial free cholesterol as well as whole cellular free cholesterol were significantly lower in SCAP-deficient cells than in WT cells (Fig. 2*D*). This reduced free cholesterol availability in mitochondria is consistent with the lower rates of steroidogenesis in SCAP-deficient cells.

To further verify the specificity of the FokI/dCas9 approach and to investigate the possible contribution of newly imported and stored cholesteryl esters to steroid hormone production, we generated cell clones deficient in HSL (*Lipe*) by using the same method (Fig. S2). The HSL-deficient cell clones h-222, h-225, and h-227 showed no HSL protein expression (Fig. 2*C*), but also showed no reduction in progesterone production in either the presence or absence of serum, compared with the ms-16 cells or WT clones (Fig. 2*B* and Fig. S3*A*). Furthermore, treatment of MA10 cells with an HSL-selective inhibitor, CAY10499, did not affect steroid production (Fig. S3*D*), consistent with the results of HSL-deficient clones.

Both Reduced de Novo Cholesterol Synthesis and Isoprenylation Contribute to Impaired Steroidogenesis in SCAP-Deficient Cells. Our results demonstrated that PDE4+8 inhibition resulted in phosphorylation of a large number of proteins, including several guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) for Rab- and Rho-type GTPases. Those small GTPases are thought to be involved in vesicle trafficking, lipoprotein uptake, and processing in MA10 cells (Table 1) (8). We therefore investigated the relative contribution of cholesterol synthesis versus mobilization through these pathways by using the HMG-CoA reductase inhibitor lovastatin and inhibitors of farnesylation (FTIs) and geranylgeranylation (GGTIs) that inhibit isoprenylation of a large number of proteins, including those involved in the uptake and intracellular vesicular transport of cholesterol. Lovastatin dose-dependently reduced steroidogenesis induced by PDE4+8 inhibitors in ms-16 cells (Fig. 2*F*). The maximum inhibitory effect of this statin was reached at 1 μM, which produced an inhibitory effect on progesterone production equivalent to that of SCAP deficiency (Fig. 2*F*). Incubation with lovastatin for 18 h caused a more marked inhibition of steroidogenesis both in the presence and absence of serum, compared with the acute 2-h incubation (compare Fig. 2*F* with Fig. S3 *B* and *C*). Addition of mevalonate completely rescued the attenuated steroidogenesis in lovastatin-treated cells, demonstrating that lovastatin did not exert off-target effects (Fig. 2*H*). We next treated the cells with GGTIs or FTIs. Each of these inhibitors significantly reduced steroidogenesis (Fig. 2*G*). Furthermore, treatment of the cells with geranylgeranyl pyrophosphate (GGPP) at 50 μM partially reversed the inhibitory effect of lovastatin on steroidogenesis in WT cells, suggesting that part, but not all, of the effect of lovastatin on steroidogenesis is mediated by de novo cholesterol biosynthesis and part by isoprenylation (lovastatin inhibits both). In SCAP-deficient cells, GGPP at 30–50 μM significantly increased steroidogenesis (Fig. 2*H*), demonstrating that in this respect SCAP-deficient cells behave like WT cells in which cholesterol biosynthesis has been inhibited by lovastatin. Furthermore, exogenous cholesterol (methyl-β-cyclodextrin cholesterol and high-density lipoprotein; HDL) augmented steroidogenesis stimulated by PDE4+8 inhibition (Fig. 2*E*). In cells treated with lovastatin, exogenous cholesterol increased steroidogenesis to a lesser extent than in cells incubated in the absence of lovastatin (Fig. 2*E*). Together, these results suggest that a significant portion of cholesterol used in SCAP-dependent steroidogenesis is provided through de novo cholesterol biosynthesis but that the SCAP-dependent pathway also regulates isoprenylation that in turn is needed for optimal steroid hormone production. It should be noted that several of the small GTPases that are regulated by the GEFs and GAPs phosphorylated in response to the PDE4 + PDE8 inhibitors likely require isoprenylation to be fully active in the cell.

Reexpression of SCAP in SCAP-Deficient Clonal Cell Lines Restores SREBP2 Expression and Steroidogenesis. As noted previously in the livers of SCAP-deficient mice (18), SCAP deficiency generated by the Cas9 and FokI/dCas9 systems in MA10 cells was characterized by a marked reduction in SREBP2 protein levels (Figs. 1*A* and 3*A*). This likely resulted from reduced SREBP2 mRNA and accelerated SREBP2 degradation. Expression of GFP-SCAP restored SREBP2 levels to wild-type levels (Fig. 3*A*). Furthermore, GFP-SCAP dose-dependently increased progesterone production (Fig. 3*B*), suggesting that a more complete transfection efficiency would also restore steroidogenesis. A SCAP-dependent restoration of SREBP2 protein levels was also demonstrated by immunocytochemistry in GFP-SCAP–expressing cells showing increased SREBP2 staining compared with the "no GFP-expressing" cells within the same microscopy field of vision (Fig. 3*C*). As a control, the ER marker PDI (protein disulfide isomerase) showed no differences in ER staining in cells expressing GFP-SCAP and cells that did not (Fig. 3*C*).

