0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(8):3924–3935

doi: 10.1210/en.2005-1204

Energized, Polarized, and Actively Respiring Mitochondria Are Required for Acute Leydig Cell Steroidogenesis

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The first and rate-limiting step in the biosynthesis of steroid hormones is the transfer of cholesterol into mitochondria, which is facilitated by the steroidogenic acute regulatory (StAR) protein. Recent study of Leydig cell function has focused on the mechanisms regulating steroidogenesis; however, few investigations have examined the importance of mitochondria in this process. The purpose of this investigation was to determine which aspects of mitochondrial function are necessary for acute cAMP-stimulated Leydig cell steroidogenesis. MA-10 cells were treated with 8-bromoadenosine 3′,5′-cyclic monophosphate (cAMP) and different site-specific agents that disrupt mitochondrial function, and the effects on acute cAMP-stimulated progesterone synthesis, StAR mRNA and protein, mitochondrial membrane potential ($\Delta\psi_m$), and ATP synthesis were determined. cAMP treatment of MA-10 cells resulted in significant increases in both cellular respiration and $\Delta\psi_m$. Dissipating $\Delta\psi_m$ with carbonyl cyanide *m*-chlorophenyl hydrazone resulted in a profound reduction in progesterone synthesis, even in the presence of newly synthesized StAR protein. Preventing electron transport in mitochondria with antimycin A significantly reduced cellular ATP, potently inhibited steroidogenesis, and reduced StAR protein levels. Inhibiting mitochondrial ATP synthesis with oligomycin reduced cellular ATP, inhibited progesterone synthesis and StAR protein, but had no effect on $\Delta\psi_m$. Disruption of intramitochondrial pH with nigericin significantly reduced progesterone production and StAR protein but had minimal effects on $\Delta\psi_m$. 22(R)-hydroxycholesterol-stimulated progesterone synthesis was not inhibited by any of the mitochondrial reagents, indicating that neither P450 side-chain cleavage nor 3β-hydroxysteroid dehydrogenase activity was inhibited. These results indicate that $\Delta\psi_m$, mitochondrial ATP synthesis, and mitochondrial pH are all required for acute steroid biosynthesis. These results suggest that mitochondria must be energized, polarized, and actively respiring to support Leydig cell steroidogenesis, and alterations in the state of mitochondria may be involved in regulating steroid biosynthesis. (*Endocrinology* 147: 3924–3935, 2006)

MITOCHONDRIA ARE a key control point for the regulation of steroid hormone biosynthesis. The first and rate-limiting step in steroidogenesis is the transfer of cholesterol across the intermembrane space from the outer mitochondrial membrane to the inner mitochondrial membrane, a process dependent on the action of steroidogenic acute regulatory protein (StAR) (1, 2). Testosterone production by Leydig cells is primarily under the control of LH, which acts via its intracellular second messenger cAMP to regulate testosterone production acutely at the level of cholesterol transport into the mitochondria via the action of StAR and chronically at the level of steroidogenic enzyme gene transcription. StAR is a nuclear encoded, mitochondrial targeted protein that is rapidly synthesized in response to intracellular pulses of cAMP. It is translated as a 37-kDa precursor, believed to be the form active in cholesterol transfer, which is imported into the mitochondrial matrix and processed to the mature 30-kDa form. Recent investigations have suggested that phosphorylation of StAR is important for modulating its activity in cholesterol transfer (3–5). LH stimulation of the Leydig cell results in the activation of StAR transcription and translation, and StAR facilitates the transfer of cholesterol into the mitochondrial matrix to cholesterol side-chain cleavage cytochrome P450/Cyp11A (P450scc), which converts cholesterol to pregnenolone (6). Pregnenolone diffuses out of the mitochondria to the smooth endoplasmic reticulum in which it is further metabolized via the action of 3β-hydroxysteroid dehydrogenase Δ^5-Δ^4-isomerase to progesterone. Progesterone in turn is converted by a two-step process to androstenedione via the action of 17α-hydroxylase/C_{17-20} lyase. The conversion of androstenedione to testosterone is catalyzed by 17β-hydroxysteroid dehydrogenase type III (for review see Ref. 7).

In addition to their importance in steroid biosynthesis, mitochondria are essential for the formation of ATP, which occurs during metabolism. During aerobic respiration, ATP is generated through oxidative phosphorylation, which involves the transport of electrons through four enzyme complexes located at the inner mitochondrial membrane. These electron transport chain enzymes including nicotinamide ad-

First Published Online May 11, 2006

Abbreviations: BCA, Bicinchoninic acid; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; $\Delta\Psi_m$, mitochondrial membrane potential; ΔpH, mitochondrial pH gradient; P450scc, P450 side-chain cleavage enzyme; R22, 22(R)-hydroxycholesterol; SDS, sodium dodecyl sulfate; SFM, serum-free medium; StAR, steroidogenic acute regulatory protein; TCA, trichloracetic acid; TMRE, tetramethylrhodamine ethyl ester dye.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

enine dinucleotide hydroxide dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome bc_1 (complex III), and cytochrome oxidase (complex IV). Transport of electrons through the enzymes yields free energy that is used to pump protons from the matrix into the intermembrane space, thereby creating a proton gradient in the mitochondria. This proton gradient generates a pH differential (ΔpH) and the mitochondrial membrane potential ($\Delta\psi_m$), which provides the proton motive force that drives ATP synthesis by the F_0/F_1 ATPase (complex V).

Historically, studies of the mitochondria have focused on elucidating the mechanism of ATP production and defining the biochemical basis of oxidative phosphorylation. More recently studies on the mitochondria have focused on its role in the intrinsic pathway of programmed cell death. Collectively these studies have provided a detailed and comprehensive understanding of mitochondrial dynamics and provide a wealth of highly specific reagents to probe aspects of mitochondrial function.

Pharmacological agents known as mitochondrial disrupters can directly affect mitochondrial activity, often by uncoupling electron transport from ATP synthesis. Ionophores such as carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) and valinomycin perturb the $\Delta\psi_m$ by disrupting the hydrogen ion gradient (8–10). Another ionophore, nigericin disrupts ΔpH by exchanging K^+ for H^+, effectively equilibrating pH without affecting $\Delta\psi_m$ (9). CCCP, valinomycin, and nigericin perturb the mitochondria and inhibit StAR mediated cholesterol transfer (5, 11–17). Previous studies have shown that during oxidative stress, reactive oxygen species, and reactive nitrogen species perturb Leydig cell mitochondria, causing an abrupt cessation to cholesterol transfer and steroid hormone production (18–20). In addition, acute inflammation results in oxidative stress of Leydig cells, which perturbs mitochondria and inhibits steroidogenesis (18, 19). Other mitochondrial disruptors such as antimycin A and oligomycin and their effects on steroidogenesis have not been reported. Antimycin A is a complex III inhibitor that prevents electron transport. Oligomycin inhibits the F_0/F_1 ATPase preventing ATP synthesis.

The purpose of the present study was to determine which aspects of mitochondrial function are necessary for acute cAMP-stimulated Leydig cell steroidogenesis. Results of these studies demonstrate that $\Delta\psi_m$, ΔpH, and mitochondrial ATP synthesis are all essential for mitochondrial steroidogenesis. This study indicates that disrupting mitochondria results in posttranscriptional inhibition of StAR and that mitochondria must be energized, polarized, and actively respiring to support Leydig cell steroidogenesis.

Materials and Methods

Materials

[α-^{32}P]Deoxy-GTP [^{35}S]-translabel were from ICN (Irvine, CA). Random primed labeling kit was from Roche Molecular Biochemicals (Indianapolis, IN). Progesterone RIA kits were purchased from Diagnostic Products Corp. (Los Angeles, CA). Oligomycin, antimycin A, CCCP, nigericin, 5-cholesten-3β, 22(R)-diol [22(R)-hydroxycholesterol], 8-bromoadenosine-cAMP (cAMP), phenylmethylsulfonyl fluoride, leupeptin, dithiothreitol, EDTA, and Nonidet P-40 were purchased from Sigma Chemical Inc. (St. Louis, MO). Waymouth's MB752/1, penicillin, and streptomycin were obtained from Gibco BRL Life Technologies (Gaithersburg, MD). Bicinchoninic acid (BCA) kit and protein A Sepharose were from Pierce (Rockford, IL). Tetramethyl rhodamine ethylester dye (TMRE) was obtained from Molecular Probes (Eugene, OR). Mouse StAR cDNA and pSport-StAR were a generous gift from Douglas M. Stocco (Texas Tech University, Lubbock, TX). The Promega ATP luminescent cell viability assay kit was purchased from Fisher Scientific (Fair Lawn, NJ). All other reagents were from sources previously described. Antibovine P450scc antisera was prepared previously, as described (21).

Cell culture

MA-10 cells were a generous gift of Dr. Mario Ascoli (University of Iowa, Iowa City, IA) and were cultured essentially as originally described (22). MA-10 cells were cultured in Waymouth's complete medium MB 752/1 (Life Technologies, Inc., Grand Island, NY) containing 15% heat-inactivated donor herd horse serum (Life Technologies). Pretreatment of confluent cell layers in culture dishes was performed in serum-free Waymouth's medium (SFM) for 1 h before the onset of experimental treatments. Cells were incubated at 37 C in 5% CO_2 in a humidified incubator.

