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# An arachidonic acid generation/export system involved in the regulation of cholesterol transport in mitochondria of steroidogenic cells

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**Abstract** **Recent studies demonstrated the importance of the mitochondrial ATP in the regulation of a novel long-chain fatty acid generation/export system in mitochondria of diabetic rat heart. In steroidogenic systems, mitochondrial ATP and intramitochondrial arachidonic acid (AA) generation are important for steroidogenesis. Here, we report that mitochondrial ATP is necessary for the generation and export of AA, steroid production and steroidogenic acute regulatory protein induction supported by cyclic 3′–5′-adenosine monophosphate in steroidogenic cells. These results demonstrate that ATP depletion affects AA export and provide new evidence of the existence of the fatty acid generation and export system involved in mitochondrial cholesterol transport.**



*Keywords:* Arachidonic acid; Steroidogenesis; Mitochondria; Cholesterol transport

## 1. Introduction

In the steroidogenic cells, the step that determines the rate of steroid synthesis is the transport of cholesterol to the inner mitochondrial membrane [1]. It is now well established that there are several hormone-regulated proteins necessary in cyclic 3′–5′-adenosine monophosphate (cAMP) mediated stimulation of cholesterol availability and particularly in the transport of cholesterol from the outer to the inner mitochondrial membrane. Three of them are mitochondrial proteins: the peripheral benzodiazepine receptor (PBR) [2], the steroidogenic acute regulatory protein (StAR) [3] and the acyl-CoA thioesterase (Acot2) [4–6].

In the steroidogenic cells, Acot2 works in concert with an acyl-CoA synthetase, acyl-CoA synthetase 4 (ACS4), regulating the acute production of steroids [4]. In this mechanism, it has been suggested that ACS4 and Acot2 may constitute a generation system of arachidonic acid (AA) into a specific intracellular compartment, e.g. the mitochondria [7,8]. Mitochondrial AA plays a crucial role mediating the induction of the StAR protein through the conversion to leucotrienes outside the mitochondria. There is a recent report of activation of a novel long-chain free fatty acid generation and export system in mitochondria of diabetic rat heart [9], where Acot2 plays an important role. Disruption of the mitochondrial function with oligomycin, preventing ATP hydrolysis, produced no effect on the rate of generation but it inhibited the rate of export of free fatty acid from the mitochondria [9].

Several studies have focused on the importance of the mitochondrial function in the regulation of steroid synthesis [10–13]. The latest report [13] indicated that antimycin A (inhibitor of electron transport) or oligomycin (inhibitor of ATP synthesis) significantly reduced cellular ATP, potently inhibited steroidogenesis, and reduced StAR protein levels. Since intramitochondrial AA is necessary for StAR induction and steroidogenesis [7], it is reasonable to hypothesize that lowering ATP may alter the function of this generation/export system of AA leading to the inhibition of StAR protein expression and steroidogenesis. For that reason the purpose of this investigation was to determine the existence of the export system of AA in the mitochondria of steroidogenic cells and its role in the regulation of cholesterol transport.

## 2. Materials and methods

### 2.1. Rat testis interstitial cells and MA-10 Leydig cell culture

Interstitial cells were obtained from rat testis following a standard and already used procedure [14]. One million cells were stimulated during 3 h with 200 μM 8Br-cAMP in a final volume of 1 ml of M199 supplemented with 0.1% fatty acid-free BSA. The stimulation was performed in the presence or absence of antimycin A (1 and 5 μM), oligomycin (0.5, 1 and 10 μM) or carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, 5 μM). In all cases, the treatments were performed in the presence or absence of 200 μM AA. Testosterone production in the incubation media was measured by radioimmunoassay [14], and data are shown as testosterone concentration in ng/ml.

The MA-10 cell line is a clonal strain of mouse Leydig tumor cells that produce progesterone rather than testosterone as the major steroid. The cells were generously provided by Dr. Mario Ascoli, University of Iowa, College of Medicine (Iowa City, IA, USA) and were handled as originally described [15]. Cells were stimulated during 3 h with 8Br-cAMP (0.2, 0.5 or 1 mM) or 5 μM 22(R)OH-cholesterol in the culture medium containing 0.1% fatty acid-free BSA in the presence or absence of 200 μM AA, antimycin (1 μM), oligomycin (1 μM) or CCCP (5 μM). Progesterone production in the incubation media was measured by radioimmunoassay [7], and data are shown as progesterone concentration in ng/ml.