Combined Inhibition of PDE4 and PDE8 Increases SREBP2 Activity. To examine whether PDE4+8 inhibition might regulate SCAP–SREBP2 activity, the cleaved mature form of SREBP2 in nuclear fractions was measured by using a rabbit anti-SREBP2 antibody recognizing the active SREBP2 N terminus. Inhibition of PDE4+8 significantly (~1.8-fold) increased the relative abundance of the mature form of SREBP2, compared with vehicle treatment (Fig. 4*A*). The full-length cytosolic SREBP2 may have been slightly (nonsignificantly) reduced by PDE4+8 inhibition. To clarify whether full-length SREBP2 in complex with SCAP is reduced by PDE inhibition, MA10 cells were transfected with GFP-tagged SCAP and treated with the combined PDE inhibitors. SREBP2–GFP-SCAP complexes were then immunoprecipitated with an anti-GFP antibody, and full-length SREBP2 in the immunoprecipitates was detected by Western blot analysis. The results demonstrated a significant reduction of full-length SREBP2 immunoprecipitated with GFP-SCAP in cells treated with PDE

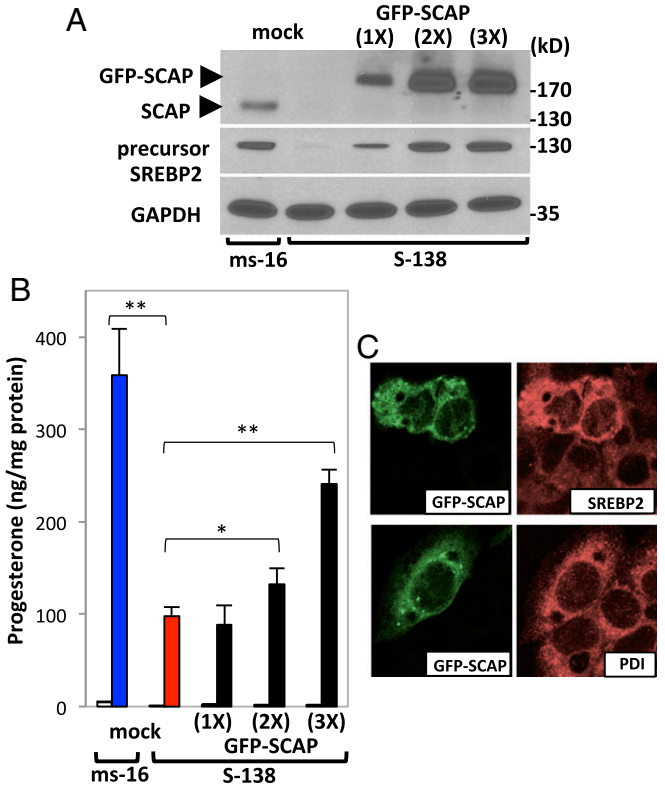

Fig. 3. Restoration of SREBP2 and steroidogenesis by reexpression of SCAP in a SCAP-deficient cell clone. The cell clones s-138 and ms-16 were transfected with GFP-SCAP (2.5, 5, or 7.5 μg DNA in 2 × 10^6 cells) or mock transfected together with a pIRS-GFP/Puro plasmid. Twenty-four hours later, puromycin was added to the medium and the cells were cultured for another 24 h. (*A*) Cell extracts were used to determine SCAP, GFP-SCAP, and SREBP2 by Western blot analysis. (*B*) The cells were treated with vehicle (unfilled column) or PDE4+8 inhibitors (filled column) for 2 h under serum-starved conditions for measurement of steroidogenesis. Each value represents mean ± SEM (n = 4). Representative results from three repeated experiments are shown. *P < 0.05 and **P < 0.01 denote statistical significance. (*C*) SCAP-deficient s-138 cells transfected with GFP-SCAP without the pIRS-GFP-Puro plasmid were immunostained with either an anti-SREBP2 antibody or an anti-PDI antibody (ER marker), as described in *SI Materials and Methods*.

inhibitors, suggesting that active PDE4 and PDE8 retard processing and subsequent activation of SREBP2 (Fig. 4*B*). In addition, endogenous SREBP2–SCAP complexes were immunoprecipitated with a mouse anti-SREBP2 antibody that recognizes both the full-length and cleaved C-terminal portion of SREBP2 (the remaining inactive portion of SREBP2 after cleavage). Total SCAP bound to SREBP2 (both the precursor and the cleaved C-terminal portion, as detected with the mouse SREBP2 C-terminal antibody) was increased in the cells treated with PDE4+8 inhibitors compared with nontreated cells (Fig. 4*C*), suggesting that more of the SREBP2 C-terminal peptide is bound to SCAP in response to PDE inhibition. Overall, these results indicate that PDE4+8 inhibition facilitates SREBP2 processing possibly by increasing SREBP2–SCAP binding.