Western blotting

Total cellular protein was obtained by placing cells in lysis buffer [PBS/0.1% sodium dodecyl sulfate (SDS)] followed by brief sonication (Ultrasonic processor GE 50 T, 50% power for ~2 sec). Protein concentrations were determined by micro-BCA (Pierce). Thirty micrograms of total protein were separated by SDS-PAGE using 10% acrylamide/SDS separating gels and transferred to nitrocellulose paper membranes as described previously (23–25). The preparation of the polyclonal antiserum to StAR and P450scc has been described previously (21, 26). A polyclonal rabbit antisera to Nur77 was obtained from Dr. Lester Lau (University of Illinois at Chicago, Chicago, IL). A phospho-specific StAR antibody that recognizes phosphorylated ser194 was provided by Dr. Steve King (Baylor University, Houston, TX) (3). Detection of bound antibody on the blot was assessed with a horseradish peroxidase-conjugated, goat antirabbit IgG antibody (Sigma), visualized by chemiluminescent detection (enhanced chemiluminescence Western blot detection kit; Amersham Pharmacia Biotech Inc., Piscataway, NJ), and quantitated after densitometry (personal densitometer, Molecular Dynamics, Sunnyvale, CA) using Imagequant software (Molecular Dynamics). Data for protein are represented as integrated OD.

Northern blotting

Total cellular RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (27) as described previously (23). The RNA was analyzed by Northern blotting as described (25, 28). Northern hybridization was performed by use of a α^{32}P-dGTP-labeled random primed cDNA probe specific for StAR. Radioactivity was visualized after exposure to a phosphor screen for 24 h. Hybridization signals were quantified and documented by use of a PhosphorImager (Storm 860TM; Molecular Dynamics).

[^{35}S]Methionine labeling of StAR and immunoprecipitation

Metabolic labeling and immunoisolation of StAR were done as described previously (29) with modifications, as described (30). Cells were grown to approximately 50% confluency and then cultured in SFM for 1 h before the initiation of labeling. Cells were transferred to methionine-free medium containing 0.5 mM cAMP and incubated for 30 min and then metabolically labeled with 100–250 μCi [^{35}S]-translabel in methionine-free control media or media that contained cAMP, or cAMP plus 5 μM CCCP or CCCP alone. Cells were labeled for 2 h and then lysed in NaPBS that contained 0.1% SDS and 1% cholate, scraped from the dish with a rubber policeman, and flash frozen on dry ice. The lysates were thawed on ice and diluted with lysate dilution buffer to a final concentration of 1.25% Nonidet P-40, 0.1% SDS, 1 mM EDTA, 10 L-methionine, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 50 μg/ml leupeptin in NaPBS. Lysates were cleared by centrifugation (12,000 × *g* for 20 min). Protein concentration of supernatants was determined by micro-BCA protein assay. Tricholoracetic acid (TCA)-precipitable radioactivity was measured by precipitating a portion of the lysate with

1 ml ice-cold TCA in the presence of 1 mg of BSA, trapping the precipitate on glass fiber filters (GF/A; Whatman, Middlesex, UK) and washing extensively in ice-cold TCA and 95% ethanol. The air-dried filters were solubilized and radioactivity determined by liquid scintillation spectroscopy. An equal amount of protein from each sample was preincubated with protein A-Sepharose for 1 h at room temperature before pelleting for 5 min at 12,000 × *g*. Supernatants were incubated with anti-StAR antiserum (1:1000 final dilution) overnight at 4 C before incubation with protein A-Sepharose for 1 h at room temperature. Pellets were collected by brief centrifugation in a tabletop microcentrifuge, the supernatant removed, and the pellet washed three times in lysate dilution buffer. The final pellet was resuspended in SDS-PAGE sample buffer, boiled for 5 min, and subjected to electrophoresis in an 10% SDS-polyacrylamide gel according to the method of Laemmli (24) and analyzed by phosphor imaging.

Analysis of $\Delta\psi_m$ with TMRE

Mitochondrial $\Delta\psi_m$ was assessed by measuring uptake and accumulation of the potentiometric dye, TMRE. MA-10 cells were grown in culture to 2.5×10^5 cells/cm^2 (~75% confluency) in a 96-well florescence assay plate (Costar 3603, Fisher Scientific). Treatment media were prepared in Waymouth's SFM. Treatment groups were analyzed in replicates of 16. After 3 h incubation at 37 C, treatment media were removed. Cells were then incubated in 200 μl of a 50-nM solution of TMRE in SFM for 20 min at 37 C. TMRE media were aspirated off and replaced with 200 μl of 0.1% PBS/BSA for analysis. Samples were analyzed for overall fluorescence using the Synergy HT microplate fluorescence reader (Bio-Tek, Winooski, VT) and the accompanying computer software (KC-4, version 3.3). TMRE fluorescence was obtained using an excitation of 550 nm (540/25 nm filter) and an emission at 590 nm (590/20 nm filter). Background fluorescence was normalized by subtracting fluorescence values of control cells (untreated, no TMRE). These corrected TMRE fluorescence values are proportional to the magnitude of $\Delta\psi_m$ and provided a quantitative assessment of the mitochondrial electrochemical gradient.

In addition, TMRE fluorescence was analyzed using fluorescence microscopy. MA-10 cells were cultured in 24-well plates and treated with or without 5.0 μM CCCP for 3 h. Cells were then incubated with 50 nm TMRE for 10 min at 37 C and examined using epifluorescence microscopy. A polarized, intact mitochondrial potential is necessary for the uptake of TMRE dye in mitochondria. Thus, analysis of TMRE fluorescence by microscopy allowed the semiquantitative assessment of $\Delta\psi_m$, as described (31). Fluorescence of the dye in Leydig cell mitochondria was visualized before treatment with CCCP. Fluorescent images were obtained using an inverted microscope equipped for fluorescent microscopy (Nikon Eclipse TE 300, 547 nm wavelength excitation, 579 nm emission, via high pressure Nikon Xenon XBO 75 W lamp; Nikon, Tokyo, Japan); a digital camera (RTE/CCD-1300 Y/HS, Roper Scientific, Trenton, NY; MicroMAX camera controller, Princeton Instruments Inc., Trenton, NY; Lambda 10–2 shutter, Sutter Instruments Co., Navato, CA), and image-processing software (IPLab, Scanalytics Inc., Fairfax, VA).

ATP Assay

Cellular ATP levels were assessed using the Promega luminescent cell viability assay (Promega G7570, Fisher Scientific). The 2.5×10^5 MA-10 cells were seeded and grown to 75% confluency in a 96-well luminescence assay plate (Costar, Fisher Scientific). Treatment media were prepared in 100 μl Waymouth's SFM. Treatment groups were analyzed in replicates of six. After 3 h treatment incubation at 37 C, cells were brought to room temperature, and 100 μl of Promega Cell Titer-Glo substrate (a mixture of Cell-Glo reagent and buffer) were added to the wells. Cells were incubated at room temperature on an orbital shaker for 2 min, followed by a 10-min standing incubation at room temperature enabling cell lysis. Samples were analyzed for overall luminescence using the Synergy HT microplate reader (Bio-Tek). These luminescent values are proportional to total cellular ATP and provided a quantitative assessment of cellular ATP levels.

RIA

After treatment, culture media were removed and boiled for 5 min and centrifuged at 2000 × g for 20 min at 4 C. The supernatant was stored at −20 C until assayed for progesterone using Coat-A-Count RIA kits (DPC, Los Angeles, CA) as previously described (20).

Assessment of mitochondrial respiration

Mitochondrial respiration was examined by measuring oxygen consumption. MA-10 cells were treated with or without 1 mM cAMP for 3 h, and control cells were incubated in SFM alone. Cells were then trypsinized and resuspended in Waymouth's complete medium and placed into a 300-μl capacity Mitocell MT200 (Strathkelvin Instruments, Glasgow, Scotland, UK) respirometry chamber and maintained at 37 C by means of a Lauda Econoline water bath. After resupension, oxygen consumption was measured over an 8-min period using a model 782 oxygen meter (Strathkelvin Instruments). To account for cell number variability, data obtained were normalized to cell count for each sample.

Statistical analysis

Data were presented as means ± SEM of three or more independent experiments. For two point data comparisons, the Mann-Whitney unpaired nonparametric two-tailed test was performed; for group comparisons, one-way ANOVA followed by a Student-Newman-Keuls multiple range test was performed, both using GraphPad InStat statistical software package (version 3.0; GraphPad Software, San Diego, CA). Differences were considered as significant at $P < 0.05$.