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*Abbreviations:* AA, arachidonic acid; StAR protein, steroidogenic acute regulatory protein; cAMP, cyclic 3′–5′-adenosine monophosphate; P450scc, side chain cleavage cytochrome P450; PBR, peripheral benzodiazepine receptor; Acot2, mitochondrial acyl-CoA thioesterase; ACS4, acyl-CoA synthetase 4; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone

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Inhibitors of mitochondrial function are widely used throughout the literature. Due to their mechanism of action (inhibition of the respiratory chain, ATP synthesis or electrochemical gradient) no evidence exist showing that they exert a different effect in tumour as compared to normal cells. The reported concentrations used in tumour and normal cells are similar and correspond to the concentration used in our study [11–13,16–18].

Quantitation of cellular ATP was performed using a luminescent assay. Cells were seeded in 96-well plates ($2.5 \times 10^5$ cells/well/100 μl serum free medium) and after stimulation in the conditions described above, cultures were treated as indicated by the manufacturer of the Cell Titer-glo luminescent cell viability assay (Promega, USA). Luminescence was determined using a Veritas microplate luminometer, Turner Biosystem.

### 2.2. [1-$^{14}$C] AA incorporation in MA-10 cells

MA-10 cells were labeled following a previously described methodology [7,19]. After labeling the cells with [$^{14}$C] AA (New England Nuclear, USA, specific activity 53.0 mCi/mmol), they were incubated in the presence or absence of 1 mM 8Br-cAMP for 3 h. When indicated, cells were incubated with antimycin A, oligomycin or CCCP together with 8Br-cAMP.

After these treatments, mitochondrial [$^{14}$C] AA content was determined as previously described [7]. The [$^{14}$C] AA-CoA formation in the post-mitochondrial fraction was evaluated by extraction from the aqueous phase according to the literature [20].

### 2.3. Statistical analysis

Statistical analysis was performed by ANOVA followed by the Student–Newman–Kuels test.

## 3. Results and discussion

To determine which properties of mitochondria are important for cAMP mediated AA generation and export, in the initial experiments, steroidogenic cells (MA-10 Leydig cell line) were treated with an inhibitor of the mitochondrial electron transport, antimycin A or an inhibitor of the ATP synthesis, oligomycin. As shown in Fig. 1 panels A and B, antimycin A and oligomycin produce a significant increase in cAMP-induced accumulation of AA into the mitochondria (Fig. 1, panels A and B, lanes 4 and 5 vs. lane 2). Treatment of MA10 cells with the $\Delta\Psi_m$ uncoupler CCCP does not affect AA accumulation induced by 8Br-cAMP (Fig. 1, panels A and B, lane 3 vs. lane 2). The effect produced by antimycin A and oligomycin on AA accumulation into Leydig cell mitochondria suggested that ATP synthesis is necessary for free fatty acid export as has been shown in rat heart mitochondria [9]. However, this result may also be explained with the theory that, the inhibitors antimycin A and oligomycin increase AA-CoA formation providing more substrate for Acot2 thus, increasing AA accumulation into the mitochondria. We measured the formation of AA-CoA in the presence of the inhibitors. As shown in Fig. 1, panel C, AA-CoA levels in the mitochondrial and post-mitochondrial fractions induced by cAMP do not increase in the presence of these two compounds.

