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The Role of Arachidonic Acid on LH-Stimulated Steroidogenesis and Steroidogenic Acute Regulatory Protein Accumulation in MA-10 Mouse Leydig Tumor Cells

Xingjia Wang, Lance P. Walsh, and Douglas M. Stocco

Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX

Metabolic pathways leading to the production of arachidonic acid (AA) and its metabolites have been reported to have modulatory effects on steroidogenesis in a number of cell types. To examine the importance of the arachidonic acid pathway in steroid production and steroidogenic acute regulatory (StAR) protein expression, luteinizing hormones (LH) or N^6-2-*o*-dibutyryl-adenosine-3:5-cyclic monophosphate-(Bt_2cAMP) stimulated MA-10 mouse Leydig tumor cells were treated with various concentrations of quinacrine (an inhibitor of arachidonic acid production). Incubation of the cells with quinacrine resulted in dose-dependent decreases in steroid production and StAR protein. Twenty micromolars quinacrine inhibited 92 and 91% of LH-induced progesterone and StAR protein, respectively, and 98 and 90% of Bt_2cAMP-induced progesterone and StAR protein. Reversal of this inhibition was obtained by incubation of quinacrine-treated cells with various levels of AA, which resulted in a dose-dependent increase in both steroid and StAR protein levels. Two hundred micromolars of AA rescued 57 and 60% of the LH-induced steroid production and StAR protein, respectively, and 52 and 89% of Bt_2cAMP-induced steroid production and StAR protein. These results suggest that the effect of AA on LH- and cAMP-stimulated steroidogenesis is associated with the modulation of StAR protein expression.

Key Words: Arachidonic acid; quinacrine; steroidogenic acute regulatory protein; StAR; steroid biosynthesis.

Introduction

Arachidonic acid (AA) is produced in the cell mainly through the activation of phospholipase A_2 (PLA_2), which catalyzes its release from different phospholipids *(1,2)*. In addition, arachidonic acid can also be generated by other mechanisms *(3,4)*. Once released, AA is generally acted on by one of three enzyme systems, namely, lipoxygenase, cyclooxygenase, or cytochrome P450-dependent epoxygenase, to produce various metabolites *(5)*. Increasing evidence has suggested an important role for AA in the modulation of hormone-induced steroidogenesis in rat Leydig cells *(6,7)*, bovine adrenal cells *(8,9)*, human corpora luteal cells *(10)*, and in ovarian cells of rats *(11)* and goldfish *(12)*. As such, AA has been proposed as a potential second messenger in signal transduction *(3)*. Cooke et al. reported a rapid release of AA in rat Leydig cells following stimulation by luteinizing hormone (LH) *(13)*. Further studies from this group indicated that PLA_2 inhibitors inhibited both arachidonic acid release and LH induced steroid production in rat Leydig cells, but did not change the level of cyclic AMP (cAMP) formation *(14)*. Also, other studies have reported that AA release occurred in a dose- and time-dependent manner and depended on LH/hCG–receptor interaction *(15)*. In addition to the effects of AA itself, the metabolites of AA have also been reported to modulate hormone-induced steroid production *(16–18)*.

The results obtained in most of these studies indicated that AA and its metabolites acted at the level of the acutely regulated step in steroid hormone biosynthesis, the transfer of the substrate for all steroid hormones, cholesterol, from the outer mitochondrial membrane to the cytochrome P450 side chain cleavage enzyme (P450scc), which is located in the inner mitochondrial membrane. They did not appear to affect the activities of the steroidogenic enzymes P450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD), since addition of the more hydrophilic cholesterol-like substrate 22(*R*)-hydroxycholesterol, which readily diffuses to the P450scc, indicated that there was no inhibitory effect of PLA_2 inhibitors on steroid production *(6,19)*. However, the mechanism for the effect of AA and its metabolites on steroidogenesis remained unknown.