Results

Effects of disrupting $\Delta\psi_m$

To examine $\Delta\psi_m$, mouse tumor Leydig cells (MA-10) were treated with the proton ionophore CCCP, which dissipates the mitochondrial electrochemical gradient. To quantitate changes in the electrochemical gradient, $\Delta\psi_m$ was measured by TMRE fluorescence because TMRE fluorescence values are proportional to the magnitude of $\Delta\psi_m$. MA-10 cells were treated with 1 mM cAMP for 3 h to acutely stimulate steroidogenesis or with cAMP plus 5.0 μM CCCP. cAMP treatment of cells significantly increased $\Delta\psi_m$, and CCCP treatment resulted in a significant decrease in $\Delta\psi_m$ (Fig. 1A). TMRE fluorescence in MA-10 cells was also qualitatively assessed using fluorescence microscopy. CCCP treatment of MA-10 cells decreased TMRE fluorescence (Fig. 1B). Cells exposed to 5.0 μM CCCP showed an overall reduction in TMRE fluorescence, and the number of brightly fluorescent cells was markedly reduced.

To examine the effects of CCCP on cAMP-stimulated Leydig cell steroidogenesis, MA-10 cells were treated with cAMP or cAMP plus 5.0 μM CCCP, and progesterone production was determined by RIA. CCCP treatment of MA-10 cells inhibited cAMP-stimulated progesterone synthesis (Fig. 2A), indicating that dissipating $\Delta\psi_m$ significantly decreases steroidogenesis. To investigate whether CCCP inhibits steroidogenesis at the level of StAR, changes in StAR protein levels and mRNA expression were analyzed by Western and Northern blotting. cAMP treatment of MA-10 cells resulted in detection of the 37-kDa precursor form of StAR protein as well as the 30-kDa processed form (Fig. 2B). However, cAMP plus 5.0 μM CCCP significantly decreased the 30-kDa StAR protein, whereas the 37-kDa form remained constant. This decrease in the 30-kDa StAR protein due to CCCP suggests that StAR import and processing is prevented by dissipating $\Delta\psi_m$.

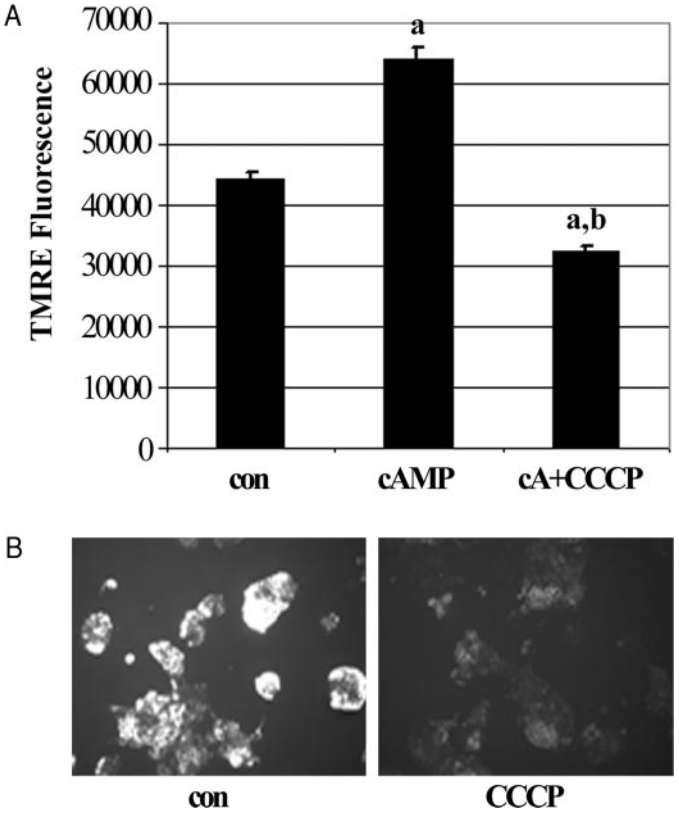

FIG. 1. Effect of CCCP on the $\Delta\psi_m$ in MA-10 cells. A, MA-10 cells were grown in a 96-well plate and treated for 3 h with 1 mM cAMP or cAMP plus 5 μM CCCP. Cells were subsequently incubated with 50 nm TMRE for 10 min, washed, and TMRE fluorescence quantitated using a fluorescence plate reader. A polarized, intact $\Delta\psi_m$ is necessary for the uptake of TMRE dye in mitochondria. Data are represented as mean ± SEM for 30 independent experiments. (n = 30); a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* cAMP. B, MA-10 cells were cultured in 24-well plates and treated with 5.0 μM CCCP for 3 h. Cells were then incubated with 50 nm TMRE dye for 10 min and examined by fluorescent microscopy. Analysis of TMRE fluorescence by microscopy allowed the semiquantitative assessment of $\Delta\psi_m$. Images of cells are representative of five independent experiments (n = 5).

In addition to StAR, P450scc protein was also examined by Western analysis; however, CCCP had no detectable effects on P450scc protein level (Fig. 2C). P450scc is localized within the matrix of mitochondria in which it catalyzes the conversion of cholesterol to pregnenolone. StAR mRNA expression was induced by cAMP, but CCCP did not alter the expression of any of the StAR mRNA transcripts (Fig. 2D), indicating that CCCP mediates its effects on StAR posttranscriptionally. To determine whether CCCP alters StAR translation, synthesis of StAR was examined by [^{35}S]methionine metabolic labeling, followed by immunoprecipitation and phosphor imaging as described in *Materials and Methods*. Two hours of cAMP treatment of MA-10 cells induced translation and incorporation of [^{35}S]methionine into newly synthesized StAR protein (Fig. 2E). Both the 37-kDa precursor and 30-kDa processed forms of StAR were radiolabeled after cAMP incubation. Treatment of MA-10 cells with cAMP plus 5.0 μM CCCP resulted in [^{35}S]methionine labeling of the 37-kDa StAR protein; however, no radiolabeled 30-kDa StAR could be detected. Thus, dissipation of $\Delta\psi_m$ with CCCP appears to affect StAR processing as evidenced in both Western blots for total StAR protein (Fig. 2B) and from the [^{35}S]methionine labeling studies for newly synthesized StAR protein (Fig. 2E). This indicates that dissipating $\Delta\psi_m$ with CCCP does not inhibit translation of StAR but instead alters StAR processing. Even though the 37-kDa StAR does not appear to accumulate due to CCCP, this is likely due to degradation of the 37-kDa form. The ratio of 37-kDa StAR to 30-kDa StAR was increased from approximately 0.25 to 5.0 after cells were treated with CCCP (Fig. 2E, *lower panel*). Of note, 5.0 μM CCCP treatment reduced $\Delta\psi_m$ by 30% *vs.* cAMP (Fig. 1A), but steroid production is reduced by over 90% (Fig. 2A). This suggests that there is a certain threshold of $\Delta\psi_m$ required to facilitate steroidogenesis and that below this threshold, cholesterol transfer and steroid biosynthesis cannot be supported. These experiments demonstrate that treatment of MA-10 cells with CCCP dissipates $\Delta\psi_m$, which profoundly inhibits acute cAMP-stimulated steroidogenesis even in the presence of newly synthesized StAR protein.

Effects of inhibiting mitochondrial electron transport at complex III

Shuttling of electrons through the four enzyme complexes at the inner mitochondrial membrane is coupled to oxidative phosphorylation and ATP synthesis. Transport of electrons through the enzymes yields free energy that is used by the complexes to pump protons into the intermembrane space, thereby generating the proton motive force that drives ATP synthesis. Antimycin A is a complex III inhibitor that prevents electron transport. To examine the effects of antimycin A on mitochondrial steroidogenesis, MA-10 cells were treated with cAMP or cAMP plus antimycin A for 3 h. These acute cAMP incubations alone induced the production of progesterone, but treatments of cAMP plus 1 μM and 10 μM antimycin A significantly inhibited steroidogenesis by over 90% *vs.* cAMP (Fig. 3A). This suggests that blocking mitochondrial electron transport at complex III potently inhibits cAMP stimulated steroidogenesis. To examine StAR protein, Western analyses were performed and StAR immunoreactivity was quantitated by scanning densitometry. A representative blot in Fig. 3B shows that cAMP robustly up-regulated the level of StAR protein. However, cAMP plus 1 μM and 10 μM antimycin A reduced StAR protein levels by 55 and 98%, respectively (n = 3). These data suggest that antimycin A inhibits steroidogenesis due to a reduction in StAR protein. To assess whether antimycin altered $\Delta\psi_m$, TMRE fluorescence in MA-10 cells was quantitated as previously described in Fig. 1. cAMP treatments alone resulted in a significant increase in $\Delta\psi_m$, and 1 μM and 10 μM antimycin reduced TMRE fluorescence by 22 and 57% *vs.* cAMP, respectively (n = 16) (Fig. 3C). These data indicate that preventing mitochondrial electron transport inhibits acute cAMP-stimulated steroidogenesis, reduces StAR protein level, and dissipates $\Delta\psi_m$.