The inhibition of AA export would reduce the steroidogenesis. This hypothesis may explain the effect of these two inhibitors on steroid synthesis as it was described recently [13]. If this is correct, one should be able to overcome the effect of oligomycin and antimycin A by adding exogenous AA to the cells. This AA would overload the cytosol and access directly to the lipoxygenases enzymes without going into the mitochondria. To test this hypothesis, steroidogenesis was measured in response to cAMP, alone or in combination with AA in the

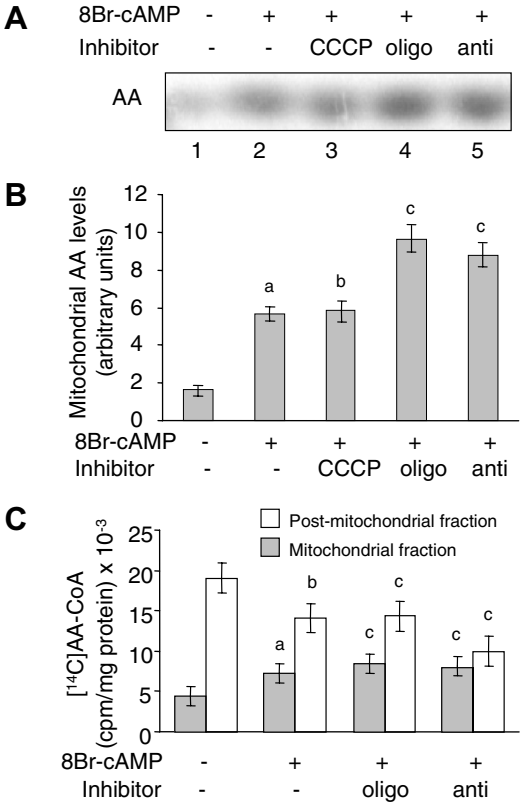


Fig. 1. Effect of antimycin A, oligomycin and CCCP on AA accumulation and on AA-CoA formation in MA-10 Leydig cells. MA-10 Leydig cells were labeled with [1-$^{14}$C] AA as described in Section 2. Then, cells were stimulated with 8Br-cAMP (1 mM) in the presence and in the absence of antimycin A (1 μM), oligomycin (1 μM) or CCCP (5 μM) for 3 h. Panel A: representative autoradiography showing AA spots in mitochondrial fraction. Panel B: autoradiography spots quantification by densitometry. The autoradiographies were quantified by densitometry and the data were normalized against the intensity of the signal of unlabeled AA stained with iodine, quantitated also by densitometry. Bars denote mitochondrial AA levels (in arbitrary units). Results are expressed as the means ± S.D. from three independent experiments. (a) $P < 0.001$ vs. control; (b) ns vs. 8Br-cAMP alone; (c) $P < 0.001$ vs. 8Br-cAMP alone. Panel C: Bars denote levels (in cpm/mg protein) of AA-CoA in mitochondrial (grey bars) and post-mitochondrial (white bars) fractions. Results are expressed as the means ± S.D. from three independent experiments. (a) ns vs. control; (b) $P < 0.1$ vs. control; c, ns vs. 8Br-cAMP alone.

presence and absence of antimycin A and oligomycin. Treatment of these two compounds significantly reduced cAMP stimulated steroid synthesis after 3 h of stimulation (Fig. 2). The inhibiting effect is observed on different concentrations of the second messenger (0.2 and 0.5 mM, data not shown). The results obtained in these experiments are identical to those published by Allen et al. [13]. Interestingly, the effect of antimycin A or oligomycin on cAMP-stimulated steroid synthesis can be reversed by the addition of exogenous AA in combination with cAMP (Fig. 2).

We next treated MA10 cells with the $\Delta\Psi_m$ uncoupler CCCP. Fig. 2 shows that this compound significantly inhibited steroid synthesis stimulated by cAMP after 3 h. In contrast to the results described above, CCCP also inhibited the biosynthesis stimulated by the combination of cAMP and AA (Fig. 2).

