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# Effects of disruption of the mitochondrial electrochemical gradient on steroidogenesis and the Steroidogenic Acute Regulatory (StAR) protein☆

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**Abstract**

The steroidogenic acute regulatory (StAR) protein, which mediates cholesterol delivery to the inner mitochondrial membrane and the P450scc enzyme, has been shown to require a mitochondrial electrochemical gradient for its activity *in vitro*. To characterize the role of this gradient in cholesterol transfer, investigations were conducted in whole cells, utilizing the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (*m*-CCCP) and the potassium ionophore valinomycin. These reagents, respectively, dissipate the mitochondrial electrochemical gradient and inner mitochondrial membrane potential. Both MA-10 Leydig tumor cell steroidogenesis and mitochondrial import of StAR were inhibited by *m*-CCCP or valinomycin at concentrations which had only minimal effects on P450scc activity. *m*-CCCP also inhibited import and processing of both StAR and the truncated StAR mutants, N-19 and C-28, in transfected COS-1 cells. Steroidogenesis induced by StAR and N-47, an active N-terminally truncated StAR mutant, was reduced in transfected COS-1 cells when treated with *m*-CCCP. This study shows that StAR action requires a membrane potential, which may reflect a functional requirement for import of StAR into the mitochondria, or more likely, an unidentified factor which is sensitive to ionophore treatment. Furthermore, the ability of N-47 to stimulate steroidogenesis in nonsteroidogenic HepG2 liver tumor cells, suggests that the mechanism by which StAR acts may be common to many cell types. 

*Keywords:* Steroidogenesis; Steroidogenic acute regulatory protein, StAR; Leydig cells; Mitochondria; Mitochondrial import

## 1. Introduction

The rate-limiting step in steroidogenesis is the delivery of cholesterol from the outer to the inner mitochondrial membrane where it is converted by the cholesterol side chain cleavage system (CSCC) to pregnenolone [1–8]. Evidence to date strongly supports that this transfer is mediated by the Steroidogenic Acute Regulatory (StAR) protein [9], which is rapidly synthesized and imported into the mitochondria of steroidogenic cells upon tropic hormone stimulation [10]. This observation is dramatically supported by studies which have demonstrated that mutations in the StAR gene cause lipoid congenital adrenal hyperplasia (lipoid CAH), a condition in which the biosynthesis of adrenal and gonadal steroids is severely impaired [11, 12].

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StAR is first synthesized as a 37 kDa precursor [13, 14], which is imported into the mitochondria and appears to be processed, at least in mouse cells, first to a 32 kDa intermediate form and finally to four 30 kDa mature forms [13–15]. Since mature StAR persists after both stimulation and steroidogenesis have ceased [13, 14], it has been proposed that the short-lived precursor is the functionally relevant form. It was further proposed that StAR might effect cholesterol transfer from the outer to the inner mitochondrial membrane during its import into the mitochondrial matrix by forming contact sites between these two membranes [13, 14]. However, more recent evidence indicates that the cholesterol transferring activity of the StAR protein resides in the C-terminal portion of the molecule and that import into the mitochondria is not required for cholesterol transfer [16].

Since the mechanism by which StAR promotes intermembrane cholesterol transfer is unclear, we have undertaken experiments to define some of the requirements for this activity. Utilizing carbonyl cyanide *m*-chlorophenylhydrazone (*m*-CCCP), a well-characterized reagent that equilibrates the proton gradient across the inner mitochondrial membrane [17, 18], *in vitro* studies have revealed that StAR-induced steroidogenesis requires maintenance of an electrochemical gradient across the inner mitochondrial membrane [19]. In the present study, this requirement was examined using *in vitro* cell systems. MA-10 mouse Leydig tumor cells were incubated with varying levels of *m*-CCCP or valinomycin, a potassium ionophore which disrupts the mitochondrial electrochemical potential ($\Delta\Psi$), and the effects on steroidogenesis and expression of the StAR gene were measured. Separately, COS-1 monkey kidney cells and HepG2 human hepatic carcinoma cells were transfected with cDNA for StAR or an N-terminally truncated mutant, N-47. Effects on steroidogenesis and import of StAR into mitochondria were measured after similar treatment of COS-1 cells with *m*-CCCP.

## 2. Materials and methods

### 2.1. Chemicals

Waymouth's MB/752 medium, Dulbecco's modified Eagle's medium (DMEM), horse serum, fetal bovine serum, antibiotics, trypsin-EDTA, and culture-quality PBS were purchased from GIBCO-BRL (Grand Island, NY) and fetal calf serum, from Biological Industries (Kibbutz Beit-Haemek, Israel). Culture-quality phosphate-buffered saline (PBS) was supplied by Oxoid Ltd. (Bassingstoke, U.K.). *m*-CCCP, aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), valinomycin, 22*R*-hydroxycholesterol (22*R*-HC), dimethylsulfoxide (DMSO) and dibutyryl cAMP ([Bu]2cAMP) were obtained from Sigma Chemical Co. (St. Louis, MO).

### 2.2. Cell culture

The MA-10 cell line used in these studies was a generous gift from Dr Mario Ascoli, University of Iowa College of Medicine (Iowa City, IA). COS-1 and HepG2 cells were purchased from the American Type Tissue Culture Co. (Rockville, MD). Both cell lines were maintained in culture utilizing methods previously described [15, 20, 21].

### 2.3. Cell staining

To examine the integrity of the mitochondrial electrochemical potential following ionophore treatment for 100 min in the presence or absence of ionophore, cells were treated with the dye JC-1 (Molecular Probes, Eugene, OR), a commonly used dye which detects the presence of $\Delta\Psi$ [22]. This dye was suspended in 2.5 mg/ml stock solution in ethanol and diluted to a final concentration of 2.5 $\mu$g/ml of media. After 20 min, cells were carefully washed twice with PBS to remove unincorporated dye and then media with or without the indicated concentration of *m*-CCCP or valinomycin was placed back on the cells. Cells were examined using a Zeiss Axiovert 135 microscope through a 40× L.D. Achroplan objective. When the electrochemical potential is intact, intense staining of mitochondria caused by the aggregation of JC-1 molecules inside the mitochondria (J-aggregates) occurs and can be visualized using a Texas red filter. If the electrochemical potential is lost, no aggregation of JC-1 occurs and the monomeric units of JC-1 which stain the cytoplasm and mitochondria nonspecifically increase in number and thus staining intensity and can be measured in the green channel using a fluorescein filter. In some cases, cells were treated with JC-1 prior to treatment with ionophore. Photography was performed using TMAX 100 film using identical exposure times.