Effects of inhibiting mitochondrial ATP synthesis at complex V

Relatively few studies have examined the requirement of ATP for steroidogenesis (11, 32). To further investigate this,

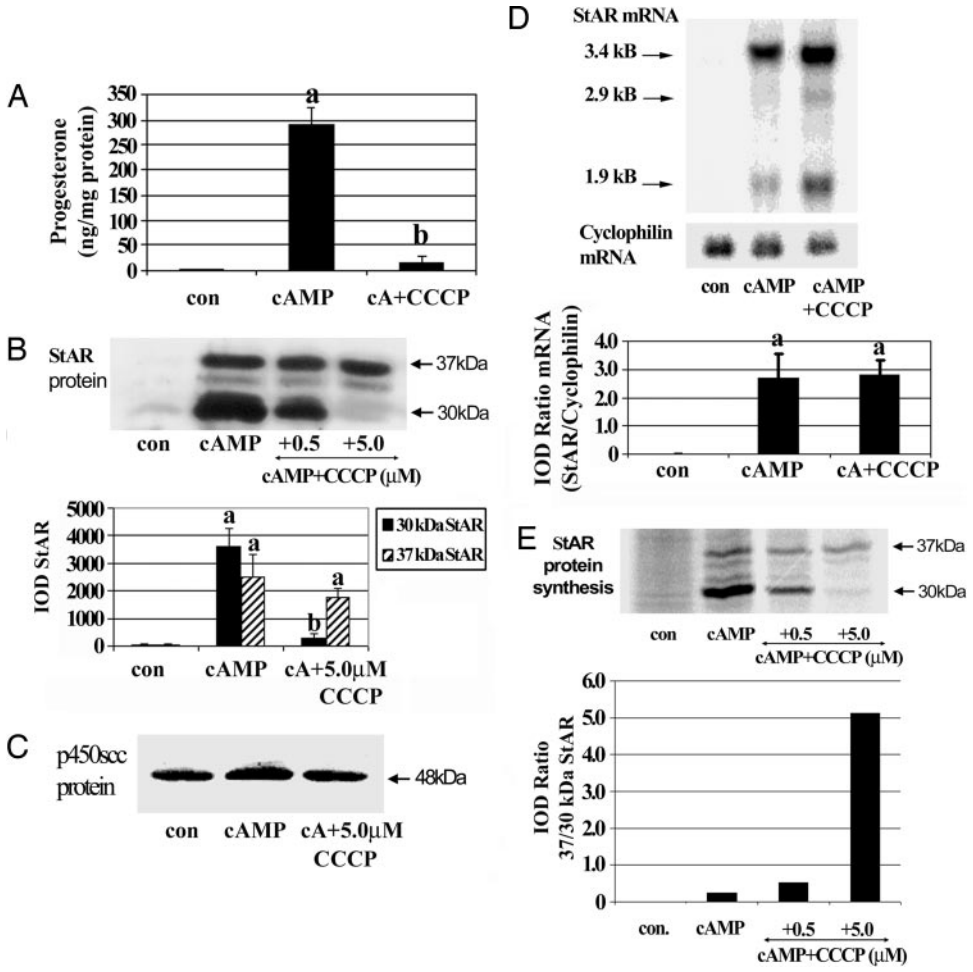

FIG. 2. Effects of CCCP on steroidogenesis and StAR expression in MA-10 cells. A, MA-10 cells were grown in culture and treated for 3 h with 1 mM cAMP or cAMP plus 5 μM CCCP. After treatment, media were collected and subjected to a progesterone RIA. Progesterone concentrations were normalized to protein concentrations for each sample and are represented as means ± SEMs for three independent experiments (n = 3). B, MA-10 cells were grown in culture and then treated for 3 h with cAMP or cAMP plus CCCP (0.5 or 5.0 μM). Cells were lysed and subjected to Western blot analysis to assess the level of StAR and P450scc proteins. A representative StAR blot is shown (*upper panel*), and pooled data from scanning densitometry (*lower panel*) are represented as mean ± SEM for three independent experiments (n = 3). C, MA-10 cells were treated and protein lysates subjected to Western blot analysis for P450scc protein. Data are representative of similar results obtained form three independent experiments. D, Northern blot analyses of CCCP-treated Leydig cells were performed to determine whether CCCP effects on steroidogenesis are at the level of StAR mRNA expression. StAR mRNA levels were normalized to that of cyclophilin and are expressed as the ratio of 3.4-kb StAR transcript to cyclophilin. Data are represented as means ± SEM for three independent experiments. (n = 3). E, To determine whether CCCP alters StAR protein synthesis, cells were metabolically labeled with [^{35}S]methionine in the presence of cAMP or cAMP plus 5 μM CCCP. Cell lysates were collected and immunoprecipitated for StAR, and radiolabeled samples were examined by phosphor imaging after electrophoresis. A representative phosphor image of [^{35}S]methionine-labeled StAR is shown (*upper panel*). The ratio of 37-kDa StAR to 30-kDa StAR was plotted (*lower panel*) to demonstrate that CCCP results in a loss of 30-kDa StAR. a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* cAMP.

MA-10 cells were treated with oligomycin, a selective inhibitor of the mitochondrial F_0/F_1 ATP synthase enzyme (complex V). MA-10 cells were treated for 3 h with cAMP or cAMP plus increasing concentrations of oligomycin (0.1–10 μM) and effects on progesterone production, StAR protein and $\Delta\psi_m$ were assessed. RIA results demonstrate that 0.1 μM oligomycin is a sufficient concentration to significantly inhibit cAMP-stimulated progesterone synthesis by nearly 75% (Fig. 4A), indicating that ongoing ATP synthesis within the mitochondria is important for steroidogenesis. Similar inhibitory effects on steroid production were observed after treating cells with concentrations of oligomycin from 0.1 to 10 μM. To examine StAR protein, Western analyses were performed and StAR immunoreactivity was quantitated by scanning densitometry. cAMP robustly up-regulated StAR protein levels, whereas oligomycin treatment resulted in reductions in both the 37- and 30-kDa forms of StAR (Fig. 4B). Oligomycin (1.0 μM) treatments reduced 30- and 37-kDa StAR protein immunoreactivity by 50% *vs.* cAMP alone (Con, 99 ± 8; cAMP, 8949 ± 920; cAMP + 1.0 μM oligomycin, 4511 ± 545; n = 3). Only at the highest concentration of oligomycin (10 μM) was StAR protein reduced to undetectable levels; however, this reduction did not decrease progesterone biosynthesis further. cAMP-induced StAR mRNA expression was unaffected by oligomycin (Fig. 4C), indicating that oligomycin mediates its effects on StAR posttranscriptionally. Next, the effects of oligomycin on $\Delta\psi_m$ were examined. cAMP treatments alone resulted in a significant increase in TMRE

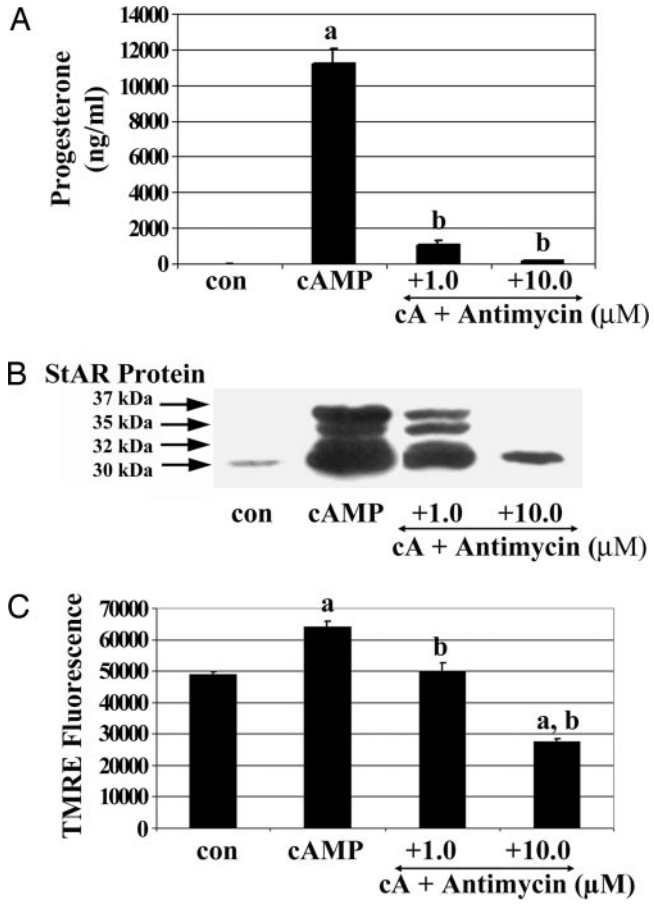

FIG. 3. Effects of antimycin A on steroidogenesis, StAR protein, and $\Delta\psi_m$ in MA-10 cells. MA-10 cells were grown in culture and treated for 3 h with 1 mM cAMP or cAMP plus increasing concentrations of antimycin A (1–10 μM). After treatment, media were collected and subjected to a progesterone RIA, and cells were lysed and subjected to Western blot analysis for StAR protein. A, Progesterone concentrations were determined by RIA, were normalized to protein concentrations for each sample, and are represented as mean ± SEM (n = 3). B, Representative Western blot for StAR protein after cAMP and antimycin treatments (n = 3). C, MA-10 cells were cultured in a 96-well fluorescence assay plate and treated as in A. Cells were subsequently incubated with 50 nM TMRE for 10 min, washed, and $\Delta\psi_m$ quantitated by TMRE fluorescence. Data are represented as mean ± SEM for 16 independent experiments (n = 16). a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* cAMP.

fluorescence (Fig. 4C). Oligomycin treatments had no effect on $\Delta\psi_m$ (n = 8) beyond the increase observed with cAMP alone. In addition, the inhibitory effects of oligomycin on cellular ATP are investigated in the results (see Fig. 8A. These data indicate that mitochondrial ATP synthesis is required for acute cAMP-stimulated steroidogenesis because oligomycin inhibited progesterone synthesis, even in the presence of adequate StAR protein and polarized mitochondria.