Fig. 3 shows the results obtained when the inhibitors were used on freshly isolated testicular cells. The graph indicates

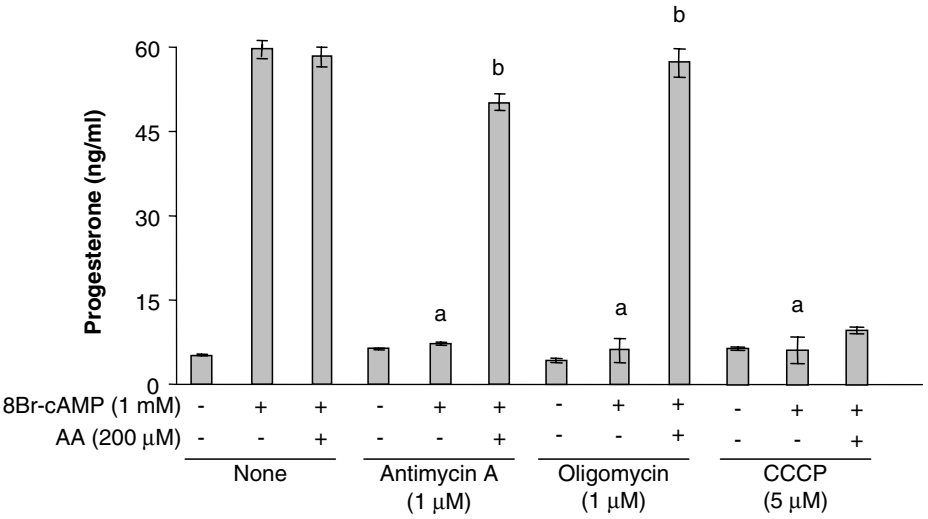


Fig. 2. Effect of AA on the action of antimycin A, oligomycin and CCCP on steroidogenesis in MA-10 Leydig cells. MA-10 Leydig cells were stimulated with 8Br-cAMP (1 mM) during 3 h in the presence and in the absence of the inhibitors (antimycin A, 1 μM; oligomycin, 1 μM; CCCP, 5 μM) and AA (200 μM) in the culture medium containing 0.1% fatty acid-free BSA. Progesterone production in the incubation media was measured by radioimmunoanalysis and data are shown as progesterone concentration (ng/ml). The results are expressed as the means ± S.D. from five independent experiments. (a) $P < 0.001$ vs. 8Br-cAMP alone; (b) $P < 0.001$ vs. 8Br-cAMP + inhibitor, alone.

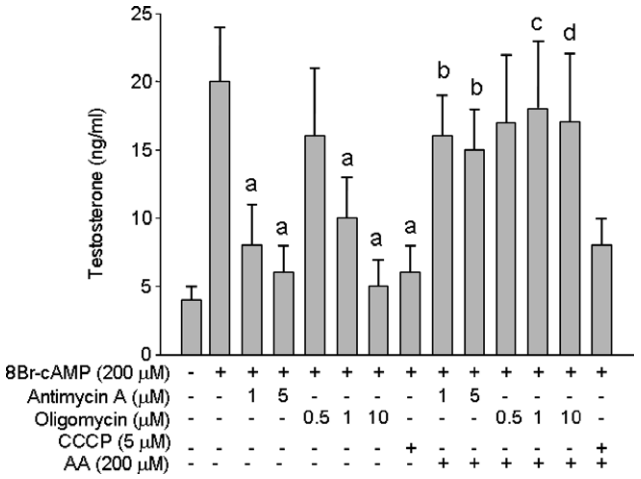


Fig. 3. Effect of AA on the action of antimycin A, oligomycin and CCCP on steroidogenesis in rat interstitial testicular cells. Interstitial testicular cells were stimulated with 8Br-cAMP (200 μM) in the presence and in the absence of the inhibitors (antimycin A, 1 and 5 μM; oligomycin, 0.1, 1 and 10 μM; CCCP, 5 μM). All the treatments were performed in the absence and in the presence of AA (200 μM) in the incubation medium containing 0.1% fatty acid-free BSA. After 3 h of stimulation, testosterone production in the incubation media was measured by radioimmunoanalysis and data are shown as testosterone concentration (ng/ml). The results are expressed as the means ± S.D. from five independent experiments. (a) $P < 0.001$ vs. 8Br-cAMP alone; (b) $P < 0.01$, (c) $P < 0.05$ and (d) $P < 0.001$ vs. 8Br-cAMP + inhibitor, alone.

that disruption of mitochondrial function affects testosterone production in a similar fashion that the inhibition observed in MA-10 cell line. Similarly, only the inhibitory effect of antimycin A and oligomycin may be reverted by the exogenous addition of AA, effect that is not observed on the inhibition produced by CCCP. Then, the interpretation of the results presented in the present paper is on the basis that the inhibitors have equal effects on both rat interstitial testicular cells and MA-10 Leydig cells. This is also supported by several previous studies performed using similar compounds in adrenal cells in vivo and in MA-10 Leydig cells, an accepted model of analysis of the regulation of Leydig cell steroidogenesis [10–13].