The steroidogenic acute regulatory (StAR) protein was demonstrated to have a critical function in the transfer of

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Author to whom all correspondence and reprint requests should be made: Dr. Douglas M. Stocco, Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX 79430. E-mail: cbbdms@ttuhsc.edu

cholesterol from the outer to the inner mitochondrial membrane during hormone-stimulated steroidogenesis *(20–22)*. Since AA appears to modulate this rate-limiting step of cholesterol transfer, we reasoned that there may be a relationship between cellular AA levels and StAR protein expression. Indeed, if AA can act as a second messenger, it may serve to modulate StAR protein expression. In the present study, we examined the relationship between AA and StAR protein by treating LH- or N^6-2-*o*-dibutyryl-adenosine-3:5-cyclic monophosphate- (Bt_2cAMP) stimulated MA-10 Leydig tumor cells with the PLA_2 inhibitor quinacrine (which prevents AA release) and to examine its effect on steroidogenesis and StAR protein expression. The results of these experiments demonstrated that inhibition of AA release resulted in an inhibition of LH- or cAMP-induced steroidogenesis, and that this inhibition probably occurs via a concomitant inhibition of StAR protein expression.

Results

Effect of the PLA_2 Inhibitor, Quinacrine, on LH- or Bt_2cAMP-Stimulated Steroidogenesis

To test the effect of inhibition of AA release on steroidogenesis, LH- or Bt_2cAMP-stimulated MA-10 cells were treated with various concentrations of the PLA_2 inhibitor, quinacrine, for 6 h. Figure 1A illustrates the effect of quinacrine on LH-stimulated progesterone production in MA-10 Leydig cells and demonstrates a dose-dependent inhibitory effect. A quinacrine concentration of 20 μ*M* inhibited 92% of the LH-stimulated progesterone production seen in control cultures. Quinacrine also inhibited Bt_2cAMP stimulated steroidogenesis in a similar manner (Fig. 1B), with 20 μ*M* quinacrine inhibiting 98% of progesterone production. To determine if the inhibitory effect of quinacrine on progesterone production might be due to inhibition of the activities of the steroidogenic enzymes P450scc and/or 3β-HSD, 22(*R*)-hydroxycholesterol was added to each treatment for a 2-h period. There was no significant difference in steroid production among the treatments when 22(*R*)-hydroxy–cholesterol was used as substrate (Fig. 1A,B), thus indicating that the observed inhibition was not a result of the impairment of these enzymes.

Effect of Inhibition of AA Release on LH- or Bt_2cAMP-Stimulated StAR Protein Expression

To determine the effect of the inhibition of arachidonic acid release on LH- or Bt_2cAMP-stimulated StAR protein expression, Western blot analysis was employed as previously described *(23)*. Incubation with increasing levels of quinacrine resulted in a dose-dependent decrease in StAR protein in both LH- and Bt_2cAMP-stimulated MA-10 cells (Fig. 2). A quinacrine concentration of 20 μ*M* reduced StAR protein to 9% of LH-induced levels and 10% of Bt_2cAMP-induced levels. Comparing Figs. 1 and 2, it can readily be seen that the inhibition of StAR protein expression is highly correlated to the inhibition of LH- or Bt_2cAMP-stimulated progesterone production. Results from statistical analysis showed that the correlation coefficient between these two parameters is 0.92 ($p < 0.05$) for LH-stimulated cells and 0.95 ($p < 0.05$) for Bt_2cAMP-stimulated cells (Table 1).