Effects of altering mitochondrial pH (ΔpH)

Another important intrinsic property of respiring mitochondria is the acidic pH gradient created within the organelle. Pumping of protons into the mitochondrial intermembrane space during electron transport results in a Δ*pH* gradient, which establishes the proton motive force during respiration that drives ATP synthesis. The ionophore nigericin disrupts ΔpH by exchanging K^+ for H^+ without affecting $\Delta\psi$m (9). To investigate whether ΔpH was important for Leydig cell mitochondrial steroidogenesis, MA-10 cells were treated with cAMP or cAMP plus increasing concentrations of nigericin (0.1–10 μM). Three hours of cAMP treatment stimulated the production of progesterone, and treatment with 1 μM and 10 μM nigericin significantly decreased cAMP-stimulated steroidogenesis by 60 and 95%, respectively, *vs.* cAMP alone (Fig. 5A). This suggests that disrupting ΔpH inhibits cAMP-stimulated progesterone synthesis. To examine StAR protein, Western analyses were performed and StAR immunoreactivity was quantitated by scanning densitometry. cAMP strongly increased StAR protein levels, and 1 and 10 μM nigericin reduced StAR protein to undetectable levels (Fig. 5B; Con, 12 ± 32; cAMP, 4090 ± 865; cAMP + 0.1 μM nigericin, 2590 ± 634; cAMP + 1 μM nigericin, 0 ± 27; cAMP +10 μM nigericin, not detectable; n = 3). To assess the effects of nigericin on $\Delta\psi_m$, TMRE fluorescence in MA-10 cells was quantitated. cAMP treatments alone significantly increased $\Delta\psi_m$, and 1 and 10 μM nigericin reduced TMRE fluorescence by 29 and 49% *vs.* cAMP, respectively (n = 23) (Fig. 5C). These data suggest that disrupting mitochondrial ΔpH with nigericin inhibits cAMP stimulated steroidogenesis, profoundly inhibits StAR protein, and dose dependently dissipates $\Delta\psi_m$.

Effects of mitochondrial disrupters on 22(R)-hydroxycholesterol (R22)-stimulated steroidogenesis

To determine whether the arrest of cAMP-stimulated steroidogenesis in response to mitochondrial disruption is exclusively due to preventing the transfer of cholesterol into mitochondria, experiments were also performed with the hydrophilic cholesterol analog R22, which freely diffuses into mitochondria, thereby bypassing the need for StAR. MA-10 cells were incubated with 40 μM R22, which induced the production of progesterone (Fig. 6). Cells were also treated with R22 plus CCCP, antimycin A, oligomycin, and nigericin at minimal concentrations that were previously shown to maximally inhibit cAMP stimulated steroidogenesis. Treatment of MA-10 cells with these mitochondrial disrupting agents did not significantly change R22-stimulated progesterone production, indicating that disruption of mitochondria with these agents does not affect P450scc or 3β-hydroxysteroid dehydrogenase enzyme activities in steroidogenesis. These data indicate that disrupting mitochondria with CCCP, antimycin A, oligomycin, or nigericin arrests steroidogenesis (Figs. 1–5) by preventing cholesterol transfer into the mitochondria, but these agents do not inhibit the conversion of cholesterol to pregnenolone or progesterone.

Effects of mitochondrial disrupters on StAR phosphorylation

Whereas the level of StAR protein alone cannot predict the steroidogenic capacity of the cell, it is notable that even in the presence of adequate StAR protein (Figs. 2–4), disruption of mitochondria prevents acute steroidogenesis. These observations could be due to decreased phosphorylation of StAR because phosphorylation is an important factor in cholesterol

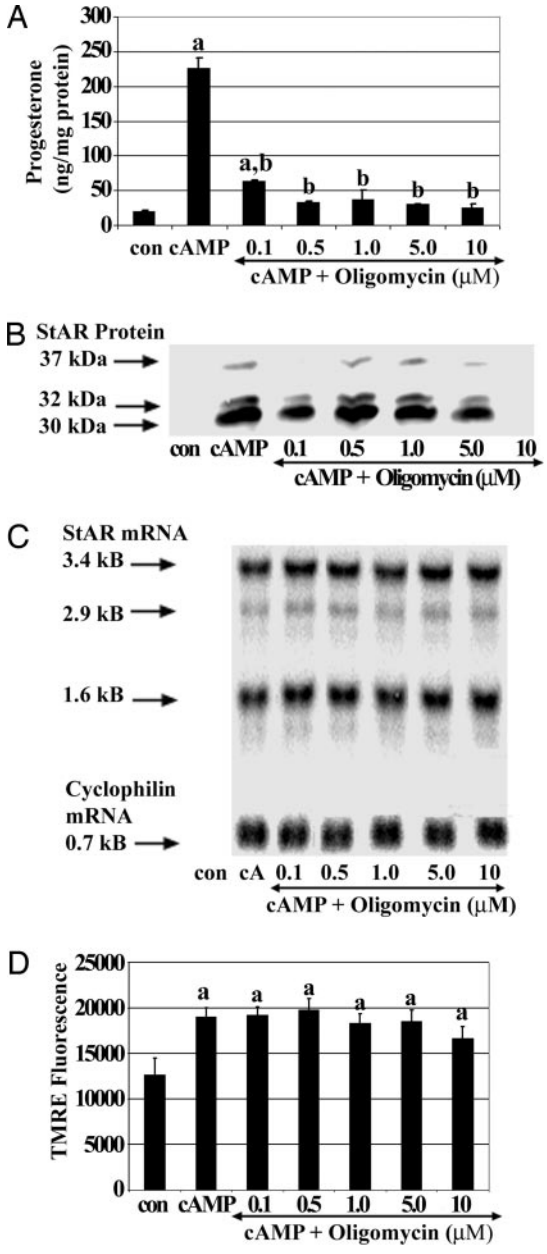

FIG. 4. Effects of oligomycin on steroidogenesis, StAR expression, and $\Delta\psi_m$ in MA-10 cells. MA-10 cells were grown in culture and treated for 3 h with 1 mM cAMP or cAMP plus increasing concentrations of oligomycin (0.1–10 μM). After treatment, media were collected and subjected to a progesterone RIA, and cells were lysed and subjected to Western or Northern blot analysis for StAR protein. A, Progesterone concentrations were determine by RIA, were normalized to protein concentrations for each sample, and are represented as mean ± SEM (n = 3). B, Representative Western blot for StAR protein after cAMP and oligomycin treatments (n = 3). C, Representative Northern blot analysis for StAR mRNA after cAMP and oligomycin treatments. MA-10 cells were treated and total cellular RNA was extracted and subjected to northern blotting for StAR. StAR mRNA levels were normalized to that of cyclophilin and are expressed as the ratio of 3.4-kb StAR transcript to cyclophilin. Data are represented as mean ± SEM for three independent experiments (n = 3). D, MA-10 cells were cultured in a 96-well fluorescence assay plate and treated and subsequently incubated with 50 nm TMRE for 10 min, washed, and $\Delta\psi_m$ quantitated by TMRE fluorescence. Data are represented as mean ± SEM for 23 independent experiments (n = 23). a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* cAMP.