PKA-Dependent Phosphorylation of SCAP in Response to PDE4+8 Inhibition. Because simultaneous inhibition of PDE4 and PDE8 resulted in increased SREBP2 processing, we hypothesized that cAMP/PKA-dependent phosphorylation events promote SREBP2/SCAP signaling as part of the response leading to increased steroidogenesis. S821 in SCAP is located within the WD domain, which is known to associate with the SREBP2 C-terminal domain (24, 25). To investigate the potential role of this phosphorylation event, a custom-made S821 phospho-specific antibody was used to confirm that the increase of S821 phosphorylation noted in the mass spectrometry data could be seen by another method. The specificity of the phospho-antibody was confirmed by Western blot (Fig. S4). A large increase in the S821 band was induced by the combination of PDE4+8 inhibitors. A smaller increase was seen with the PDE8 inhibitor alone (Fig. 5*A*) and essentially no effect was seen with the PDE4 inhibitor alone, mimicking the effect on steroidogenesis previously reported (5) and the mass spectrometry data (8). Furthermore, stimulation of the cells with either LH (10 ng/mL) or 8Br-cAMP (300 μM) also increased SCAP S821 phosphorylation. The PKA inhibitors H89 (10 μM) and Rp-CPT-cAMPS (0.5 mM) both partially blocked the effect of PDE4+8 inhibition (Fig. 5*A*), indicating that this phosphorylation event was likely to be largely

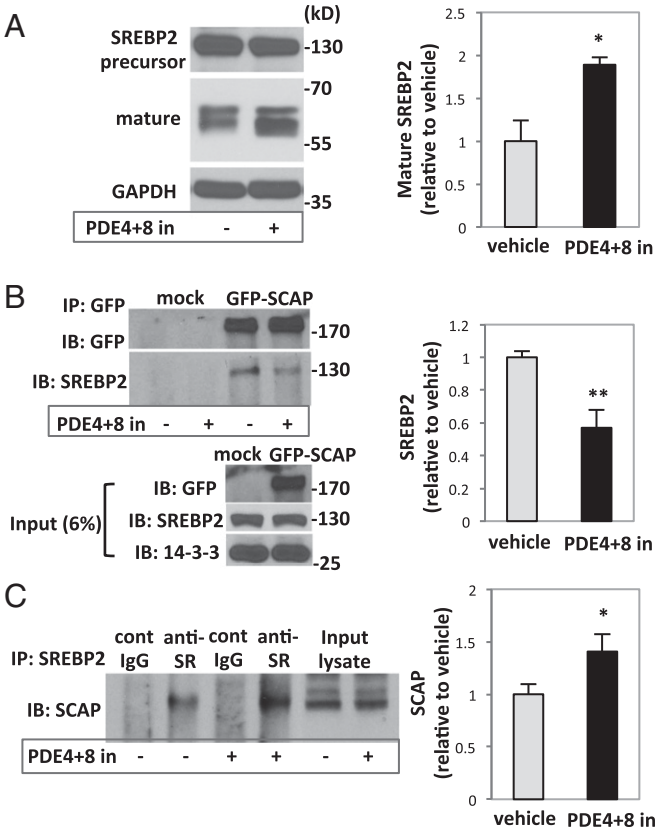

Fig. 4. PDE4+8 inhibition increases SREBP2 activity. (*A*) MA10 cells were serum-starved for 3 h and then treated with vehicle or PDE4+8 inhibitors. Nuclear and cytosolic fractions were isolated and used to determine mature SREBP2 and precursor SREBP2, respectively, by using a rabbit anti-SREBP2 antibody detecting both full-length and cleaved SREBP2. The values in the bar graph represent mean ± SEM of densitometric data obtained from three separate experiments. (*B*) MA10 cells transfected with GFP-SCAP were treated with vehicle or PDE4+8 inhibitors for 2 h, and cell extracts were used for immunoprecipitation with an anti-GFP antibody. Immunoprecipitated samples and the lysate (6%) were used to determine SREBP2 levels (rabbit SREBP2 antibody) and expressed GFP-SCAP. 14-3-3 protein was used as a loading control. The values in the bar graph represent mean ± SEM of densitometric data obtained from three separate experiments. IB, immunoblotting; IP, immunoprecipitation. (*C*) MA10 cells were treated with vehicle or PDE4+8 inhibitors for 2 h, and cell extracts were then used for immunoprecipitation with a mouse anti-SREBP2 antibody (anti-SR) recognizing full-length SREBP2 and the C-terminal fragment of cleaved SREBP2, or control IgG (cont IgG). Immunoprecipitated samples and the lysate (6%) were used to determine SCAP levels. The values in the bar graph represent mean ± SEM of densitometric data obtained from two separate experiments each performed in triplicates. *P < 0.05 and **P < 0.01 denote statistical significance.