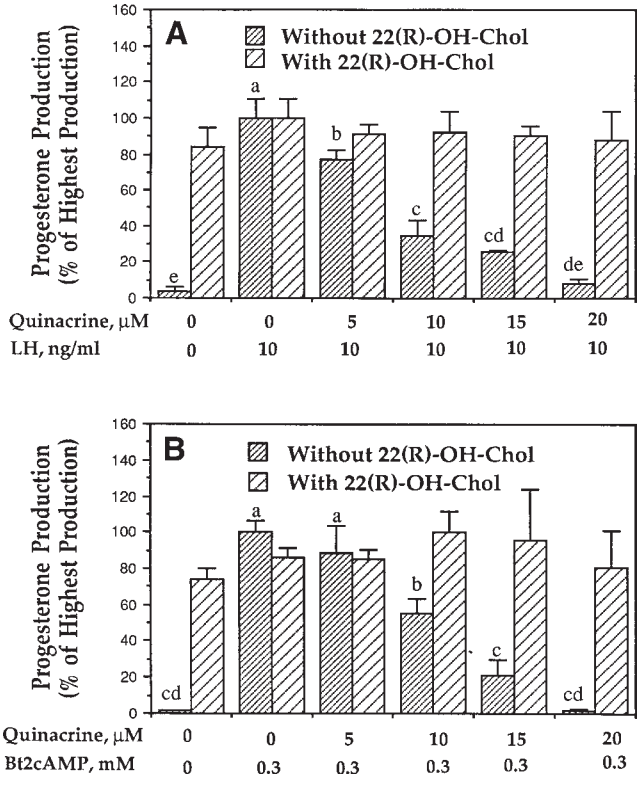

Fig. 1. Inhibitory effect of the PLA_2 inhibitor, quinacrine, on LH- or Bt_2cAMP-stimulated steroid production in MA-10 Leydig cells. MA-10 cells were cultured in serum-free Waymouth's MB/752 medium containing various concentrations of quinacrine, for 30 min as indicated in the figure, then stimulated with 10 ng/mL of LH (**A**) or 0.3 m*M* of Bt_2cAMP (**B**) for 6 h. To test the effect of quinacrine on activities of P450scc and 3β-HSD, 25 μ*M* of 22(*R*)-hydroxycholesterol were added to each treatment for a 2-h culture. Media from both 6- and 2-h cultures were collected and assayed for progesterone production by RIA. The progesterone production was expressed as percentage of the highest production among the treatments. Highly significant differences in progesterone production ($p \leq 0.01$) following quinacrine treatment are indicated in the groups not having the same letter.

Quinacrine-Induced Inhibition of Steroidogenesis is Reversed by AA

In order to determine if the inhibitory effects of 20 μ*M* quinacrine on steroidogenesis could be reversed, increasing concentrations of AA were added to the quinacrine-treated cultures of LH- and Bt_2cAMP-stimulated MA-10 cells. As shown in Fig. 3, addition of AA reversed and partially rescued the quinacrine-inhibited progesterone production induced by LH or Bt_2cAMP. The reversal of quinacrine-inhibited steroidogenesis was also dose-dependent, attaining 57% of progesterone production in LH-

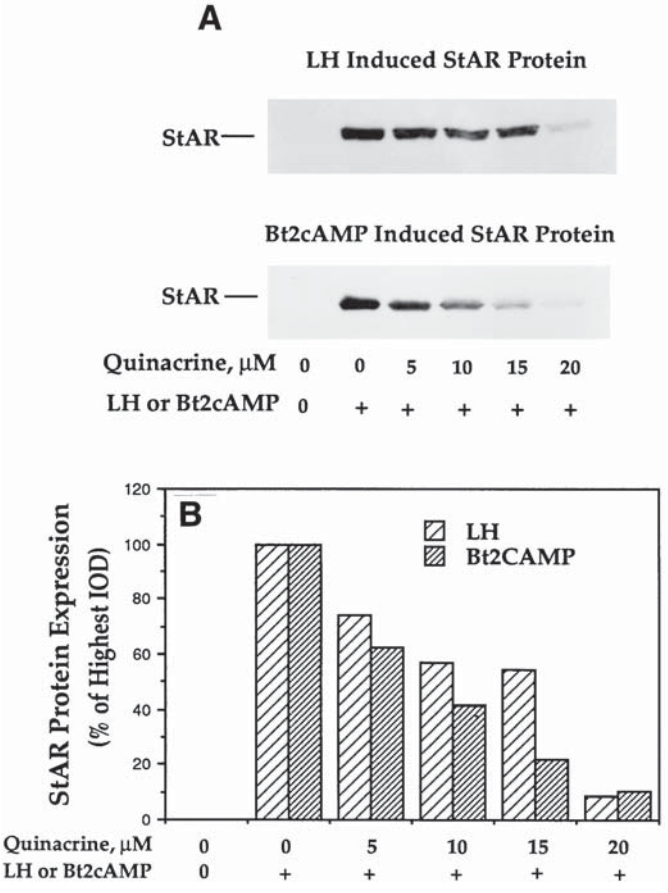

Fig. 2. Inhibitory effect of PLA_2 inhibitor on LH- or Bt_2cAMP-stimulated StAR protein expression in MA-10 Leydig cells. MA-10 cells were cultured in serum-free Waymouth's MB/752 medium containing various concentrations of quinacrine for 30 min as described in the figure, and then stimulated with 10 ng/mL of LH or 0.3 m*M* of Bt_2cAMP. After 6 h of culture, the cells were collected, and mitochondria were isolated. **(A)** Western blot analysis was performed with isolated mitochondia. The StAR protein specific bands detected by Western blot were quantitated using the BioImage Visage 2000 and integrated optical density (IOD). **(B)** The StAR protein was expressed as percentage of the highest IOD obtained among the treatments. Shown are the results of one experiment that was performed twice with the same results.