### 2.4. Steroid production

MA-10 cells were stimulated for 2 h with 1 mM [Bu]2cAMP in Waymouth's media with or without *m*-CCCP or valinomycin at the indicated concentrations. After cells were harvested, progesterone concentration in the media was measured by radioimmunoassay (RIA). As a test for total P450scc activity, cells were incubated for 2 h with or without the ionophore and 25 $\mu$M 22*R*-HC. In all experiments, the final concentration of solvent was below that which is inhibitory for steroidogenesis [19, 23].

### 2.5. Transient expression of StAR mutants in COS-1 cells

Murine StAR cDNA and mutant StAR lacking the N-terminal 19 (N-19) and 47 (N-47) or the C-terminal 28 (C-28) amino acids were previously subcloned into the expression vector, pCMV5 [9, 16, 24]. The F2 plasmid, which encodes a fusion protein consisting of components of the CSCC enzyme system (cytochrome P450scc, adrenodoxin, and adrenodoxin reductase), was kindly provided by Dr Walter Miller (University of California, San Francisco, CA) [25].

Cells were transfected using LipofectAMINE (GIBCO-BRL) [15] or by electroporation [26]. Using the former method, HepG2 cells were transfected with F2 and N-47. COS-1 cells were first transfected with F2 and then at 24 or 48 h posttransfection with pCMV[StAR] or vector alone. COS-1 cells were incubated in DMEM with 0.5% serum with or without *m*-CCCP as indicated in Section 3. Media were collected for pregnenolone RIA [27] and cells were used for Western or Northern blot analysis.

COS-1 cells ($2\times10^6$ cells/0.8 ml) were transfected in suspension by electroporation using 30 $\mu$g of plasmid and then aliquotted into wells of a 24-well plate (Nunc, Denmark), and incubated in DMEM plus 0.5% serum with or without 5 $\mu$M *m*-CCCP for 48 h. The drug was replenished once, 24 h after initiation of treatment. Cells were subsequently collected for Western blot analysis.

### 2.6. Protein synthesis

The effects of the ionophores on protein synthesis were measured essentially as detailed elsewhere [28]. Briefly, $5\times10^4$ MA-10 cells/well were grown overnight in 96-well dishes then incubated in the presence of 50 $\mu$Ci EXPRE$^{35}$S$^{35}$S protein labeling mix (DuPont NEN, Boston, MA) per 100 $\mu$l serum-free media for 0 or 2 h. Media were removed and cells were washed with PBS and dissolved in 0.2 M NaOH overnight at 37°C. Protein synthesis was determined by collecting acid-precipitable material on filter paper and, following extensive washing, radioactivity assayed using liquid scintillation counting (Scintiverse, Fisher Scientific, Fair Lawn, NJ).

### 2.7. Western blot analysis

Cells were harvested in TSE buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4). Mitochondria were isolated by mechanical homogenization and differential centrifugation as detailed by Clark *et al.* [9]. Western analysis was then performed using total cellular or mitochondrial protein essentially as previously described [9, 15].

COS-1 cells transfected by electroporation were extracted by adding 30 $\mu$l of RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS and 0.1 mM of freshly added phenylmethylsulfonylfluoride) to each well. After a 30 min incubation on ice, extracts from triplicate wells were pooled, vortexed, and centrifuged for 5 min at 14,000$\times$ *g* to remove DNA and cellular debris. The supernatant was separated and protein concentration determined by a modification of the protein assay as specified by Bradford [29]. Cell extracts were solubilized in sample buffer [15], heated for 5 min at 95°C, and stored at −20°C.

For each sample, 8.5 $\mu$g protein was electrophoresed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% minigel [15, 30] then electrophoretically transferred to Optitran BA-S 85 nitrocellulose membranes (Schleicher&Schuell, Dassel, Germany) using a semi-dry electrotransfer apparatus [E&K Scientific Products, Saratoga, CA] for 45 min at 3 mA/cm$^2$ in buffer containing 48 mM Tris base, 39 mM glycine, 0.04% SDS and 20% methanol [31]. The membrane was incubated for 30 min in blocking buffer (0.1% Tween 20 and 5% nonfat dry milk in PBS buffer), followed by overnight incubation with antiserum against recombinant murine StAR [32]. After 3$\times$5 min washes with PBS-Tween buffer, membranes were incubated with peroxidase-conjugated AffiniPure goat anti-rabbit IgG [H + L] (Jackson ImmunoResearch Inc., West Grove, PA) for 1 h. Immunoreactive bands were detected by chemiluminescence utilizing LumiGlo (New England BioLabs, Beverly, MA).

Quantitation of chemiluminescent signals on X-ray films was performed as follows. Fluorograms were scanned with a Power Macintosh computer-based scanner (AV 6120, Avision, Inc., Hsinchu, Taiwan) using a green filter, 150 dpi resolution, 256 gray levels, 10% brightness, 30% contrast, and $\gamma = 2$. Quantification of scanned images was performed as outlined in the NIH Image Program (http://rsb.info.nih.gov/nih-image/).

### 2.8. Northern blot hybridization

Total RNA was isolated from MA-10 cells using the acid guanidinium thiocyanate–phenol–chloroform extraction method [33] with RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX). The resulting RNA pellet was dissolved in water. Ten $\mu$g of total RNA were separated on 2.2 M formaldehyde denaturing agarose gels and transferred onto Hybond-N nylon membrane (Amersham), followed by UV-crosslinking [34]. After preincubation in rapid hybridization solution [Amersham] with 10 $\mu$g/ml of denatured salmon testes DNA, the blot was further incubated for 4 h at 65°C in the presence of [$^{32}$P]-labeled mouse StAR cDNA.