PKA-dependent. However, we cannot rule out the possibility of some contribution of another kinase. Immunostaining using the phospho-S821 SCAP antibody detected signals only in cells treated with PDE inhibitors (Fig. 5*B*).

Discussion

The data in our previous phosphoproteomic study (8) and this study strongly suggest that cAMP/PKA coordinates not just one but rather a large number of different processes that operate together to provide sufficient cholesterol for maximal cAMP-stimulated steroidogenesis. This conclusion is based mostly on the identity and known roles of the large number of different proteins phosphorylated on PKA consensus sites in response to what should be a relatively pure cAMP signal (i.e., a signal caused by inhibition of PDEs 4 and 8). Two of the phosphoproteins seen were SCAP and HSL/cholesteryl ester hydrolase (Table 1).

In this study, we have used CRISPR-Cas9–mediated gene inactivation to investigate in some detail the SCAP/SREBP pathway, and found a heretofore unrecognized and important role for cAMP/PDE4+8/PKA in the SCAP/SREBP pathway that likely contributes to the large increase in steroidogenic response to cAMP and LH.

We decided to knock down SCAP by using two different CRISPR gene-editing protocols [CRISPR-Cas9 and FokI/dCas9 (23)] because standard RNAi and shRNA gene-depletion protocols usually are quite difficult in these cells, as they are hard to fully transfect. As previously seen in the liver of SCAP knockout mice (18), MA10 cells with SCAP deficiency had greatly reduced levels of SREBP2. Importantly, these cells showed markedly attenuated steroid production in response to either PDE4+8 inhibition or to LH. SCAP deficiency also attenuated mRNA levels of the SREBP2 targets *Hmgcr*, *Hmgcs*, *Fdps*, *Cyp51*, and *Ldlr*, as previously reported in different tissues and cell types (18). Furthermore, overexpression of exogenous SCAP restored both attenuated SREBP2 and steroidogenesis, suggesting that SCAP is necessary for optimal steroidogenesis in these cells.

We then focused on examining whether cAMP/PKA, through inhibition of PDE4 and PDE8, could directly modulate SREBP2 processing. The PDE inhibitors increased the presence of cleaved mature SREBP2 in the nuclear fraction while reducing the amount of full-length SREBP2 bound to SCAP, clearly indicating that PDE4+8 inhibition caused SREBP2 activation. One explanation for this effect could be that PDE inhibition increased formation of SCAP–SREBP2 complexes and/or SCAP recycling after SREBP cleavage (26), thereby increasing the pool of SREBP2 available for subsequent cleavage and activation. Thus, immunoprecipitation with a C-terminal anti-SREBP2 antibody resulted in more SCAP bound in cells treated with the PDE inhibitors, compared with untreated cells. It also is possible that PKA reduces Insig availability (27) and/or results in recruitment of unknown adapter proteins to either SCAP or SREBP2. Such processes might allow formation of more SREBP2–SCAP complexes.

Our phosphoproteomic study demonstrated an increased phosphorylation of SCAP S821 in response to PDE4+8 inhibition (Table 1). Interestingly, S821 is located in SCAP's WD domain, which directly binds to the C terminus of SREBP2. We confirmed that PKA activation through PDE4+8 inhibition increased the phosphorylation of S821 in SCAP by developing a phospho-S821–specific antibody. S821 phosphorylation was clearly increased by cAMP agonists as well as the PDE inhibitors and was sensitive to PKA inhibitors (H89 and Rp-8-CPT-cAMPS) in MA10 cells. Interestingly, our recent phosphoproteomic study (8) identified only 54 phosphorylation sites as significant regulatory sites in the response to PDE8 inhibition alone, compared with 749 sites regulated by the combination of PDE4 and PDE8 inhibitors. S821 was one of the 54 regulatory sites, which might be characterized as low-threshold for cAMP and/or be location-specific, possibly Golgi-specific in the case of SCAP, because PDE8B also is localized in the Golgi apparatus (5).