stimulated cells and 52% in Bt_2cAMP-stimulated cells at 200 μ*M* AA. Once again, there was a very high level of correlation between the concentration of AA in the incubation medium and progesterone production. The coefficient of correlation was 0.97 ($p < 0.01$) in LH-stimulated cells and 0.99 ($p < 0.01$) in Bt_2cAMP-stimulated cells (Table 1).

Reversal of Quinacrine-Induced StAR Protein Expression Inhibition by AA

Figure 4 shows the effect of AA addition on 20 μ*M* quinacrine-inhibited StAR protein expression induced by LH or Bt_2cAMP. As shown in Fig. 2, StAR protein expression in MA-10 cells stimulated with LH was inhibited by quinacrine. This inhibition was reversed by incubation of quinacrine treated cells with arachidonic acid as shown in Fig. 4. Similar effects were observed in Bt_2cAMP-stimulated cells, also as indicated in Fig. 4. The reversal of quinacrine-inhibited StAR protein levels by arachidonic acid was dose-dependent, with 200 μ*M* rescuing 60% of the StAR protein induced by LH and 89% of the StAR protein induced by Bt_2cAMP. As seen for the reversal of steroid production, the reversal of quinacrine-inhibited StAR protein was also highly correlated with the levels of AA in the incubation. The coefficient of correlation is 0.97 ($p < 0.01$) in LH-stimulated cells and 0.99 ($p < 0.01$) in Bt_2cAMP-stimulated cells. It should also be noted that in the present studies, 200 μ*M* AA alone had only a slight stimulatory effect on progesterone production and did not induce a detectable increase in StAR protein using the methods described.

Discussion

cAMP is generally accepted as the second messenger in LH-stimulated steroidogenesis in Leydig cells. Increasing evidence has also suggested that AA may represent another second messenger *(3)*. Modulatory effects of AA on hormone-induced steroidogenesis have been reported *(24,25)*. In support of AA acting as a messenger, it was reported that AA was released rapidly following LH stimulation *(13,15)*. Phospholipase A_2 (PLA_2) is a key enzyme in the AA release from phospholipids. The results obtained in the present study indicate that this enzyme plays an important role in LH or Bt_2cAMP-stimulated steroidogenesis in MA-10 Leydig tumor cells. Inhibition of PLA_2 activity almost completely inhibited LH- or Bt_2cAMP-stimulated steroid production. The inhibitory effect of quinacrine on LH- or Bt_2cAMP-induced steroidogenesis was clearly not the result of toxic effects of quinacrine on the cells or inhibition of the activities of the enzymes P450scc or 3β-HSD. When 22(*R*)-hydroxycholesterol was added to quinacrine-treated cells, there was no significant difference in steroid production in any of the cultures, indicating that the cells were viable and that these enzymes were unaffected by this inhibitor. This observation is also strengthened by the finding that quinacrine-inhibited steroidogenesis could be rescued to significant levels by treatment of the cells with arachidonic acid. This suggests that the inhibitory effect of quinacrine on steroidogenesis was the result of inhibition of AA release from phospholipids. These results are in essential agreement with observations reported previously *(6,14)*. It should be noted that although the reversal of quinacrine inhibition was significant, it failed to restore steroid production and StAR protein induced by LH or Bt_2cAMP to levels seen in control cultures. This may be a reflection of the inability of cultured cells to take up adequate amounts of exogenous AA from the medium in that a serum-free medium that lacks a binding protein for this compound was used. Regardless, a significant dose-dependent reversal of both quinacrine-inhibited progesterone production and StAR expression by AA addition was observed in these studies.

