An arachidonic acid-preferring acyl-CoA synthetase is a hormone-dependent and obligatory protein in the signal transduction pathway of steroidogenic hormones

Fabiana Cornejo Maciel, Paula Maloberti, Isabel Neuman, Florencia Cano, Rocío Castilla, Fernanda Castillo, Cristina Paz and Ernesto J Podestá

Department of Biochemistry, School of Medicine, University of Buenos Aires, Paraguay 2155, 5th (C1121ABG) Buenos Aires, Argentina

Abstract

We have described that, in adrenal and Leydig cells, the hormonal regulation of free arachidonic acid (AA) concentration is mediated by the concerted action of two enzymes: an acyl-CoA thioesterase (MTE-I or ARTISt) and an acyl-CoA synthetase (ACS4). In this study we analyzed the potential regulation of these proteins by hormonal action in steroidogenic cells. We demonstrated that ACS4 is rapidly induced by adrenocorticotropin (ACTH) and cAMP in Y1 adrenocortical cells. The hormone and its second messenger increased ACS4 protein levels in a time and concentration dependent way. Maximal concentration of ACTH (10 mIU/ml) produced a significant effect after 15 min of treatment and exerted the highest increase (3-fold) after 30 min. Moreover, 35S-methionine incorporation showed that the increase in ACS4 protein levels is due to an increase in the de novo synthesis of the protein. On the contrary MTE-I protein levels in Y1 and MA-10 cells did not change after steroidogenic stimuli. In contrast with the effect observed on protein levels, stimulation of both cell lines did not change ACS4 RNA levels during the first hour of treatment, indicating that the effect of both stimuli is exerted at the level of ACS4 protein synthesis.

StAR protein induction has a key role on the activation of steroidogenesis since this protein increases the rate of the limiting step of the whole process. In agreement with the fact that the inhibition of ACS4 activity by triacsin C blocks cAMP-stimulated progesterone production by MA-10 Leydig cells, here we demonstrated that ACS4 inhibition also reduces StAR protein levels. Moreover, exogenous AA was able to overcome the effect of triacsin C on both events, StAR induction and steroidogenesis. These results were confirmed by experiments using ACS4-targeted siRNA which result in a reduction in both ACS4 and StAR protein levels. The concomitant decrease in steroid production was overcome by the addition of AA to the knocked-out cells. In summary, this study suggests that in adrenal and Leydig cells the hormonal action prompts the synthesis of a labile protein, ACS4, which activity is involved in the regulation of AA release and is essential for steroidogenesis and StAR protein induction.

Introduction

In the different steroidogenic tissues the appropriate trophic hormone activates the mitochondrial conversion of cholesterol to pregnenolone. This reaction is catalyzed by the side-chain splitting cytochrome P450 enzyme (P450 scc) located in the matrix side of the inner mitochondrial membrane. The access of free cholesterol to the active site of P450 scc, and not the enzymatic activity itself, is the true rate-limiting step in immediate steroidogenesis (Crivello & Jefcoate 1980, Privalle et al. 1983). Numerous reports indicate that this event is controlled by the steroidogenic acute regulatory protein (StAR protein) (Clark et al. 1994, Stocco & Clark 1996, Wang et al. 2000). In addition, it is well known that steroid biosynthesis requires de novo protein synthesis of a labile factor that has a short half-life, and the process is inhibited by cycloheximide (CHX) (Garren et al. 1965, Cooke et al. 1975). In this regard, the further characterization of StAR has revealed that this protein may be considered as the ‘labile’ factor that mediates the steroidogenic response to hormone action in adrenal and Leydig cells, as well as in other steroidogenic systems studied to date. Thus, StAR is rapidly synthesized in response to the respective trophic hormones, it is cycloheximide-sensitive, its active form has a very short half-life and it rapidly increases the transport of cholesterol to the site of P450 scc action (Stocco & Clark 1996, Reinhart et al. 1999).

In adrenal and Leydig cells, the increase of 3′, 5′-cyclic-adenosine monophosphate (cAMP) levels and cAMP-dependent protein kinase (PKA) phosphorylation events are accepted as intermediate steps in the response of the adrenocorticotropin (ACTH) and luteinizing hormone (LH) action (Dufau et al. 1977, Sala et al. 1979). It is also accepted that cAMP and PKA regulate arachidonic acid...
(AA) release. Following LH stimulation, AA is released within one minute in rat testicular Leydig cells (Dix et al. 1984, Didolokar & Sundaram 1987, Cooke et al. 1991). Also, AA release occurs in a dose and time dependent manner in human chorionic gonadotropin (hCG)-stimulated Leydig (Moraga et al. 1997) and ACTH-stimulated adrenal cells (Dada et al. 1996). In addition, previous studies have reported that inhibition of AA release abrogates the effect of LH- (Mele et al. 1997) and ACTH-stimulated steroid production (Solano et al. 1988). Recently, we have presented results suggesting that in steroidogenic tissues the release of AA involves the action of two enzymes, an acyl-CoA synthetase and an acyl-CoA thioesterase (Finkielstein et al. 1998, Maloberti et al. 2002). The acyl-CoA thioesterase, MTE-I (Svensson et al. 1998) or ARTISt (Finkielstein et al. 1998), is a mitochondrial phosphoprotein included in a family of acyl-CoA thioesterases that displays preferential activity towards very long chain acyl-CoA (Svensson et al. 1998). The protein was first identified by its capacity to increase mitochondrial steroidogenesis in a cell-free assay (Paz et al. 1994). It is present in adrenal and testicular Leydig cells, and ovary, brain and placenta among other tissues (Finkielstein et al. 1996), and its activity would be regulated by ACTH (Maloberti et al. 2002).