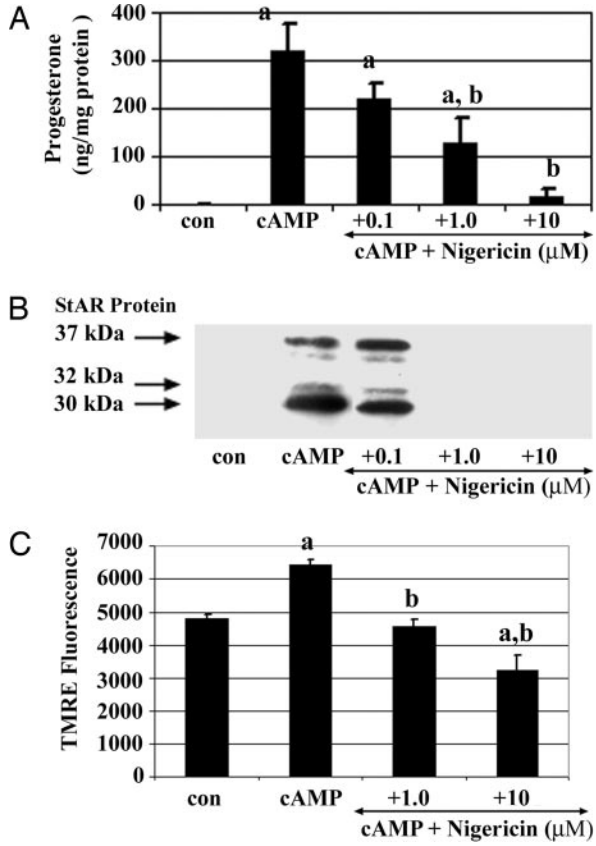

FIG. 5. Effects of nigericin on steroidogenesis, StAR protein, and $\Delta\psi_m$ in MA-10 cells. MA-10 cells were grown in culture and treated for 3 h with 1 mM cAMP or cAMP plus increasing concentrations of nigericin (0.1–10 μM). After treatment, media were collected and subjected to a progesterone RIA, and cells were lysed and subjected to Western blot analysis for StAR protein. A, Progesterone concentrations were determined by RIA, were normalized to protein concentrations for each sample, and are represented as mean ± SEM (n = 3). B, Representative Western blot for StAR protein after cAMP and nigericin treatments (n = 3). C, MA-10 cells were cultured in a 96-well fluorescence assay plate and treated as in A. Cells were subsequently incubated with 50 nm TMRE for 10 min, washed, and $\Delta\psi_m$ quantitated by TMRE fluorescence. Data are represented as means ± SEM for 23 independent experiments (n = 23); a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* cAMP.

transfer (3, 5). To test the hypothesis that cAMP-stimulated phosphorylation of StAR is decreased in response to mitochondrial disruption, a phospho-specific StAR antibody that recognizes phosphorylated serine 194 was used in Western blots. MA-10 cells were treated with 1 mM cAMP or cAMP plus minimal concentrations of the inhibitors, which maximally prevented acute cAMP-stimulated steroidogenesis. CCCP, antimycin A, and nigericin treatments reduced immunoreactivity for phosphorylated StAR equivalent to the reductions observed for total StAR protein (Fig. 7, A and 7B), indicating these agents do not preferentially reduce phosphorylated StAR.

For the ATP synthase inhibitor oligomycin, two concentrations were tested, 0.1 and 1.0 μM, which both similarly decreased progesterone synthesis by 75% *vs.* cAMP alone (Fig. 4A). cAMP plus 0.1 μM oligomycin treatments had no effect on either total StAR or ser 194 phosphorylated StAR *vs.* cAMP alone. However, 1.0 μM oligomycin significantly re-

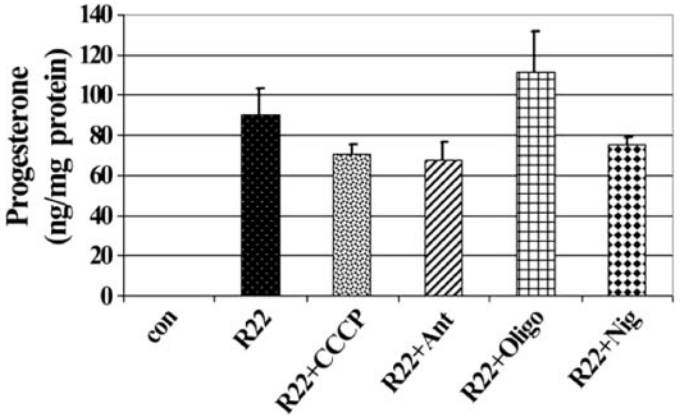

FIG. 6. Effects of mitochondrial disruptors on R22-stimulated steroidogenesis. MA-10 cells were cultured in a 96-well plate and incubated for 3 h with 40 μM R22 or R22 plus 5.0 μM CCCP, 1 μM antimycin A (Ant), 10 μM oligomycin (Oligo), or 1 μM nigericin (Nig). After treatment, media were collected and subjected to a progesterone RIA. Progesterone concentrations were normalized to protein concentrations for each sample and are presented as means ± SEM for two independent experiments performed in triplicate (n = 2). Data are representative of similar results obtained from three independent experiments.

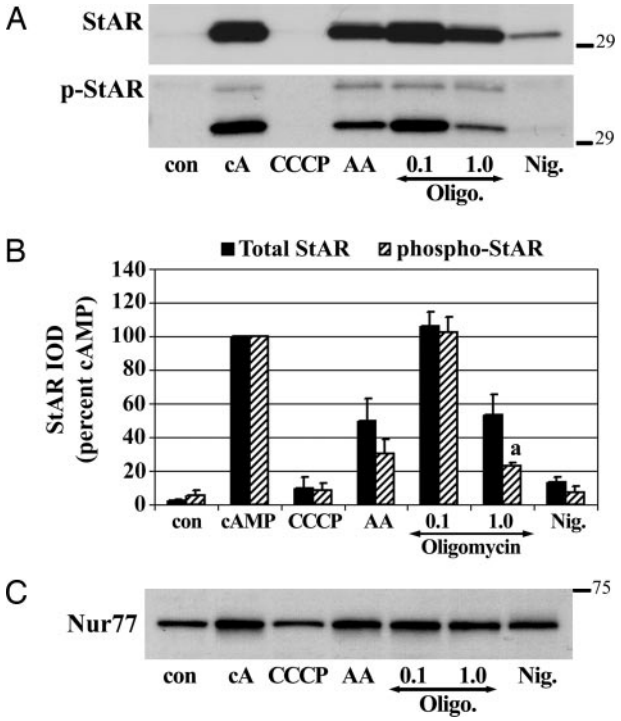

FIG. 7. Effects of mitochondrial disrupters on total StAR, Ser194 phosphorylated StAR, and Nur77 protein levels. MA-10 cells were cultured and treated for 3 h with 1 mM cAMP (cA) or cAMP plus 5.0 μM CCCP, 1 μM antimycin A (AA), 0.1 or 10 μM oligomycin (Oligo.), and 1 μM nigericin (Nig.). After treatment, cells were lysed and subjected to Western blot analysis using antibodies against Nur77, total StAR, or Ser194 phosphorylated StAR using a phospho-specific antibody. A, Representative Western blot for total StAR protein (*top panel*) or Ser194 phosphorylated StAR (*lower panel*) before and after treatments. B, Immunoblots were quantitated by scanning densitometry and are expressed as StAR or phospho-StAR integrated OD (IOD). Data are normalized to percent of cAMP ± SEM for three independent experiments (n = 3). C, Representative Western blot for Nur77 protein before and after treatments. Data are representative of similar results obtained from three independent experiments. Approximate migration of molecular mass markers are indicated in kilodaltons. a, $P \leq 0.05$ *vs.* total StAR protein for each treatment group.

duced phosphorylated StAR protein by approximately 80%, whereas total StAR protein was reduced by only 50% *vs.* cAMP alone (Fig. 7, A and B). These data indicate that whereas higher concentrations of oligomycin (1.0 μM) preferentially reduces phosphorylated StAR, there is no reduction at the lower concentration of 0.1 μM, a concentration that significantly reduced progesterone synthesis (Fig. 4A). These data indicate that ongoing mitochondrial ATP synthesis is required for cAMP-stimulated steroidogenesis and the observed reduction in progesterone by oligomycin cannot be attributed to reductions in total StAR or phosphorylated StAR.

Effects of mitochondrial disrupters on Nur77 protein

As an additional control for the agents tested, the protein level of the transcription factor Nur77 was assessed. Nur77 protein is significantly up-regulated in MA-10 cells in response to acute cAMP treatments (33). Cells were treated with cAMP plus CCCP, antimycin A, oligomycin, and nigericin at minimal concentrations that were previously shown to maximally inhibit cAMP-stimulated steroidogenesis. Immunoblotting using a Nur77 antibody detected a single band near the predicted molecular mass of 60 kDa. Blots were analyzed by scanning densitometry and Nur77 ODs were expressed as percent cAMP. cAMP treatments alone increased Nur77 immunoreactivity by nearly 60%, and only CCCP treatments significantly reduced this up-regulation (Fig. 7C; data expressed as percent cAMP: Con, 42 ± 5; cAMP, 100 ± 0; cAMP + 5.0 μM CCCP, 50 ± 6*; cAMP + 1 μM antimycin A, 79 ± 5; cAMP + 0.1 μM oligomycin, 76 ± 9; cAMP + 1.0 μM oligomycin, 86 ± 4; cAMP + 1.0 μM nigericin, 90 ± 11; n = 3, *, $P \leq 0.05$ *vs.* cAMP). These data indicate that of the several mitochondrial disrupting agents studied in this report, only CCCP prevented cAMP-stimulated Nur77 protein, reducing it to control levels.