Table 1

Linear Correlation Coefficient of AA with StAR Protein Synthesis and Steroid Production in LH- or Bt_2cAMP-Stimulated MA-10 Cells

Parameter 1	Parameter 2	Coefficient	Probability
LH-stimulated cells			
StAR protein[a]	Progesterone	0.92	0.025
AA	StAR protein	0.97	0.007
AA	Progesterone	0.97	0.005
Bt2cAMP-stimulated cells			
StAR protein[a]	Progesterone	0.95	0.014
AA	StAR protein	0.99	0.002
AA	Progesterone	0.99	0.001

[a]StAR protein was detected by Western blot analysis as described in Materials and Methods and quantitated using the BioImage Visage 2000 *(21)*.

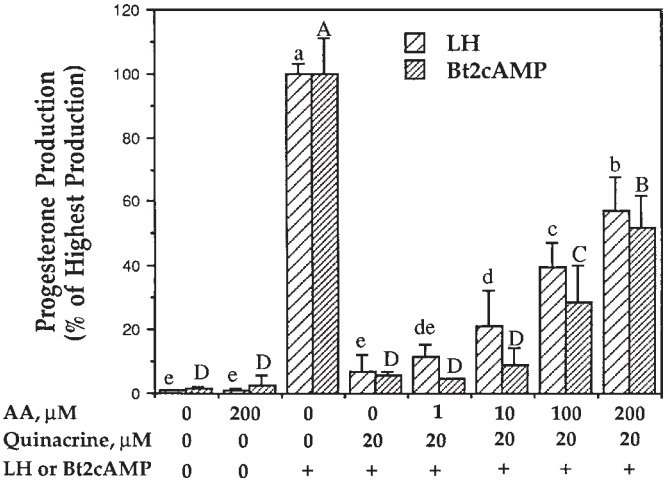

Fig. 3. Effect of coincubation of AA with the PLA_2 inhibitor, quinacrine, on steroid production in MA-10 Leydig cells stimulated by LH or Bt_2cAMP. MA-10 cells were cultured in serum-free Waymouth's MB/752 medium containing various concentrations of AA and 20 μ*M* of quinacrine for 30 min, as indicated in the figure. The cells were then stimulated with 10 ng/mL of LH or 0.3 m*M* of Bt_2cAMP. One group was treated with 200 μ*M* AA only. The media were collected after 6 h of culture, and progesterone production was assayed by RIA and expressed as percentage of the highest production among the treatments. Significant differences in progesterone production ($p \leq 0.05$) are indicated in the groups not having the same letter.