Other phosphorylation sites likely to be of direct importance in the SCAP/SREBP activation pathway were also seen in the phosphoproteomic studies (Table 1). For example, Sec23-interacting protein (S748), oxysterol-binding protein-related protein 11 (S194), and Sec22b (S137) are likely to be involved in the production and transport of the SCAP/SREBP-containing vesicles (28). In fact, a PKA inhibitor has been reported to prevent the recruitment of Sar1 and Sec23/24 to the sites forming the COPII coat complex, thereby preventing ER export of cargo (29). These findings suggest that the phosphorylation on SCAP S821 may be just one of the modifications needed in order for cAMP/PKA to exert an effect on SCAP function. Similarly, the recycling of SCAP back to the ER is likely to involve ARF1-mediated pathways, and several small GTPase regulatory proteins important to these pathways were also phosphorylated on consensus PKA sites by the combination of PDE4 and PDE8 inhibitors (Table 1). The relative importance of these other sites remains to be elucidated in future work.

Our study also suggests that a major source of cholesterol used for steroidogenesis in serum-starved Leydig cells is provided through de novo synthesis, consistent with earlier studies demonstrating that primary Leydig cells and MA10 tumor Leydig cells do not depend heavily on extracellular cholesterol uptake (30, 31), at least acutely. Thus, the HMGCoA reductase inhibitor lovastatin inhibited steroidogenesis in WT cells to levels similar to those seen in SCAP-deficient cells. Isoprenylation of proteins provided by farnesyl pyrophosphate and geranylgeranyl pyrophosphate through the cholesterol synthesis pathway is likely to contribute as well, because addition of inhibitors of farnesyl or geranylgeranyl transferases led to significant impairment of cAMP-dependent steroidogenesis, which was partially reversed by geranylgeranyl pyrophosphate. Furthermore, addition of exogenous cholesterol

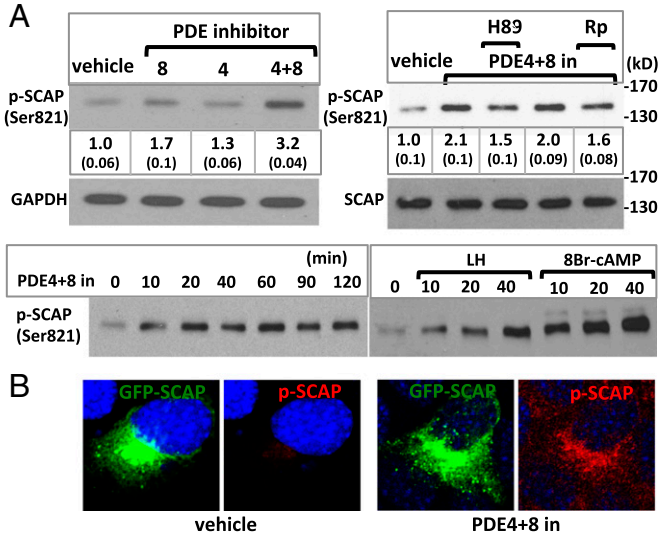

Fig. 5. PDE4+8 inhibitors and PKA activators increase Ser821 phosphorylation of SCAP. (*A*) MA10 cells were serum-starved for 3 h and then treated with vehicle, PDE8 inhibitor (200 nM), PDE4 inhibitor (10 μM), or PDE4+8 inhibitors for the indicated incubation times. H89 (10 μM) or Rp-8-CPT-cAMPS (Rp; 0.5 mM) was added to the cells 30 min before addition of PDE4+8 inhibitors (20-min stimulation). The intensity of p-SCAP was densitometrically measured and is shown as an average (SD) ($n = 3$). Serum-starved MA10 cells were also treated with LH (20 ng/mL) or 8Br-cAMP (300 μM) for 10–40 min. The cell extracts were used to determine the phosphorylated SCAP at S821. Total SCAP or GAPDH was used as a loading control. (*B*) MA10 cells transfected with GFP-SCAP were used for immunostaining with the phospho-SCAP–specific antibody (Ser821). Phospho-SCAP immunoreactivity was labeled with an anti-rabbit secondary antibody conjugated with Alexa 546 (red). TOPRO3 was used as a nuclear counterstain (blue).

did not fully restore progesterone production in lovastatin-treated cells. It is therefore likely that prenylation of proteins involved in trafficking of cholesterol from the plasma membrane or intracellular compartments by mechanisms that may rely on prenylated proteins also contribute to steroidogenesis (32). In addition, we found that levels of active RhoA were increased by the PDE4+8 inhibitor treatment in MA10 cells (Fig. S5), and significant phosphorylation events in Arhgap17 and Arhgef2, which are known regulators of Rho activity (33–35), were identified in the phosphoproteomic study (Table 1). Importantly, these findings strongly suggest that PKA activation could modify the activities of these GAPs and GEFs (in addition to regulating prenylation), thereby resulting in a faster activation cycle of Rho-type GTPases that are involved in vesicle transport and microfilament arrangement (36). These molecular events might also be expected to influence cholesterol transport to mitochondria for steroidogenesis (37). A similar argument can be made for several different GEFs and GAPs that regulate Rab proteins, which are also highly implicated in regulation of vesicle trafficking (Table 1).