This cDNA was obtained by restriction digestion of pSPORT1[Δ20 StAR] [15] with *Sac*I and *Bgl*II followed by agarose gel purification (QIAEX II kit, Qiagen, Chatsworth, CA) and radiolabeled by random primer labeling (Prime-a-Gene Labeling System, Promega, Madison, WI) [35]. The blot was washed twice with 2X standard saline citrate solution (SSC), 0.1% SDS for 20 min at 24°C and once with 0.1× SSC, 0.1% SDS for 30 min at 65°C before exposure to X-ray film. For normalization of RNA loading, a 26-mer oligonucleotide derived from 28*S* rRNA was end-labeled and hybridized to the blot [36].

## 3. Results

### 3.1. m-CCCP and valinomycin inhibit [Bu]2cAMP-stimulated steroidogenesis

MA-10 cells were stimulated for 2 h with 1 mM [Bu]2cAMP in the presence of varying concentrations of *m*-CCCP or valinomycin. Five hundred nM and 5 μM *m*-CCCP inhibited stimulated steroidogenesis by 85% and 95%, respectively, and 10 nM valinomycin by 77%, ($p < 0.05$) (Fig. 1). Five hundred nM and 5 μM *m*-CCCP inhibited 22*R*-HC-supported P450scc activity by approximately 25%, while 10 nM valinomycin had no significant effect. Protein synthesis was not reduced in the presence of 500 nM *m*-CCCP or 10 nM valinomycin and only slightly reduced in the presence of 5 μM *m*-CCCP (data not shown).

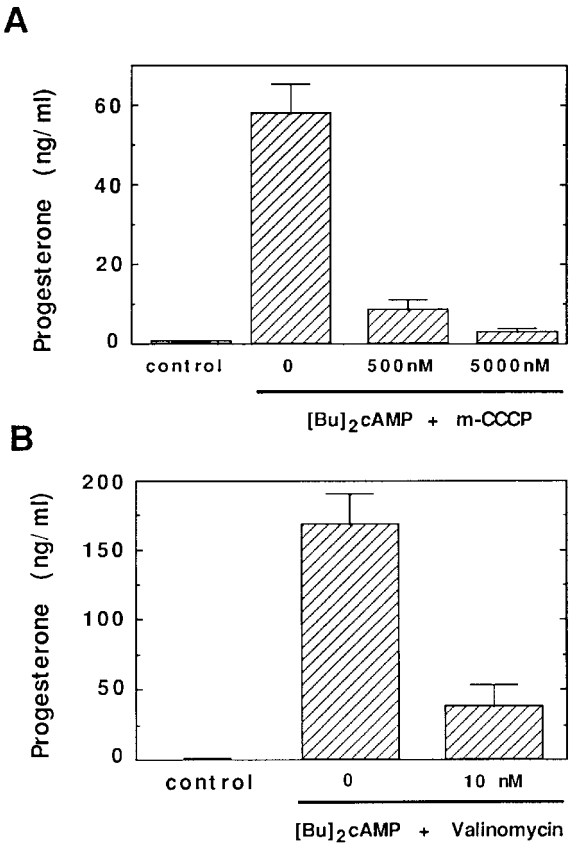


Fig. 1. Effects of *m*-CCCP and valinomycin on [Bu]2cAMP-stimulated steroidogenesis in MA-10 cells. (A) and (B), steroid production by stimulated MA-10 cells in the presence or absence of 500 nM or 5 μM *m*-CCCP and 10 nM valinomycin, respectively. These values are all the means ± S.D. of quadruplicate samples. Results are representative of at least three separate experiments.

### 3.2. m-CCCP and valinomycin decrease the electrochemical potential

To more directly examine the effects of the ionophores on the mitochondrial electrochemical potential, the dye JC-1 was utilized. Intense red staining of the mitochondria was indicated by punctate staining of cells, indicative of an intact electrochemical potential (Fig. 2A). However, this pattern of staining decreased in intensity in the presence of either 500 nM *m*-CCCP or 10 nM valinomycin (Fig. 2B and C). Also, while the level of green staining in control cells was low and displayed a "pock-marked" pattern (Fig. 2D), this level was significantly higher in ionophore-treated cells (Fig. 2E and F). This was also seen using 5 μM *m*-CCCP or 50 nM valinomycin. Moreover, ionophore-induced loss of $\Delta\Psi$ occurred quickly upon treatment. Approximately 5 min following the addition of 500 nM *m*-CCCP to cells pretreated with JC-1, mitochondrial staining began to decrease. Ionophore treatment had no observable effect on cell survival nor morphology when compared to control cells (Fig. 2G). In fact, after incubation in serum-free medium in the presence of 500 nM *m*-CCCP for 24 h, mitochondrial staining was once again observed to be at near control levels, perhaps indicating that a compensation in cellular metabolism had occurred. It was not feasible to measure both red and green fluorescence in the same field due to photobleaching which sharply diminishes signal strength. Film exposure times for each channel were identical for all treatments.

### 3.3. m-CCCP and valinomycin inhibit processing of StAR

To explore possible reasons for the reduction in steroidogenesis, levels of StAR mRNA in MA-10 cells stimulated for 2 h with [Bu]2cAMP in the presence of *m*-CCCP or valinomycin, were assayed by Northern analysis. Similarly, after stimulation for 6 h, the level of StAR protein was examined by Western analysis.