These results suggest that the inhibition of steroidogenesis by antimycin A and oligomycin is due to their indirect action on AA export and that ATP synthesis is necessary in an event located upstream of AA release/metabolism, while $\Delta\Psi_m$ is necessary in an event located downstream of AA release/metabolism.

The stimulation of steroid synthesis induced by 22(R)–OH–cholesterol was reduced less than 10% when antimycin A, oligomycin, or CCCP were present (data not shown), in coincidence with previously published results [13]. Given that 22(R)-OH-cholesterol is a permeable analog of cholesterol, those findings indicate that the inhibitors work on the regulation of cholesterol transport, upstream of side chain cleavage cytochrome P450 (P450scc).

The inhibitory effect of antimycin A and oligomycin on steroidogenesis can be overcome by exogenously added AA. Given that these two compounds affect the cellular content of ATP, AA could act by restoring the normal levels of this nucleotide. Fig. 4 shows that this is not the case since exogenous AA cannot reverse the action of the inhibitors on ATP synthesis. Thus, it can be proposed that exogenous AA would easily reach the lipoxygenase enzyme to produce active metabolites to induce StAR protein. The results depicted in Fig. 4 panel B and Fig. 5 support this hypothesis. Fig. 4 (panel B) shows that the effect of exogenous AA on steroidogenesis is blocked by NDGA, an inhibitor of the lipoxygenase, indicating that exogenous AA needs to be metabolized by the lipoxygenase to stimulate steroid synthesis, and this effect is exerted even in the presence of these two compounds, as demonstrated by Fig. 2. This result supports the suggestion that the action of the free fatty acid is exerted through its effect on StAR induction. Then, we next studied the effect of exogenously added AA on cAMP induced StAR protein levels in MA10 cells treated