Finally, the data strongly suggest that PKA has multiple known targets in both the cholesterol biosynthetic and uptake/mobilization pathways, some of which appear to be partly independent of SCAP. For example, the SREBP2-regulated genes *Hmgcr*, *Fdps*, *Cyp51*, and *Ldlr* were all significantly up-regulated by the PDE inhibitors in both WT and SCAP-deficient cells, although the absolute levels were lower in SCAP-deficient cells. Some portion of these effects could be due to PKA-mediated activation of the transcription factors CREB, SF-1 (*Nr5a1*), GATA4, and NF-γ/p300, because maximal transcriptional activation by SREBPs requires activation of multiple transcription factors (38–41). In addition to the facilitation of cholesterol biosynthesis, the up-regulation of *Hmgcr* and *Fdps* by PKA should also increase the availability of isoprenoids that are necessary to locate small GTPases to their proper targets.

cAMP elevation and PKA activation are known to stimulate several critical steps in steroid production (42). PKA activation leads to activation of HSL (5, 43), which releases cholesterol and free fatty acids from cholesteryl ester storage and perhaps other sites in the cell. However, we demonstrate using HSL-deficient cell clones that HSL appears not to contribute significantly to cAMP-dependent steroidogenesis under the experimental conditions studied. Perhaps stimulation of HSL activity is only important under certain conditions of cholesterol availability. Ultimately, the liberated free cholesterol from all sources is transported into mitochondria by StAR protein, which is also reported to be phosphorylated and activated by PKA (44, 45). Once in mitochondria, cholesterol is converted to pregnenolone by CYP11A1 and then further processed to progesterone and testosterone. Both StAR and CYP11A1 expression are induced by PKA, as demonstrated previously and in the present study, and are generally considered as rate-limiting steps in steroidogenesis (46, 47). However, the results of the present study suggest that other PKA substrates can also be regulatory with regard to cAMP activation of steroid synthesis, and add PKA-mediated regulation of SCAP function as a likely critical step for cAMP-dependent regulation of steroidogenesis.

In summary, we demonstrate that SCAP deficiency attenuates Leydig cell steroidogenesis in response to LH and cAMP elevation, and that simultaneous inhibition of PDE4 and PDE8 results in facilitated processing and activation of SREBP2. These results highlight an important role for SCAP and the cholesterol biosynthetic pathway in steroidogenesis in response to LH and cAMP elevation by PDE4 and PDE8 inhibition, and may open new avenues for modulation of SCAP-dependent processes by PDE inhibitors in other tissues.

Materials and Methods

A detailed description of experimental procedures and reagents is provided in *SI Materials and Methods*. In short, phosphorylation of proteins in response to cAMP elevation by combined inhibition of PDE4 and PDE8 in MA10 Leydig cells was determined by mass spectrometry. Two CRISPR-Cas9 approaches were used to knock down expression of SCAP, one of the proteins involved in cellular cholesterol handling identified by the phosphoproteomic screen. In some experiments, SCAP was reexpressed in SCAP-deficient cells by transfection by a GFP-SCAP plasmid. Activation of the SCAP–SREBP2 pathway in response to PDE4+8 inhibition was measured by immunoprecipitation of the SCAP–SREBP2 complex, detection of nuclear mature SREBP2, real-time PCR to evaluate downstream gene expression, and progesterone production. The relative contribution of substrate for steroidogenesis provided by de novo cholesterol synthesis and isoprenylation-dependent mechanisms was evaluated by inhibitors of cholesterol synthesis and prenylation and exogenous addition of substrates for these pathways.