In control unstimulated MA-10 cells, there was no StAR mRNA detected, consistent with previous observations (Fig. 3) [37]. All previously described forms of mouse StAR mRNA (3.4, 2.7 and 1.6 kb), were pre-

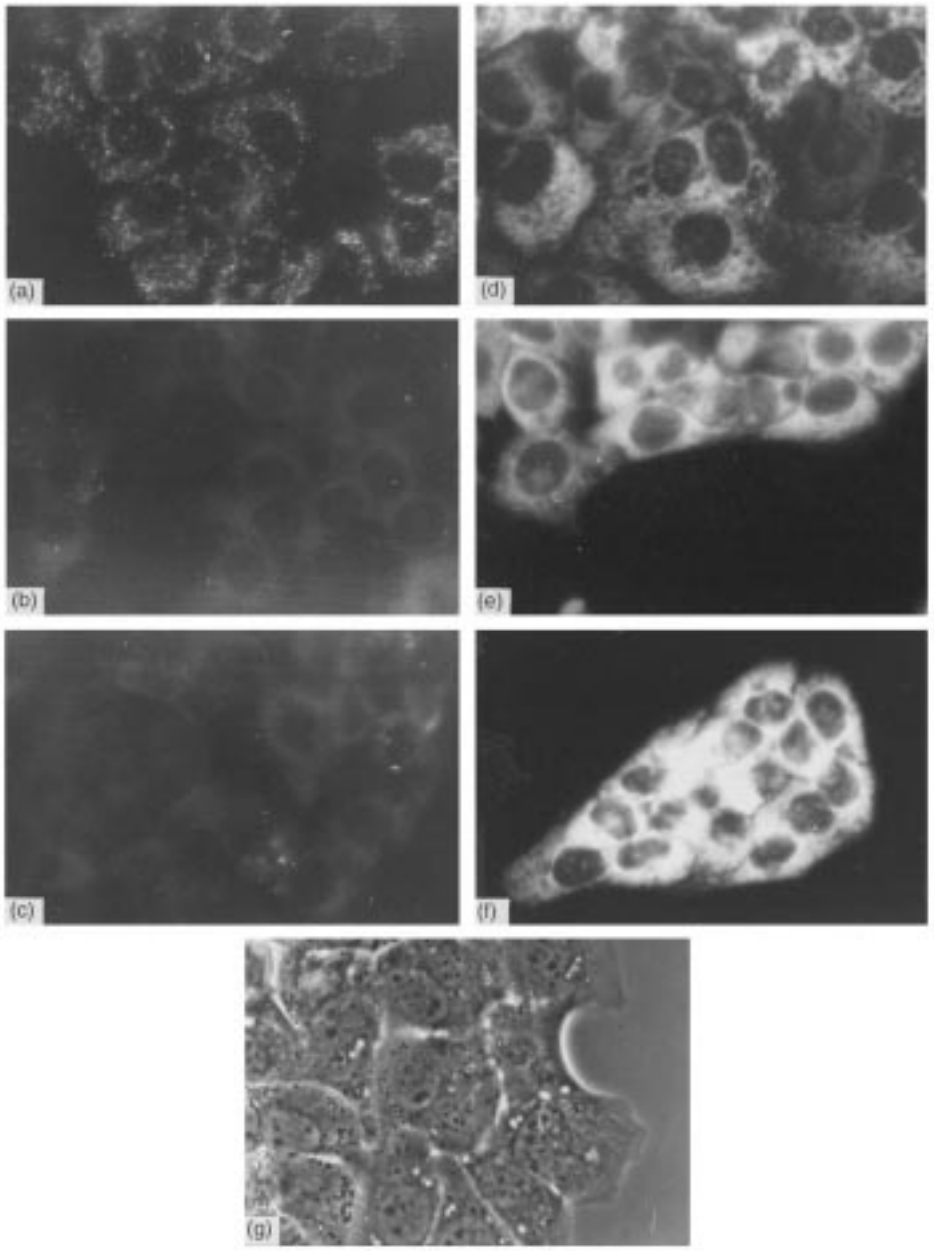


Fig. 2. Five hundred nM *m*-CCCP and 10 nM valinomycin decrease the mitochondrial electrochemical potential. (A), (B) and (C), mitochondrial staining of MA-10 cells by JC-1 as observed in the red channel in the absence of ionophores or in the presence of *m*-CCCP or valinomycin, respectively. (D), (E) and (F), cytoplasmic and mitochondrial staining of MA-10 cells by JC-1 as observed in the green channel in the absence of ionophores or in the presence of *m*-CCCP or valinomycin, respectively. (G), Phase contrast photograph of control MA-10 cells. Cells that are out of focus result from the clumping of MA-10 cells in clusters. Pictures are representative of observed staining patterns for all cells in each experiment and are typical of the results of three separate experiments.

sent in hormone stimulated cells and were found to be decreased following ionophore treatment. Treatment with *m*-CCCP resulted in 40, 37 and 15% decreases in the 3.4, 2.7 and 1.6 kb forms of StAR mRNA respectively. Treatment with valinomycin similarly resulted in decreases of 49, 30 and 14% in the 3.4, 2.7 and 1.6 kb forms of StAR mRNA. The levels of StAR mRNA were adjusted for loading using 28*S* RNA.

StAR protein levels were demonstrated to be more dramatically affected by 10 nM valinomycin and 500 nM *m*-CCCP (Fig. 4). As would be predicted from the cell staining patterns, import of StAR appeared to be affected. It was difficult to detect the rapidly turning over 37 kDa precursor form of StAR, although, consistent with previous studies [13], the level of this precursor appeared to increase with ionophore treatment (data not shown). This is also consistent with previous work which showed that *m*-CCCP inhibited processing of StAR [13]. In those studies, Western analysis of 2-dimensional SDS-PAGE gels showed that 500 nM *m*-