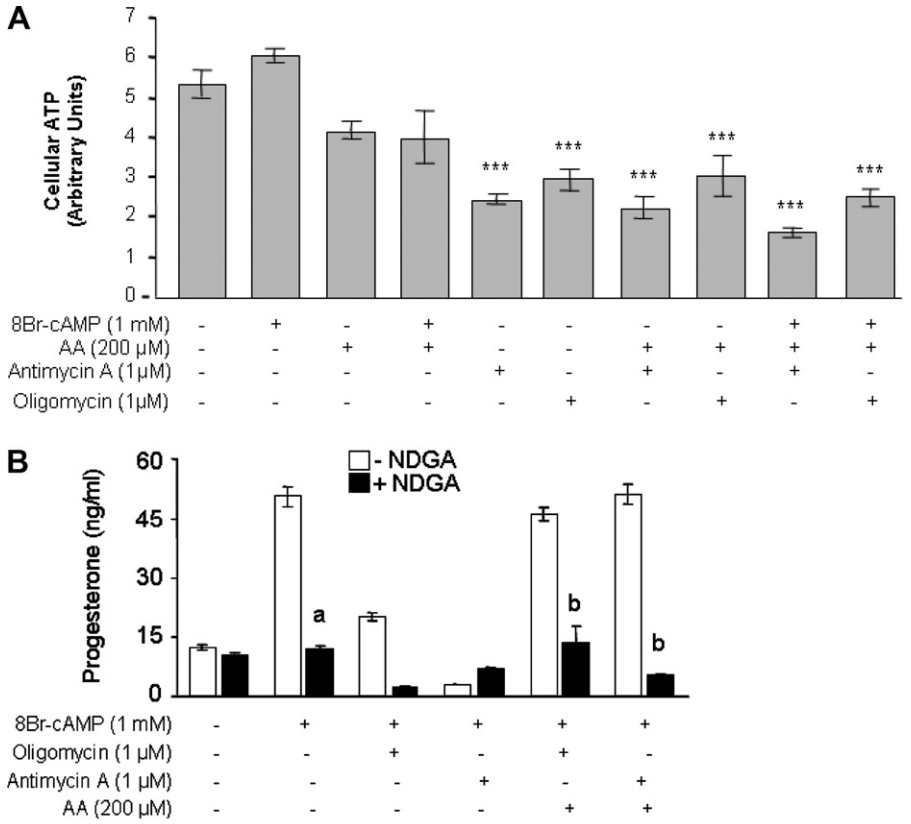


Fig. 4. Panel A: Effect of AA on the action of antimycin A and oligomycin on ATP cellular levels. MA-10 Leydig cells were stimulated with 8Br-cAMP (1 mM) in the presence and in the absence of antimycin A (1 μM) or oligomycin (1 μM) and AA (200 μM) in the culture medium containing 0.1% fatty acid-free BSA. Cell lysates were obtained and a luminescence method was used to determine ATP concentration. Bars denote ATP content in arbitrary units, expressed as the means ± S.D. from three independent experiments. $^{***}P < 0.001$ vs. control. Panel B: Effect of NDGA on the action of exogenous AA on steroidogenesis in MA-10 Leydig cells in the presence of antimycin A and oligomycin. MA-10 Leydig cells were stimulated with 8Br-cAMP (1 mM) in the presence and in the absence of the inhibitors (antimycin A, 1 μM; oligomycin, 1 μM;) and AA (200 μM) in the culture medium containing 0.1% fatty acid-free BSA. When indicated, cells were incubated with 10 μM NDGA for 30 min prior to the stimulation with 8Br-cAMP. Progesterone production in the incubation media was measured by radioimmunoanalysis and data are shown as progesterone concentration (ng/ml). The results are expressed as the means ± S.D. from five independent experiments. (a) $P < 0.001$ vs. 8Br-cAMP alone; (b) $P < 0.001$ vs. 8Br-cAMP + inhibitor + AA alone.

with both inhibitors. Since AA reversed the action of both inhibitors on steroidogenesis, we expected the same effect on StAR protein levels. Indeed, as shown in Fig. 5, inhibition of electron transport and ATP synthesis does not allow cAMP to increase StAR protein mitochondrial levels (Fig. 5, lanes 3 and 4 vs. lane 2), in accordance with Allen et al. [13]. Exogenous AA was able to overcome the inhibition produced by antimycin A and oligomycin on cAMP-induced StAR protein levels (Fig. 5 lanes 8 and 9 vs. lanes 3 and 4, respectively), indicating that neither electron transport nor ATP synthesis is directly necessary for the expression of StAR protein. Rather, the inhibition in StAR expression is a consequence of the inhibition in AA export.

Next, we studied the effect of CCCP on StAR protein levels induced by cAMP, alone or in combination with AA. There is an inhibitory effect of CCCP on the 30 kDa form of StAR induced by cAMP (Fig. 5, lane 5 vs. lane 2). In accordance with the results showing that CCCP acts downstream of AA, this fatty acid was not able to reverse the effect of CCCP on StAR protein levels (30 kDa form) (Fig. 5, lane 7 vs. lane 5). As described before [21], Acot2 does not change under cAMP stimulation and neither under the treatment of the cells with antimycin A, oligomycin, or CCCP (Fig. 5B).

It has been suggested that mitochondrial production of ATP is necessary for StAR translation [13]. The results presented here demonstrate that mitochondrial ATP is necessary for AA export as it was demonstrated for rat heart mitochondria [9], since AA can restore StAR induction and steroidogenesis in the presence of an ATP synthase inhibitor. Thus, in the absence of ATP, StAR induction is affected due to the absence of AA metabolization. The addition of exogenous AA seems to overload the cells, reaching the compartment where the lipoxygenase is acting [8].