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1. Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: Molecular regulation to clinical use. *Pharmacol Rev* 58(3):488–520.
2. Maurice DH, et al. (2014) Advances in targeting cyclic nucleotide phosphodiesterases. *Nat Rev Drug Discov* 13(4):290–314.
3. Tsai LC, Beavo JA (2011) The roles of cyclic nucleotide phosphodiesterases (PDEs) in steroidogenesis. *Curr Opin Pharmacol* 11(6):670–675.
4. Tsai LC, Shimizu-Albergine M, Beavo JA (2011) The high-affinity cAMP-specific phosphodiesterase 8B controls steroidogenesis in the mouse adrenal gland. *Mol Pharmacol* 79(4):639–648.
5. Shimizu-Albergine M, Tsai LC, Patrucco E, Beavo JA (2012) cAMP-specific phosphodiesterases 8A and 8B, essential regulators of Leydig cell steroidogenesis. *Mol Pharmacol* 81(4):556–566.
6. Vasta V, Shimizu-Albergine M, Beavo JA (2006) Modulation of Leydig cell function by cyclic nucleotide phosphodiesterase 8A. *Proc Natl Acad Sci USA* 103(52):19925–19930.
7. Oki N, Takahashi SI, Hidaka H, Conti M (2000) Short term feedback regulation of cAMP in FRTL-5 thyroid cells. Role of PDE4D3 phosphodiesterase activation. *J Biol Chem* 275(15):10831–10837.
8. Golkowski M, Shimizu-Albergine M, Suh HW, Beavo JA, Ong S-E (2016) Studying mechanisms of cAMP and cyclic nucleotide phosphodiesterase signaling in Leydig cell function with phosphoproteomics. *Cell Signal* 28(7):764–778.
9. Brown MS, Goldstein JL (1997) The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 89(3):331–340.
10. Steck TL, Lange Y (2002) SCAP, an ER sensor that regulates cell cholesterol. *Dev Cell* 3(3):306–308.
11. Sun LP, Seemann J, Goldstein JL, Brown MS (2007) Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: Insig renders sorting signal in Scap inaccessible to COPII proteins. *Proc Natl Acad Sci USA* 104(16):6519–6526.
12. Radhakrishnan A, Goldstein JL, McDonald JG, Brown MS (2008) Switch-like control of SREBP-2 transport triggered by small changes in ER cholesterol: A delicate balance. *Cell Metab* 8(6):512–521.
13. Brown AJ, Sun L, Feramisco JD, Brown MS, Goldstein JL (2002) Cholesterol addition to ER membranes alters conformation of SCAP, the SREBP escort protein that regulates cholesterol metabolism. *Mol Cell* 10(2):237–245.
14. DeBose-Boyd RA, et al. (1999) Transport-dependent proteolysis of SREBP: Relocation of site-1 protease from Golgi to ER obviates the need for SREBP transport to Golgi. *Cell* 99(7):703–712.
15. Espenshade PJ, Cheng D, Goldstein JL, Brown MS (1999) Autocatalytic processing of site-1 protease removes propeptide and permits cleavage of sterol regulatory element-binding proteins. *J Biol Chem* 274(32):22795–22804.
16. Espenshade PJ, Li WP, Yabe D (2002) Sterols block binding of COPII proteins to SCAP, thereby controlling SCAP sorting in ER. *Proc Natl Acad Sci USA* 99(18):11694–11699.
17. Horton JD, et al. (2003) Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc Natl Acad Sci USA* 100(21):12027–12032.
18. Matsuda M, et al. (2001) SREBP cleavage-activating protein (SCAP) is required for increased lipid synthesis in liver induced by cholesterol deprivation and insulin elevation. *Genes Dev* 15(10):1206–1216.
19. Grevengoed TJ, Klett EL, Coleman RA (2014) Acyl-CoA metabolism and partitioning. *Annu Rev Nutr* 34:1–30.
20. Kanter JE, Tang C, Oram JF, Bornfeldt KE (2012) Acyl-CoA synthetase 1 is required for oleate and linoleate mediated inhibition of cholesterol efflux through ATP-binding cassette transporter A1 in macrophages. *Biochim Biophys Acta* 1821(3):358–364.
21. Maloberti P, et al. (2005) Silencing the expression of mitochondrial acyl-CoA thioesterase I and acyl-CoA synthetase 4 inhibits hormone-induced steroidogenesis. *FEBS J* 272(7):1804–1814.