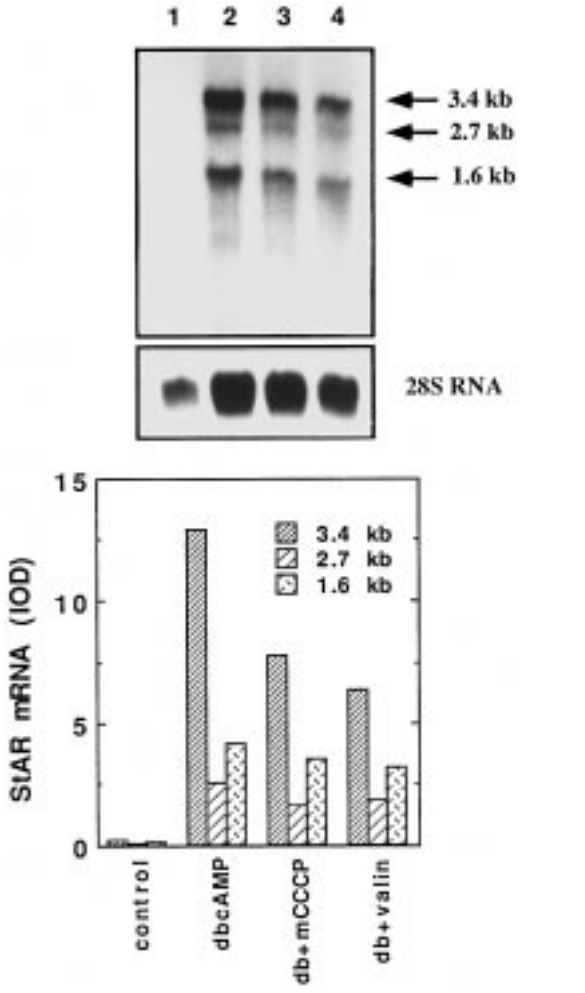


Fig. 3. Effects of ionophores on StAR mRNA levels in stimulated MA-10 cells. StAR mRNA was detected by Northern analysis and bands were quantitated as described in Section 2. Arrows indicate different species of StAR mRNA. The above autoradiogram depicts control (lane 1) or [Bu]2cAMP-stimulated MA-10 cells without (lane 2) or with 500 nM *m*-CCCP (lane 3) or 10 nM valinomycin (lane 4). The level of control mRNA was lower overall; however, the absence of detectable StAR mRNA is consistent with previously published data [37]. Compared to 28S RNA levels (lower panel), ionophore treatment reduced stimulated cell StAR mRNA levels (upper panel).

CCCP did not prevent StAR protein from being phosphorylated.

### *3.4. m-CCCP inhibits steroidogenesis in transfected COS-1 cells*

Since ionophore treatment affected StAR activity, experiments were performed with COS-1 cells transfected first with the F2 plasmid, and 24 h later, with StAR cDNA. Treatment with *m*-CCCP lowered the steroidogenic capacity of the system. Since the F2 protein must be imported into the mitochondria for activity, inhibition of mitochondrial import by *m*-CCCP would account for this observed decrease in intrinsic P450scc activity. Therefore, steroid production was normalized to the level of steroid produced in control cells without *m*-CCCP for each treatment. Since it was earlier determined that the presence of serum appeared to reduce the degree of inhibition of steroidogenesis by 500 nM *m*-CCCP in both MA-10 and transfected COS-1 cells, a higher concentration of ionophore (5 $\mu$M *m*-CCCP) was used for experiments in which serum was present.

StAR induced a 2.4-fold increase in steroid production above control, but in the presence of 5 $\mu$M *m*-CCCP, this increase was inhibited (Fig. 5). The level of 22*R*-HC-supported pregnenolone production within treatments was similar, but *m*-CCCP did depress P450scc activity by approximately 30% as seen in MA-10 cells (data not shown). This was determined by comparing 22*R*-HC-supported steroid synthesis in cells treated long-term with *m*-CCCP to that obtained from further incubation with 22*R*-HC and without *m*-CCCP.

### *3.5. m-CCCP inhibits processing of StAR in COS-1 cells*

The level of StAR protein expressed in transfected COS-1 cells was investigated (Fig. 5). In the absence of serum, 500 nM *m*-CCCP inhibited accumulation of mature StAR protein but had no effect on the level of the precursor. Visual inspection of the cultures showed few, if any, detached cells compared to control cultures, indicating that cell mortality was unaffected over 24 to 48 h. This is consistent with the results of studies employing the dye JC-1.

At 5 $\mu$M *m*-CCCP, the concentration used for steroid production measurements, similar results were obtained (Fig. 6A and B). Although 0.5% to 1% serum partially mitigated the effects of *m*-CCCP, protein synthesis was still affected at 5 $\mu$M *m*-CCCP. At this concentration, the level of StAR precursor was very low and importantly, there was no detectable mature StAR.

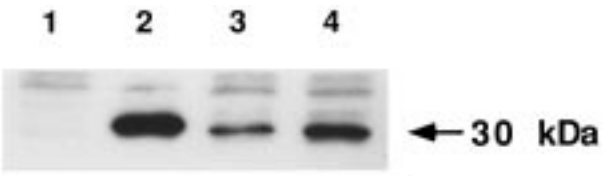


Fig. 4. Western analysis of StAR protein in MA-10 cells treated with *m*-CCCP or valinomycin. Lane 1, cell lysate from MA-10 cells; Lane 2, cell lysate from MA-10 cells stimulated for 6 h with [Bu]2cAMP. Lane 3, cell lysate from MA-10 cells stimulated for 6 h with [Bu]2cAMP with 500 nM *m*-CCCP. Lane 4, cell lysate from MA-10 cells stimulated for 6 h with [Bu]2cAMP with 10 nM valinomycin. Arrow indicates the expected position of the 30 kDa mature form of StAR.