Effects of mitochondrial disrupters on cellular ATP

The end result of oxidative metabolism in mitochondria is the production of ATP, and uncoupling electron transport or disrupting properties of mitochondria such as $\Delta\psi_m$ is associated with decreased ATP generation. To test whether the pharmacological agents used in these experiments altered cellular ATP, MA-10 cells were treated with doses of the agents that were maximally effective in inhibiting mitochondrial steroidogenesis. Luminescent values obtained are proportional to total cellular ATP and provided a quantitative assessment of cellular ATP levels. Of the several mitochondrial disrupting agents studied in this report, only antimycin A (1 and 10 μM) and oligomycin (10 μM) significantly decreased cellular ATP *vs.* control or cAMP (Fig. 8A). These results suggest that the inhibitory effects of antimycin A and oligomycin on steroidogenesis and StAR may be in part attributable to decreases in ATP. In contrast, these data also indicate that dissipation of $\Delta\psi_m$ with CCCP or disrupting

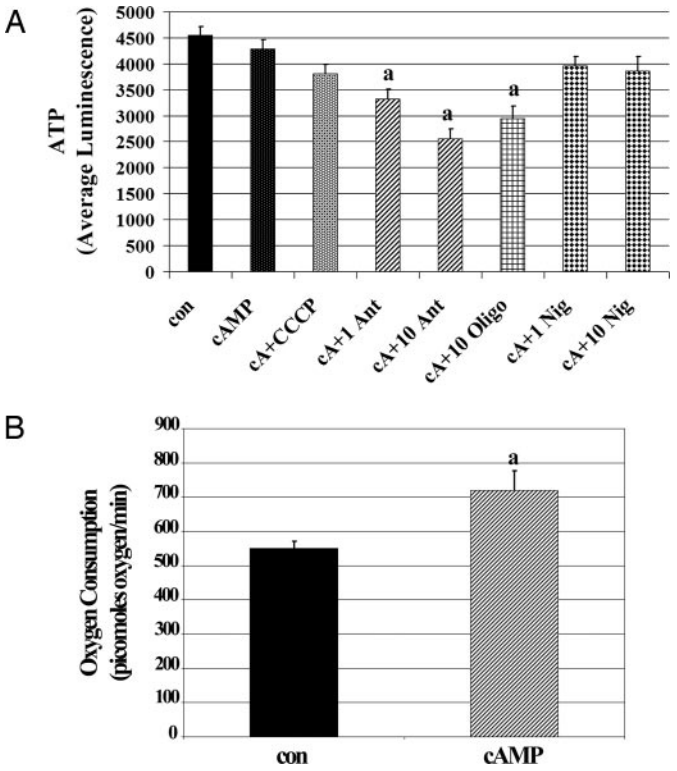

FIG. 8. A, Effects of mitochondrial disruptors on cellular ATP. MA-10 cells were cultured in a 96-well fluorescence assay plate and treated for 3 h with 1 mM cAMP or cAMP plus 5.0 μM CCCP, 1 or 10 μM antimycin A (Ant), 10 μM oligomycin (Oligo), and 1 or 10 μM nigericin (Nig). Cells were subsequently incubated with Cell Titer-Glo substrate (Promega), and samples were analyzed for overall luminescence using a microplate reader. The luminescent values are proportional to total cellular ATP, and provide a quantitative assessment of cellular ATP levels. Data are represented as means ± SEM for three independent experiments performed in replicates of six. (n = 3); a, $P < 0.05$ *vs.* cAMP. B, Effects of cAMP on respiration. MA-10 cells were cultured and treated with or without 1 mM cAMP for 3 h; cells were then resuspended in complete media and oxygen consumption determined. Data shown are represented as means ± SEM from four independent experiments performed in triplicate (n = 4); a, $P < 0.05$ *vs.* control.

ΔpH with nigericin, and the associated inhibitory effects of these agents are not due to changes in cellular ATP.

Effects of cAMP on respiration

The consistent observation that acute cAMP treatment results in a polarization of mitochondria and an increase in $\Delta\psi_m$ (Figs. 1–5) indicates that the rate of mitochondrial oxidative phosphorylation is altered in response to cAMP. To further examine this phenomenon, MA-10 cells were treated with cAMP for 3 h, and oxygen consumption was subsequently determined using a Strathkelvin oxygen meter. Treatment of MA-10 cells with cAMP significantly increased oxygen consumption by approximately 40% above control (Fig. 8B; con, 522 ± 0.21; cAMP, 718 ± 0.56; n = 4). These results indicate that cAMP enhances respiration.

Discussion

This study indicates that disrupting mitochondria results in posttranscriptional changes in StAR, and that mitochondria must be energized, polarized, and actively respiring to support cAMP-stimulated Leydig cell steroidogenesis. Using MA-10 cells and pharmacological approaches, this investigation demonstrates that several intrinsic properties of mitochondria are important for steroidogenesis. Disrupting $\Delta\psi_m$ with CCCP (Fig. 1) results in a significant inhibition in cAMP-stimulated progesterone synthesis, even in the presence of newly synthesized StAR protein (Fig. 2), indicating that $\Delta\psi_m$ is essential for steroid biosynthesis. Preventing electron transport in mitochondria with antimycin A significantly reduced cellular ATP (Fig. 8A), potently inhibited cAMP-stimulated steroidogenesis, reduced StAR protein, and dose-dependently dissipated $\Delta\psi_m$ (Fig. 3), results consistent with Kowal and Harano (34), who determined that inhibiting complex I with Amytal profoundly inhibits ACTH-stimulated corticosteroid synthesis in adrenal cells. Similarly, preventing ATP synthesis with oligomycin reduced cellular ATP (Fig. 8A) and dose-dependently inhibited cAMP-induced progesterone synthesis and StAR protein but had no effect on $\Delta\psi_m$ (Fig. 4). These data indicate that preventing mitochondrial ATP synthesis with antimycin A or oligomycin inhibits steroidogenesis. Lastly, disruption of intramitochondrial pH with nigericin inhibited cAMP-stimulated progesterone production and StAR protein but had minimal effects on $\Delta\psi_m$ (Fig. 5). These results indicate that $\Delta\psi_m$, mitochondrial ATP synthesis, and mitochondrial pH are all essential to support cAMP-stimulated Leydig cell steroidogenesis and that even in the presence of adequate StAR protein, mitochondria must be compliant and functioning to support steroid biosynthesis.

Recent study of Leydig cell function has focused on the molecular and cellular mechanisms regulating steroidogenesis; however, few investigations have examined the importance of mitochondria *per se* in this process. Several reports using CCCP or valinomycin, both of which dissipate $\Delta\psi_m$, have established $\Delta\psi_m$ is required for steroidogenesis and StAR function (5, 11, 12, 14–17). Results using CCCP in this study are generally consistent with previous reports and further confirm the importance of $\Delta\psi_m$ for mitochondrial steroidogenesis. A new contribution in this study is the quantitative assessment of the effects of mitochondrial disrupting drugs on $\Delta\psi_m$ by direct measurement of TMRE fluorescence, enabling the detection of subtle changes in $\Delta\psi_m$ due to mitochondrial depolarization. Other $\Delta\psi_m$ dye indicators such as JC-1, although useful, only enable qualitative measurements. This quantitative assessment of $\Delta\psi_m$ in actively respiring MA-10 cells demonstrates that cAMP increases $\Delta\psi_m$.

An intriguing effect of dissipating $\Delta\psi_m$ with CCCP is the lack of the 30-kDa StAR protein, whereas the newly synthesized 37-kDa precursor remains constant (Fig. 2D), indicating there is a $\Delta\psi_m$-dependent component in steroidogenesis independent of the 37-kDa StAR. The effects of CCCP on StAR protein did not reduce the level of the matrix localized steroidogenic enzyme P450scc (Fig. 2C); however, it did reduce cAMP-induced Nur77 to control levels (Fig. 7C). The observed lack of the 30-kDa StAR due to CCCP suggests that StAR import into the mitochondrial matrix and its subsequent processing is dependent on an intact $\Delta\psi_m$. The import and processing of many matrix targeted proteins are dependent on $\Delta\psi_m$ and ATP hydrolysis (35), and results of these

CCCP experiments indicate that import and processing of StAR also requires $\Delta\psi_m$. The most straightforward interpretation of these experiments is that CCCP dissipates $\Delta\psi_m$, which prevents StAR import and processing, cholesterol transfer, and thus steroid synthesis; however, this effect is likely much more complex. The mechanism by which StAR facilitates cholesterol transfer is as yet unknown, and the involvement of StAR import into the mitochondria to enable cholesterol transfer is debated. StAR is thought to act in cholesterol transfer on the outside of the mitochondria. When N-terminally truncated forms of StAR lacking mitochondrial targeting sequences are overexpressed, they facilitate steroidogenesis in COS cells (15, 36) and MA-10 cells (15), suggesting that StAR import and processing is not necessary for cholesterol transfer. In contrast, other reports have indicated that newly synthesized, phosphorylated StAR requires import to enable cholesterol transfer (4) and that StAR import and processing is central to cholesterol transfer. One interpretation of the data in this report is that preventing StAR import and processing prevents steroidogenesis (Fig. 2E). It is generally accepted that the 37-kDa precursor form of StAR is the active form in cholesterol transfer, and intramitochondrial processing is believed to inactivate the protein enabling a rapid turnover and off mechanism during steroid production. Notably, after CCCP treatments, cAMP-stimulated progesterone synthesis was completely inhibited, even in the presence of the newly synthesized 37-kDa StAR protein (Fig. 2E), indicating there is a $\Delta\psi_m$-dependent component in steroidogenesis independent of the 37-kDa StAR.