22. Cornejo Maciel F, et al. (2005) An arachidonic acid-preferring acyl-CoA synthetase is a hormone-dependent and obligatory protein in the signal transduction pathway of steroidogenic hormones. *J Mol Endocrinol* 34(3):655–666.
23. Tsai SQ, et al. (2014) Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. *Nat Biotechnol* 32(6):569–576.
24. Brown MS, Goldstein JL (1999) A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc Natl Acad Sci USA* 96(20):11041–11048.
25. Sakai J, et al. (1997) Identification of complexes between the COOH-terminal domains of sterol regulatory element-binding proteins (SREBPs) and SREBP cleavage-activating protein. *J Biol Chem* 272(32):20213–20221.
26. Shao W, Espenshade PJ (2014) Sterol regulatory element-binding protein (SREBP) cleavage regulates Golgi-to-endoplasmic reticulum recycling of SREBP cleavage-activating protein (SCAP). *J Biol Chem* 289(11):7547–7557.
27. Goldstein JL, DeBose-Boyd RA, Brown MS (2006) Protein sensors for membrane sterols. *Cell* 124(1):35–46.
28. Lehto M, Olkkonen VM (2003) The OSBP-related proteins: A novel protein family involved in vesicle transport, cellular lipid metabolism, and cell signalling. *Biochim Biophys Acta* 1631(1):1–11.
29. Aridor M, Balch WE (2000) Kinase signaling initiates coat complex II (COPII) recruitment and export from the mammalian endoplasmic reticulum. *J Biol Chem* 275(46):35673–35676.
30. Freeman DA, Ascoli M (1982) Studies on the source of cholesterol used for steroid biosynthesis in cultured Leydig tumor cells. *J Biol Chem* 257(23):14231–14238.
31. Nagy L, Freeman DA (1990) Cholesterol movement between the plasma membrane and the cholesteryl ester droplets of cultured Leydig tumour cells. *Biochem J* 271(3): 809–814.
32. Hu J, Zhang Z, Shen WJ, Azhar S (2010) Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. *Nutr Metab (Lond)* 7:47.
33. Bos JL, Rehmann H, Wittinghofer A (2007) GEFs and GAPs: Critical elements in the control of small G proteins. *Cell* 129(5):865–877.
34. Meiri D, et al. (2009) Modulation of Rho guanine exchange factor Lfc activity by protein kinase A-mediated phosphorylation. *Mol Cell Biol* 29(21):5963–5973.
35. Cherfils J, Zeghouf M (2013) Regulation of small GTPases by GEFs, GAPs, and GDIs. *Physiol Rev* 93(1):269–309.
36. Li D, Sewer MB (2010) RhoA and DIAPH1 mediate adrenocorticotropin-stimulated cortisol biosynthesis by regulating mitochondrial trafficking. *Endocrinology* 151(9): 4313–4323.
37. Ikonen E (2008) Cellular cholesterol trafficking and compartmentalization. *Nat Rev Mol Cell Biol* 9(2):125–138.
38. Halder SK, Fink M, Waterman MR, Rozman D (2002) A cAMP-responsive element binding site is essential for sterol regulation of the human lanosterol 14alpha-demethylase gene (CYP51). *Mol Endocrinol* 16(8):1853–1863.
39. Edwards PA, Tabor D, Kast HR, Venkateswaran A (2000) Regulation of gene expression by SREBP and SCAP. *Biochim Biophys Acta* 1529(1–3):103–113.
40. Rome S, et al. (2008) Microarray analyses of SREBP-1a and SREBP-1c target genes identify new regulatory pathways in muscle. *Physiol Genomics* 34(3):327–337.
41. Bergeron F, Nadeau G, Viger RS (2015) GATA4 knockdown in MA-10 Leydig cells identifies multiple target genes in the steroidogenic pathway. *Reproduction* 149(3):245–257.
42. Eacker SM, et al. (2008) Hormonal regulation of testicular steroid and cholesterol homeostasis. *Mol Endocrinol* 22(3):623–635.
43. Holm C (2003) Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Biochem Soc Trans* 31(Pt 6):1120–1124.
44. Clark BJ, Ranganathan V, Combs R (2000) Post-translational regulation of steroidogenic acute regulatory protein by cAMP-dependent protein kinase A. *Endocr Res* 26(4):681–689.
45. Dyson MT, Kowalewski MP, Manna PR, Stocco DM (2009) The differential regulation of steroidogenic acute regulatory protein-mediated steroidogenesis by type I and type II PKA in MA-10 cells. *Mol Cell Endocrinol* 300(1–2):94–103.
46. Stocco DM (2001) StAR protein and the regulation of steroid hormone biosynthesis. *Annu Rev Physiol* 63:193–213.
47. Stocco DM, Clark BJ (1996) Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. *Biochem Pharmacol* 51(3):197–205.
48. Liu J, Li H, Papadopoulos V (2003) PAP7, a PBR/PKA-RIalpha-associated protein: A new element in the relay of the hormonal induction of steroidogenesis. *J Steroid Biochem Mol Biol* 85(2–5):275–283.
49. Dyson MT, et al. (2008) Mitochondrial A-kinase anchoring protein 121 binds type II protein kinase A and enhances steroidogenic acute regulatory protein-mediated steroidogenesis in MA-10 mouse Leydig tumor cells. *Biol Reprod* 78(2):267–277.
50. Hornbeck PV, et al. (2015) PhosphoSitePlus, 2014: Mutations, PTMs and recalibrations. *Nucleic Acids Res* 43(Database issue):D512–D520.
51. Poderoso C, et al. (2008) A mitochondrial kinase complex is essential to mediate an ERK1/2-dependent phosphorylation of a key regulatory protein in steroid biosynthesis. *PLoS One* 3(1):e1443.