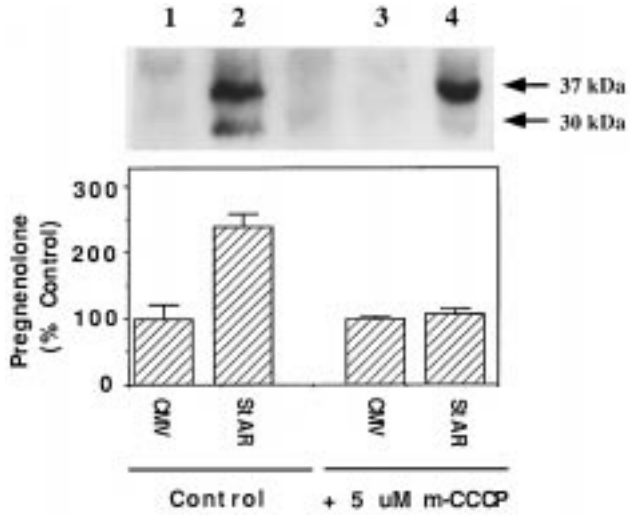


Fig. 5. Effects of *m*-CCCP on transfected COS-1 cell steroidogenesis and StAR protein levels. COS-1 cells were co-transfected with the F2 plasmid and then with pCMV[StAR] or vector alone and treated with *m*-CCCP for 24 h. Top panel, Western blot analysis of transfected COS-1 cells treated with *m*-CCCP. Lanes 1–2, cell lysate from transfected COS-1 cells transfected with pCMV or pCMV[StAR], respectively. Lanes 3–4, cell lysate from COS-1 cells transfected with pCMV or pCMV[StAR], respectively, and treated with 500 nM *m*-CCCP. Arrows indicate 37 and 30 kDa forms of StAR. Lower panel, 5 $\mu$M *m*-CCCP inhibited StAR-induced steroid production by 95% ($p < 0.05$). Levels were corrected for differences in steroidogenic capacity by normalizing data to the control level for each treatment (2212 pg/ml and 1019 pg/ml $\pm$ *m*-CCCP, respectively, for this experiment) and are expressed as a percent of control $\pm$ S.D. This experiment was repeated two times with similar results.

### 3.6. C-28 and N-19 mutants are imported into COS-1 cells

Lysates from COS-1 cells transfected with C-28 cDNA were analyzed by Western blot. C-28 precursor did not accumulate to a higher degree than wild-type, and appeared to be specifically processed and partially degraded. Variability in the relative levels of the mature and the precursor forms was observed in Fig. 6. In Fig. 6C, overexpression of C-28 resulted in both precursor and mature forms, but no degraded products were observed as in Fig. 6A and 6B. In this experiment, it should be noted that the mature form was expressed at an unusually high level. The estimated size of the C-28 precursor was 34 kDa and the mature form was 27 kDa. The level of StAR precursor was lower than typically observed, possibly due to a low transfection efficiency.

In some cases, when COS-1 cells were transfected by electroporation with C-28 cDNA, there was little observed accumulation of mature forms seen along with lower bands indicative of degradation products (Fig. 6A and B). The reasons for this observation are not clear at this time.

Generation of the mature form of the N-19 protein and the lower molecular weight forms of C-28 was preferentially inhibited by treatment with *m*-CCCP (Fig. 6A and B). Both N-19 and C-28 thus appeared to be processed in an ionophore-sensitive manner, indicating they are imported and their processing and degradation occur inside the mitochondria.

Treatment with 5 $\mu$M *m*-CCCP resulted in an overall reduction in N-47 levels, probably due to a slight inhibition of protein synthesis as noted (Fig. 6A and B). Quantitation of bands indicated that accumulation of C-28, N-47 and N-19 precursor protein was inhibited by *m*-CCCP (Fig. 6B). Unlike observations with wild-type StAR, therefore, the lower molecular weight form of N-47 may not reflect an imported, mature form as previously noted, but rather the result of translation from an alternate initiation site [38].

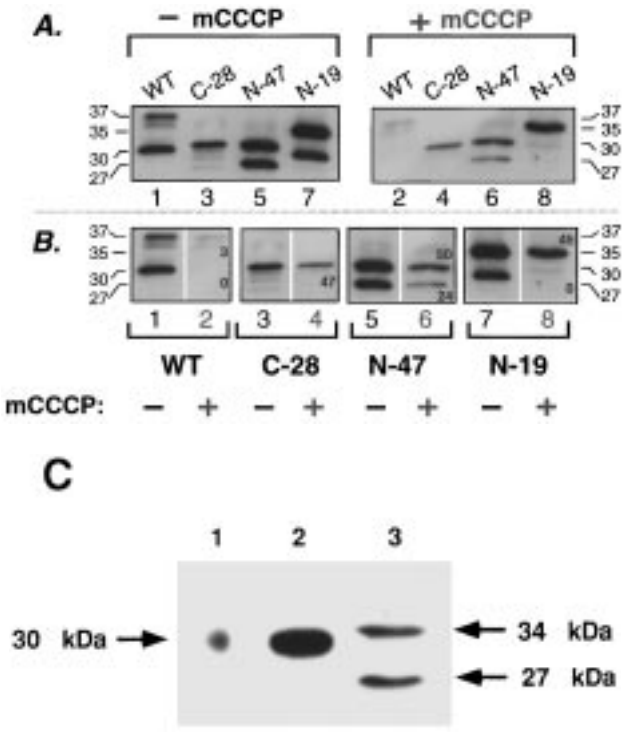


Fig. 6. Effect of *m*-CCCP on the fate of StAR mutants expressed in COS-1 cells. (A) and (B), wild-type StAR (WT), C-28, N-47 and N-19 cDNA were expressed in COS-1 cells after electroporation and treated with or without 5 $\mu$M *m*-CCCP as described in Section 2. (A), comparison of the level of StAR derivatives in the absence (-*m*-CCCP) and presence of *m*-CCCP (+*m*-CCCP). (B), rearrangement of the lanes depicted in (A) with quantitation of StAR signals. StAR signals obtained in the absence of *m*-CCCP (lanes 1, 3, 5 and 7) was determined as 100%, while corresponding levels of StAR products upon *m*-CCCP treatment are given in lanes 2, 4, 6 and 8. Similar results were obtained in two additional experiments of this kind. (C), Western analysis of transfected COS-1 cells. Lane 1, mitochondrial protein from stimulated MA-10 cells showing a strong staining band for 30 kDa StAR. Lanes 2 and 3, lysate from COS-1 cells transfected with StAR or C-28, respectively. Arrows with estimated mol wt indicate forms of StAR and C-28.