It is known that the lipoxygenase enzyme is located in the endoplasmic reticulum and is also associated with the mitochondria. The contacts between mitochondria and endoplasmic reticulum play an important function in cell metabolism; for example, they secure a direct calcium transmission from the endoplasmic reticulum to the mitochondria [22]. This could be also the case in the export of AA from the mitochondria to secure a direct transmission of AA to the site of action of the lipoxygenase and may be an explanation to the question as how the cells drive AA via the cycloxygenase pathway or via the lipoxygenase pathway. It is reasonable to speculate that each pathway will have different sources of free AA triggered by different signal transduction pathways.

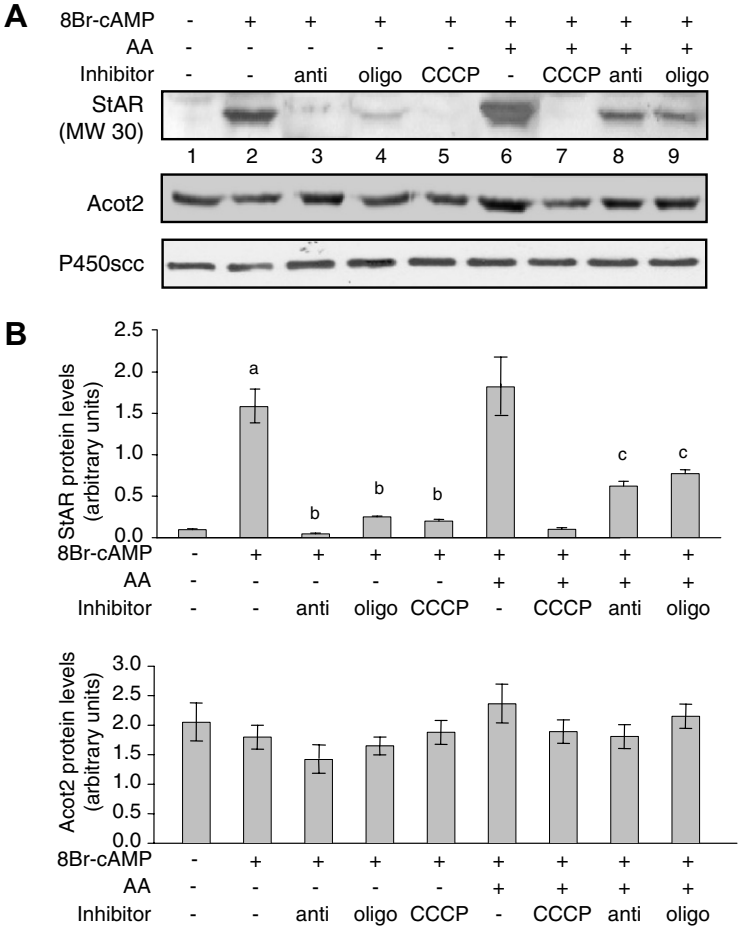


Fig. 5. Effect of AA on the action of antimycin A, oligomycin and CCCP on StAR protein levels in MA-10 Leydig cells. MA-10 Leydig cells were stimulated with 8Br-cAMP (1 mM) in the presence and in the absence of antimycin A (1 μM), oligomycin (1 μM) or CCCP (5 μM) and AA (200 μM) in the culture medium containing 0.1% fatty acid-free BSA. StAR (30 kDa) and Acot2 protein levels were evaluated by Western blot using anti-StAR and anti-Acot2 antibodies; P450scc protein levels were used as loading control, as previously described [23]. Panel A: Representative autoradiography of StAR, Acot2 and P450scc protein levels in cells stimulated with cAMP alone or in combination with AA in the presence and in the absence of CCCP, antimycin A or oligomycin. Panel B: Autoradiography quantification by densitometry. Bars denote StAR and Acot2 protein levels relative to P450scc protein levels in arbitrary units, expressed as the means ± S.D. from three independent experiments. (a) $P < 0.001$ vs. control; (b) $P < 0.001$ vs. 8Br-cAMP alone; (c) $P < 0.001$ vs. 8Br-cAMP + inhibitor, alone.

In conclusion, these results give a new insight on the role of mitochondrial function in StAR induction and steroidogenesis, demonstrating that the energized and actively respiring mitochondria are required for AA, export and metabolism which in turn will affect StAR protein levels and steroidogenesis.

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