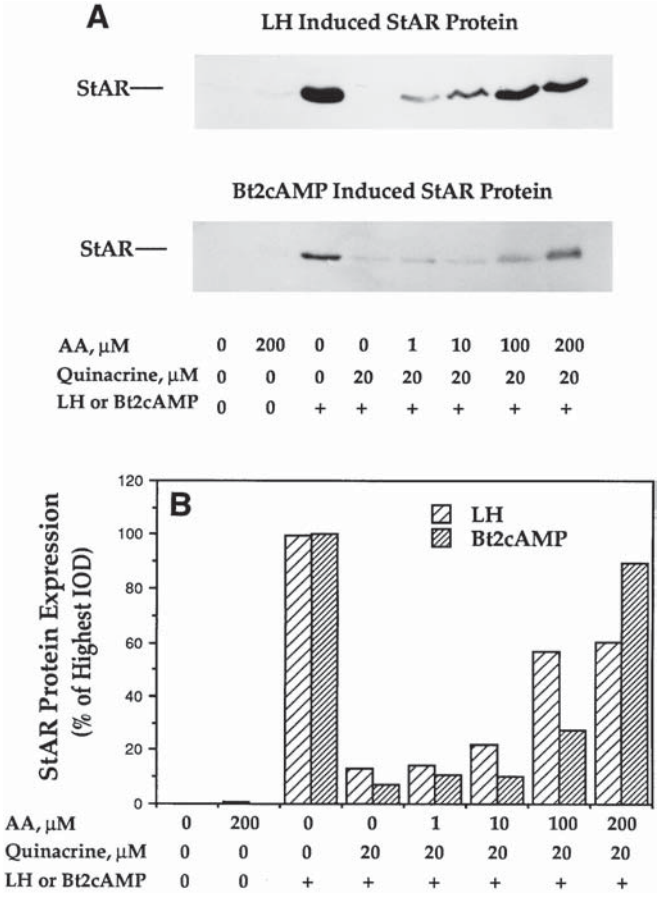

Fig. 4. Effect of coincubation of arachidonic acid with the PLA_2 inhibitor, quinacrine, on StAR protein expression in MA-10 Leydig cells stimulated by LH or Bt_2cAMP. MA-10 cells were cultured in serum-free Waymouth's MB/752 medium containing various concentrations of AA and 20 μ*M* of quinacrine for 30 min, as indicated in the figure, and then stimulated with 10 ng/mL of LH or 0.3 m*M* of Bt_2cAMP, except for control and a group receiving 200 μ*M* AA only. After 6 h of culture, the cells were collected and mitochondria were isolated. **(A)** Western blot analysis was performed with isolated mitochondia. The StAR protein specific bands detected by Western blot were quantitated using the BioImage Visage 2000 as IOD. **(B)** The StAR protein was expressed as percentage of the highest IOD among the treatments. Shown are the results of one experiment that was performed twice with the same results.

The mechanism responsible for the effect of AA and its metabolites on hormone-induced steroidogenesis is far from clear. The study described here suggests that the modulatory effect of AA on LH- or Bt_2cAMP-induced steroidogenesis is associated with modulation of StAR protein expression. This is readily seen in the results which demonstrated a highly significant dose-dependent inhibition of both steroid production and StAR protein by the PLA_2 inhibitor, quinacrine. Twenty micromolars of quinacrine treatment concomitantly inhibited virtually all of the progesterone production and StAR protein expression in LH- or Bt_2cAMP-stimulated MA-10 Leydig tumor cells.

The probability that StAR protein may be involved in this mechanism is further enhanced by the observation that addition of AA to the quinacrine treated cells resulted in a dose-dependent increase of steroid production and StAR protein levels. The high correlation between the levels of AA in the incubation, and the increase of progesterone production and StAR protein indicate that StAR is involved in the action of AA. Once again, this is in complete agreement with previous studies, which indicated that AA acted at the step of cholesterol transfer to the P450scc enzyme without affecting the activities of the P450scc and/or 3β-HSD *(6,14)*. Since earlier studies have shown that the presence of StAR is indispensable in the transfer of cholesterol to the P450scc *(27–30)*, it follows that arachidonic acid effects may be mediated through its modulation of StAR expression.

How AA and its metabolites modulate steroidogenesis and StAR protein expression is unknown. Abayasekara et al. *(14)* reported that PLA_2 inhibitors inhibited LH-stimulated steroid production, but had no effect on cAMP formation in LH-stimulated Leydig cells. Based on similar experimental results, Cooke et al. *(13)* proposed a model in which LH and cAMP control steroid production via PLA_2 activation. In this model, they speculated that cAMP may modulate PLA_2 activity, resulting in the release of arachidonic acid and subsequent stimulation of steroidogenesis *(13,14)*. However, incubation of MA-10 cells with 200 μ*M* of arachidonic acid alone without LH- or Bt_2cAMP-stimulation did not stimulate large increases in progesterone production or StAR protein as tested by Western blot analysis. Although the uptake of exogenous arachidonic acid by the cells and the sensitivity of StAR detection by Western analysis may be questioned, it appears to be clear that addition of AA by itself cannot solely result in high levels of steroid production and StAR protein. Again this is reflected in the observation that addition of AA to quinacrine-treated, stimulated cells was able to reverse a large portion of the steroid and StAR levels seen in cells not treated with the inhibitor. Apparently, sufficient AA was able to be taken up by these cells to reverse the effects of quinacrine and hence, one would expect to see an effect if exogenous AA were able to support significant increases in steroid production. Although yet to be determined, it is possible that after release of arachidonic acid by LH or cAMP, the two messengers, cAMP and AA acting through different transduction pathways, act to comodulate steroidogenesis and StAR protein expression. In this scenario, both messengers and pathways are required for full activity, but neither one alone is sufficient for maximal LH- or cAMP-stimulated steroid production and StAR protein expression. Thus, StAR protein expression may be under the comodulation of these two systems. The involvement of AA and its metabolites has also been implicated in the stimulation of steroid production in non-cAMP-mediated systems *(31,32)*. In one of these studies, Sonalo et al. *(32)* reported that lipoxygenase products of arachidonic acid could be the common intermediate in the action of angiotensin II and ACTH on steroid production. It will be interesting to determine if the AA pathway is a pathway that can serve to modulate both cAMP-dependent and cAMP-independent systems in the regulation of StAR protein expression.