Within the mitochondrial matrix, P450scc catalyzes the conversion of cholesterol to pregnenolone; thus, alterations in mitochondrial function may also effect P450scc activity. To examine this, experiments were conducted using the freely diffusible cholesterol analog R22 (Fig. 6). None of the agents tested inhibited R22-mediated progesterone synthesis, indicating that P450scc activity was unaffected by disrupting the mitochondria. In addition, treatment with CCCP (Fig. 2C) or any of the other agents used in this report did not alter the level of P450scc protein as detected in Western blots (data not shown). These R22 experiments suggest that disruption of mitochondria with the drugs tested in this report specifically prevents cholesterol import and StAR function.

Steroidogenesis is driven by cAMP, which activates numerous processes in Leydig cells such as transcription and translation of steroidogenic proteins and phosphorylation of StAR. An unexpected finding from the present study was the observation that cAMP significantly increases $\Delta\psi_m$ (Fig. 1) and increases respiration/O_2 consumption (Fig. 7B), without affecting total cellular ATP (Fig. 7A). The mechanism by which cAMP increases $\Delta\psi_m$ and respiration in Leydig cells is unknown but is likely due to an increase in ADP produced as a result of ATP use during cAMP-driven steroidogenic processes. The rate of respiration is governed by the availability of ADP and inorganic phosphate; thus, increased ADP would result in a higher rate of respiration. In other tissues such as pancreatic acinar cells, cAMP has no effect on $\Delta\psi_m$, whereas in prostate epithelial cells cAMP decreases $\Delta\psi_m$ (37, 38). This suggests that cAMP-mediated increases in $\Delta\psi_m$ is unique to the Leydig cell and perhaps other steroidogenic cells. The earliest report of gonadotropin action on respiration in steroidogenic tissues (circa 1949) demonstrated that FSH (presumably via cAMP) significantly enhanced respiration in the chicken ovary (39). To the best of our knowledge, our investigation is the first to demonstrate that cAMP increases both respiration and $\Delta\psi_m$. This increase in the membrane potential and respiration would be expected to further drive steroidogenesis and could influence the kinetics of steroid production. Presumably the reason that cAMP increases $\Delta\psi_m$ and respiration without increasing total cellular ATP (Fig. 7) is that the increased ATP generated is consumed during phosphorylation reactions and transcription/translation during steroidogenesis.

We speculate that increased $\Delta\psi_m$ due to cAMP would result in an acidification within the mitochondria, which could be important for StAR function in cholesterol transfer. In support of this, disrupting the mitochondrial pH gradient with 1 μM nigericin markedly inhibits progesterone synthesis, reduces StAR protein, does not alter ATP, and minimally affects $\Delta\psi_m$, reducing it to control levels (Figs. 5 and 7A). At the higher concentration of 10 μM, nigericin disrupts both ΔpH and $\Delta\psi_m$, resulting in complete inhibition of StAR and progesterone. These results indicate that nigericin counteracts the effect of cAMP by disrupting ΔpH and that ΔpH is important for steroidogenesis and possibly StAR function in cholesterol transfer. This conclusion contrasts a previous report by King *et al.* (13); however, that report did not investigate higher concentrations of nigericin nor did it assess the effects on $\Delta\psi_m$. Results from the present study are consistent with a ΔpH-dependent component in acute cAMP-stimulated steroidogenesis.

One of the theories proposed about StAR mechanism of action is that at low pH, StAR adopts a molten globule conformation, creating a hydrophobic pocket that facilitates cholesterol transfer into the mitochondria (40, 41). In a cell-free system, a pH of 3.5 is required to induce the molten globule conformational change in StAR. It has been proposed that the acidic head groups of polar lipids of the outer mitochondria may provide a highly localized acidic environment to enable StAR to assume this conformation (40, 42). Thus, dissipating ΔpH with nigericin may cause a loss of this acidic microdomain and perturb StAR conformation, thus preventing the cholesterol transfer complex from being formed.

Whereas the level of StAR protein alone cannot predict the steroidogenic capacity of the cell, a consistent result with all mitochondrial disrupting agents tested is that StAR protein is highly sensitive to alterations in mitochondria. The effects of the agents tested appear to specifically reduce StAR protein because P450scc protein was unaffected (Fig. 2C). Moreover, the cAMP-induced expression of the transcription factor Nur77 was unaffected by the mitochondrial disrupting drugs, except for CCCP, which reduced Nur77 to control levels (Fig. 7C). This is an intriguing observation, considering the known association between Nur77 and mitochondrially targeted Bcl-2 proteins (43). These effects indicate that StAR is highly sensitive to alterations in mitochondria. These results suggest that mitochondrial disruption results in either degradation of StAR protein or a posttranscriptional inhibition of StAR. The reduction in ATP due to antimycin A likely inhibits cAMP-stimulated steroidogenesis due to reduced

StAR translation, which is dependent on ATP. However, dissipating $\Delta\psi_m$ with CCCP is ATP independent (Fig. 7A), and CCCP did not prevent synthesis of StAR (Fig. 2E), indicating that other processes are involved, such as activation of proteases resulting in StAR degradation. CCCP appears to decrease the half-life of the 30- and 32-kDa forms of StAR and activate proteases (16, 17), supporting the notion that degradation of intramitochondrial StAR may occur due to disruption of $\Delta\psi_m$. In addition, these alterations in protease activity at mitochondria could explain the observation that the 37-kDa StAR level remains constant during CCCP treatments but does not accumulate (Fig. 2, B and E). The observation that CCCP and antimycin similarly reduce $\Delta\psi_m$ (Figs. 2 and 3) but have differential effects on StAR protein suggests that ATP is required to maintain the level of StAR protein, whereas $\Delta\psi_m$ is important for StAR import and processing. Thus, oxidative phosphorylation is necessary to maintain StAR protein, probably due to the need for ATP, and an intact $\Delta\psi_m$ is necessary to facilitate import and processing of StAR.

StAR is phosphorylated at serine residues that enhances StAR activity (3, 5), and newly synthesized, phosphorylated StAR is important for cholesterol transfer (4). Hypothetically, ongoing mitochondrial ATP synthesis could be required for StAR phosphorylation and thus cholesterol transfer activity; however, this hypothesis is not entirely supported because only the higher concentrations of oligomycin (1.0 μM) significantly reduced the phosphorylation of StAR protein, yet 0.1 μM oligomycin, which inhibits steroidogenesis, had no affect on total or phospho-StAR (Fig. 7, A and B). It is likely that other factors, *i.e.* peripheral-type benzodiazepine receptor (44), or other unidentified factors essential for steroidogenesis are dependent on ongoing mitochondrial ATP synthesis. Another intriguing observation is that the highest concentration of oligomycin tested, 10 μM, reduced StAR protein to undetectable levels; however, a basal level of steroid synthesis was maintained similar to control (Fig. 4, A and B). This suggests that cAMP-stimulated steroidogenesis is dependent on ATP; however, StAR protein alone may not be essential for maintaining basal steroidogenesis. Similar results were observed with nigericin, which reduced StAR protein to undetectable levels, yet cAMP-stimulated steroidogenesis was maintained (Fig. 5), results consistent with findings by King *et al.* (13). Overall, these results indicate that even in the presence of adequate StAR protein, mitochondria must be compliant and functioning to support steroid biosynthesis.

Steroidogenic cells have evolved a strategy to exploit the unique membrane compartmentation of the mitochondria to regulate steroid synthesis. The hydrophilic barrier of the mitochondrial intermembrane space separates P450scc from cholesterol, and bridging this gap is a key control point for enabling steroidogenesis. Results from this study suggest that the state of the mitochondria itself is an important determinant in steroidogenesis. We suggest that mitochondria must be energized, polarized, and actively respiring to support Leydig cell steroidogenesis, and alterations in the state of mitochondria may be involved in regulating steroid biosynthesis.

Acknowledgments

The authors thank John Choi, Eileen Burkhardt, and Cassie Mahon for their expert technical assistance. We thank Dr. Steve King for providing us with the phospho-StAR antibody used in these studies. We also sincerely thank Dr. Doug Stocco, Dr. Steve Kin, and Dr. Bayard Storey for critical reading of the manuscript.

Received September 20, 2005. Accepted May 3, 2006.

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This work was supported by National Institutes of Health (NIH) Research Training Grant T32 HL07692 (to J.A.A), a Postdoctoral Fellowship Grant Deutschen Forschungsgemeinschaft Di 723/1-1 (to T.D.), and NIH Grants HD-27571 and HD-35544, and American Cancer Society Grant ACS 04010 (to D.B.H.).

This work was previously presented in preliminary form at the 37th Annual Meeting of the Society for the Study of Reproduction, July 2004, and at the 18th North American Testis Workshop, April 2005.

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***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**