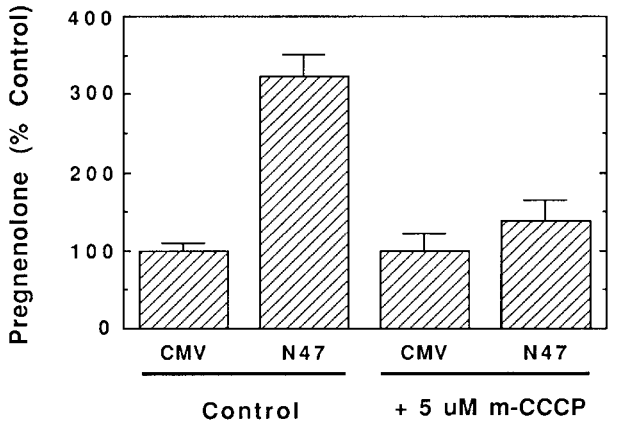


Fig. 7. Steroid production was assayed in COS-1 cells transfected with N-47 in the presence and absence of 5 $\mu$M *m*-CCCP treatment. Steroid production in COS-1 cells was normalized to control values as performed in Fig. 6A. This result is representative of four experiments with similar results.

### *3.7. m-CCCP inhibits steroidogenesis in N-47-transfected COS-1 cells*

To determine if N-47-induced steroidogenesis was susceptible to ionophore treatment, COS-1 cells were transfected with N-47 cDNA and the F2 plasmid and incubated with 0.5% serum in the presence or absence of 5 $\mu$M *m*-CCCP as before. Ionophore treatment reduced the level of N-47-stimulated steroidogenesis by 83% ($p < 0.05$) (Fig. 7). As in Fig. 6A and B, Western analysis revealed a reduction in N-47 protein at this level of protonophore treatment (data not shown), but it did not appear to correlate with the magnitude of loss in steroidogenesis.

### *3.8. N-47 induces steroidogenesis in non-StAR-expressing cells*

Since previous work has indicated that StAR is expressed in the kidney [39], the ability of N-47 to induce steroid production in HepG2 cells, a different nonsteroidogenic cell line was tested. Transfection of HepG2 cells with N-47 was found to indeed result in over a three-fold increase in steroid production ($p < 0.05$) (Fig. 8).

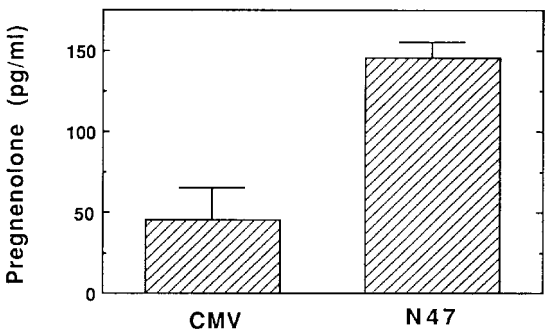


Fig. 8. The capacity of the N-47 StAR construct to induce steroid production in transfected HepG2 cells was determined. The data shown is representative of the results obtained in three separate experiments.

## 4. Discussion

Evidence to date has demonstrated that delivery of cholesterol from the outer to the inner mitochondrial membrane and the CSCC, in response to tropic hormone stimulation, is mediated by StAR [10]. However, the mechanism by which StAR promotes intermembrane cholesterol transfer is unclear. Recent work in *in vitro* systems concluded that an electrochemical gradient across the inner mitochondrial membrane is required for StAR function [19] and import [13, 38].

The present data confirm and extend these observations in cellular systems and further elucidate the role the electrochemical gradient plays in StAR function. Disruption of the electrochemical gradient and specifically, the electrochemical potential by the protonophore *m*-CCCP, was demonstrated by the pattern of JC-1 staining and the resultant loss of import of StAR. This disruption inhibited [Bu]2cAMP-stimulated steroidogenesis in MA-10 cells at concentrations that did not affect protein synthesis and minimally impaired enzyme activity. *m*-CCCP also blocked StAR-induced steroidogenesis in transfected COS-1 cells and had only minor effects on P450scc activity.

The potassium ionophore, valinomycin, was used since the electrochemical gradient is composed of both a proton motive force, and an inner membrane potential ($\Delta\Psi$), which is critical for protein import [17, 40–44]. Valinomycin specifically dissipates $\Delta\Psi$ by equilibrating potassium ions across the inner membrane and, therefore, made an attractive candidate to determine the role of $\Delta\Psi$ in steroidogenesis [42, 43, 45]. As with *m*-CCCP, 10 nM valinomycin decreased the electrochemical potential, StAR import, and [Bu]2cAMP-stimulated MA-10 cell steroidogenesis without significantly inhibiting cellular protein synthesis and P450scc activity. Thus, maintenance of an electrochemical gradient, and more specifically, $\Delta\Psi$, is required for steroidogenesis and StAR import.

We examined whether this inhibition of steroidogenesis could in part be explained by effects on StAR expression. Northern analysis revealed that while both compounds had an inhibitory effect on transcription of the StAR gene, the observed decreases in StAR mRNA could not fully account for the lowered level of mature StAR accumulation nor the dramatic impairment in steroid production. The fate of StAR precursor was difficult to follow since it does not accumulate at high levels in stimulated MA-10 cells trea-

ted with ionophore [13], consistent with the observation that precursors can be less stable than mature proteins [46]. However, overexpression in COS-1 cells did result in accumulation of both 37 and 30 kDa forms and in the presence of *m*-CCCP the level of mature protein was significantly reduced. Thus, as with most mitochondrial proteins which require maintenance of an electrochemical potential for import, StAR import was blocked by ionophore treatment and this is undoubtedly the reason for the loss of mature protein accumulation in the mitochondria.

In general, the ionophore concentrations used did not completely dissipate the electrochemical gradient since import of StAR still proceeded, although at a much lower level, and JC-1 mitochondrial staining was not completely inhibited. Visual observation of ionophore-treated cells revealed no apparent increase in cell mortality, and since 22*R*-HC-supported steroid synthesis was only marginally affected, it is apparent that mitochondrial function was not critically impaired.

ATP levels were probably not a limiting factor in treated cells, since phosphorylation and protein synthesis were not dramatically affected [45]. Rather, the metabolic state of the cells might have been accelerated to maintain ATP concentration. This would be of importance since StAR activity requires ATP and phosphorylation for maximal activity [19, 47]. A secondary decline in matrix ATP levels due to uncoupling, which would inhibit StAR import [17, 40], also seems unlikely because matrix and cytosolic ATP levels equilibrate when $\Delta\Psi$ is neutralized [48].