Materials and Methods

Chemicals

AA, quinacrine, Bt_2cAMP, 22(*R*)-hydroxycholesterol, and bovine serum albumin (BSA, fraction V) were purchased from Sigma (St. Louis, MO). Human LH was obtained from NIADDK-NIH (Bethesda, MD). Waymouth's MB/752 medium, horse serum, trypsin-EDTA, antibiotics, and phosphate-buffered saline (PBS) were purchased from Gibco-BRL Life Technologies (Gaithersburg, MD). Rabbit antisera generated against a StAR fusion protein was a generous gift from D. B. Hales (Department of Physiology and Biophysics, University of Illinois, Chicago, IL). Donkey antirabbit IgG antibody conjugated with horseradish peroxidase was purchased from Amersham (Arlington Heights, IL). Other common chemicals used in these studies were obtained from either Sigma or Fisher Chemicals (Pittsburgh, PA).

Cell Culture

The MA-10 mouse Leydig tumor cells were a generous gift from Mario Ascoli (Department of Pharmacology, University of Iowa, College of Medicine, Iowa City) and were grown in 100-mm tissue-culture dishes in Waymouth's MB/752 medium containing 15% horse serum as previously described (33). For experiments, when cells were 75% confluent, the medium was removed and replaced with serum-free Waymouth's MB/752 medium containing 0.1% BSA for 24 h and then changed to serum-free Waymouth's MB/752 medium without BSA immediately before the experiments. For the incubation times described in each figure legend, the cells were placed in incubators at 37°C and 5% CO_2.

Steroid Production

MA-10 cells were cultured in serum-free Waymouth's MB/752 medium containing various concentrations of AA or quinacrine (as described in the figure legends) for 30 min and then stimulated with 10 ng/mL LH or 0.3 μ*M* Bt_2cAMP for 6 h. To test the effects of AA or quinacrine on the activities of P450scc and 3β-HSD, 25 μ*M* of 22(*R*)-hydroxycholesterol were added to each treatment and cultured for 2 h. The medium and cells were collected at the end of each experiment and stored at –20°C. Progesterone concentrations in the medium were determined by radio-immunoassay (RIA) *(34)*.

Western Blot Analysis

The MA-10 cell mitochondria were isolated as described previously *(35)*. The mitochondria from each treatment were tested for StAR protein expression by Western analy-

sis as described previously *(20)*. The samples were solubilized in sample buffer (25 m*M* Tris/HCl, pH 6.8, 1% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 1 m*M* EDTA, 4% glycerol, and 0.01% bromophenol blue), boiled for 5 min and loaded onto a 12% SDS- polyacrylamide gel electrophoresis (PAGE) mini-gel (MiniProtean II System; Bio-Rad, Richmond, CA). Electrophoresis was performed at 200 V for 45 min using a standard SDS-PAGE running buffer (25 m*M* Tris, 192 m*M* glycine, 0.1% SDS, pH 8.3). The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad) at 100 V for 2 h at 4°C using a transfer buffer containing 20 m*M* Tris, 150 m*M* glycine, and 20% methanol at pH 8.3. The membrane was incubated in blocking buffer (PBS buffer containing 4% Carnation nonfat dry milk and 0.2% Tween-20) at room temperature for 1 h followed by incubation with a primary antibody against StAR protein for 30 min. The membrane was washed with PBS containing 0.2% Tween-20 three times for 10 min each. After incubation with the second antibody, donkey antirabbit IgG conjugated with horseradish peroxidase, the membrane was washed five times for 10 min each. Specific protein bands were detected by chemiluminescence using the Renaissance Kit (Dupont New England Nuclear, Wilmington, DE), and quantitated using the BioImage Visage 2000 *(21)*. Western analysis experiments were performed at least twice, and the results of one representative experiment shown.

Statistical Analysis

Each experiment in which progesterone measurements were made was repeated at least three times. Statistical analysis of the data were performed using ANOVA. The linear correlation coefficients were analyzed using Stat View SE system (Abacus Concepts, Berkeley, CA).

Acknowledgments

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