Import has been proposed to serve as an ''off switch'' for cholesterol transfer, based on studies showing that mutants lacking the N-terminal mitochondrial signal sequence, retain considerable activity in transfected COS-1 cells [16]. This model is supported by earlier data in which mature StAR protein appeared to increase steroidogenesis in isolated MA-10 mitochondria [28]. Thus, StAR may bind to an outer membrane effector to promote cholesterol transfer to the inner mitochondrial membrane. Although such a binding process would not require $\Delta\Psi$ [41], N-47-induced steroidogenesis was found to still be affected by *m*-CCCP. A functional role for import cannot yet be dismissed since immuno-electron microscopy data have shown that both N-47 and human N-62 StAR can be imported, though at a low rate (approximately 1% of wild-type StAR for N-47) [16, 24], and the degree of import required for maximal steroidogenesis in COS-1 cells is unclear.

The present results may also indicate that a factor which is sensitive to ionophore treatment is required for functionality. For instance, increases in mitochondrial $Ca^{2+}$ rely on the maintenance of an electrochemical gradient and have been proposed to be critical for angiotensin II-induced steroidogenesis in bovine adrenal glomerulosa cells [49]. The presence of $Ca^{2+}$ may be essential in a functionally important increase in contact site number [50, 51]. However, a broad role for mitochondrial $Ca^{2+}$ increase in cholesterol transfer remains ill-defined and may not occur in response to PKA activation. Instead, mitochondrial $Ca^{2+}$ may simply serve to modulate cytosolic $Ca^{2+}$ levels [52]. A calcium requirement for StAR function may rely on, at least, the presence of normal mitochondrial $Ca^{2+}$ concentrations. A model which proposes that a separate factor is involved would predict that it should be possible to find a condition in which import of StAR can be separated from steroid production in stimulated steroidogenic cells.

Since there is a shared sensitivity to disruption of the electrochemical gradient, the mechanism of StAR action in nonsteroidogenic cells may be similar to that in steroidogenic cell lines. Aside from COS-1 cells, N-47 induced steroidogenesis in transfected HepG2 hepatic tumor cells and preliminary data indicate that this induction is sensitive to *m*-CCCP as well (King, S. R., unpublished observations). If StAR promotes intermembrane cholesterol transfer by the same mechanism in both steroidogenic and nonsteroidogenic cells, other components important for this process would not be unique to steroidogenic tissue. Therefore, a common mechanism for mitochondrial intermembrane cholesterol and cholesterol-derivative transfer might be utilized by StAR, or StAR-like proteins, across different cell types. For instance, since StAR stimulates liver mitochondrial cholesterol 27-hydroxylase activity in transfected COS-1 cells [53], its expression in hepatocytes should increase the synthesis rate of the bile acid chenodeoxycholic acid [54]. However, it should be noted that the amount of steroid made in nonsteroidogenic cells is much less than in steroidogenic cells.

The C-28 mutant has little activity [11] and was previously thought to be inefficiently imported or processed [11, 16]. However, immuno-electron microscopy data have shown that C-28 is imported into the mitochondria [24]. Here we show that C-28 can be both imported and processed to a mature form of a size that reflects removal of the signal sequence as shown before [11]. Additionally, processing and degradation of C-28 proceeds in a *m*-CCCP-sensitive manner. Like wild type, there are high levels of precursor C-28; thus, the typically lower accumulation of mature C-28 is possibly due to reduced stability in the mitochondria. It should be noted that in accord with other previously published reports [11, 12, 24], there was a high degree of variability in the level of ''correctly'' processed mature and nonspecifically degraded forms of C-28. Since C-28 may be improperly folded in the mitochondria [19], the degraded forms may be due to errant processing of the protein. Interestingly, removal

of the first 19 amino acids of StAR (N-19) yields a protein that is still imported as previously shown [16] and sensitive to *m*-CCCP treatment. Therefore, the N-terminus is sufficient for import, although the precise region required for import appears to be flexible, while the C-28 mutation solely impacts the functional region of StAR. Thus, contrary to initial suggestions [16], C-28 is mitochondrially imported, processed and degraded, although mitochondrial processing appears to be carried out inefficiently.

StAR has extensive structural conservation across species and higher order structures have been hypothesized as important for StAR function [19]. For example, single amino acid substitutions that may solely alter protein structure cause lipoid CAH [11, 55]. If StAR must fold properly to effect cholesterol transfer and the ''off switch'' for this activity is import, which typically requires unfolding of mitochondrial proteins [40], then a model in which folding and function of StAR compete with unfolding and import is required.

A physiological system that may parallel observed experimental behavior, is the aging cell, in which both steroidogenesis and the mitochondrial electrochemical gradient have declined [56–60]. Since this reduction in steroid synthesis is thought to be due in part to a lower rate of cholesterol delivery to P450scc [58], the dependence of StAR activity on an electrochemical gradient could provide an explanation for age-related decreases in steroidogenic activity.

In summary, *m*-CCCP and valinomycin reduce the mitochondrial electrochemical potential in MA-10 cells as well as [Bu]2cAMP-stimulated steroidogenesis and accumulation of mature StAR protein. Furthermore, *m*-CCCP reduces StAR-induced steroid production and mature StAR protein levels in transfected COS-1 cells. A primary site of action by these ionophores is inhibition of the mitochondrial import of StAR. Furthermore, *m*-CCCP affects steroidogenesis induced by N-47, which is typically imported at a greatly reduced rate compared to wild-type StAR. In conjunction with other work, we show that C-28 is, in fact, imported and processed and the observed loss of function is due to the deletion of the functional region of StAR. These results show for the first time in whole cells that StAR activity requires a mitochondrial membrane potential and indicates that there exists a functionally required factor that is sensitive to the presence of this potential. Furthermore, since N-47 can induce steroidogenesis in transfected HepG2 cells, the mechanism by which StAR acts is probably common to many cell types.

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