The second enzyme involved in AA release in steroidogenic tissues, the acyl-CoA synthetase, is an enzyme designed ACS4 that belongs to a five-member family. ACS4 shares 68% of its amino acid sequence with ACS3, another member of this family, although this sequence is poorly related to the other family members (Kang et al. 1997). The purified enzyme utilizes arachidonate as substrate more preferentially among other C8-C22 saturated fatty acids and C4-C22 unsaturated fatty acids. The striking feature of ACS4 is its abundance in steroidogenic tissues, especially adrenal gland and ovary. ACS4 immunoreactivity was detected in the zona fasciculata (ZF) and reticularis of the adrenal cortex, in the corpus luteum and stromal luteinized cells of the ovary and in the Leydig cells of the testis (Kang et al. 1997).

Given that AA release is linked to hormone-dependent StAR induction and steroidogenesis (Wang et al. 2000), the present study was undertaken to investigate whether AA release in steroidogenic tissues by hormone action involves the regulation of the levels of the acyl-CoA synthetase and/or thioesterase.

In the present study the results demonstrate that hormone treatment of adrenal and Leydig cells does not modify MTE-I protein levels while it induces ACS4 protein. This enzyme appears rapidly in response to the respective trophic hormone and seems to have a short half-life. The data are consistent with the presence of a new factor, ACS4, which is essential for StAR induction and steroidogenesis.

Materials and methods

Materials

ACTH was a kind gift of Elea Laboratories (Buenos Aires, Argentina). Dexamethasone (9-fluoro-11β, 17,21-trihydroxy-16α-methyl-pregna-1,4-diene-3,20-dione) was a kind gift from Ciba Geigy (Basel, Switzerland). Media, sera, antibodies, Trypsin-EDTA, TriZol reagent and Lipofectamine 2000 were from Gibco-Life Technologies (Carlsbad, CA, USA); plastic flasks and dishes were provided by Corning-Costar (Corning, NY, USA). Acrylamide, bis-acrylamide, cycloheximide (CHX), 8Br-cyclic-adenosine-monophosphate (8Br-cAMP), arachidonic acid (AA), trypsin from bovine pancreas, soybean trypsin inhibitor, agarose, formaldehyde, bovine serum albumin (BSA) and fatty acid-free BSA were obtained from Sigma-Aldrich Fine Chemicals (St Louis, MO, USA). Triacsin C was bought from ICN (Aurora, OH, USA). Polyclonal antibodies against MTE-I were previously obtained at our laboratory (Maloberti et al. 2002). Anti-ACS4 antibodies were obtained from immunizing rabbits with recombinant ACS4 protein. The protein was obtained in E.coli (BL21) transfected with a plasmid (pGEX-4T3) containing ACS4 sequence, kindly provided by Dr T Yamamoto (University of Tohoku, Sendai, Japan). In Y1 and MA-10 cells, Western blot analysis utilizing this antiserum results in only one band of the expected molecular weight (74 kDa), which is not detected by pre-immune serum or when the antiserum is pre-adsorbed with the immunogen. Moreover, its specificity is demonstrated by the fact that in adult cardiac tissue, where ACS4 is not expressed, the antibody does not reveal any band, although this tissue expresses ACS3, another synthetase isoform. Anti-StAR antibody was generously provided by Dr Douglas Stocco (Texas Tech University, Lubbock, Texas, USA). Anti-β-tubulin monoclonal antibody was bought from Upstate (Lake Placid, NY, USA). Electrophoresis supplies, polyvinylidendifluoride membrane and secondary antibody (horseradish peroxidase conjugated goat antibody) were bought to Bio-Rad Laboratories Inc. (Hercules, CA, USA). ECL kit and Hybond N+ membrane were provided by Amersham Pharmacia Biotech (Buckinghamshire, UK). All other chemicals were commercial products of the highest grade available.

Animals

The use of animals complies with the guidelines approved by the Animal Welfare and Health Committee of the University of Buenos Aires. Male Wistar rats (90-day-old) were used throughout. Adrenal gland or ZF cells were obtained from animals supplied with dexamethasone (10 µg/ml, ad libitum) in the drinking water for 16 h before they were killed, as previously...
described (Neher et al. 1982). Animals were killed by decapitation and adrenal glands were excised and kept on ice.

Adrenal in vivo stimulation

Following dexamethasone treatment, animals were injected s.c. with 18 IU ACTH per kg body weight and killed at the indicated times. Adrenal glands were removed, zona glomerulosa (ZG) and ZF were obtained as described (Solano et al. 1988) and homogenized in 270 mM mannitol, 10 mM Tris pH 7·4 containing a cocktail of inhibitors (100 mM NaF, 1 µg/ml pepstatin, 200 µM leupeptin, 2 µg/ml aprotinin, 2 mM phenylmethyl-sulfonyl fluoride (PMSF)). Cellular proteins of the homogenates were analyzed by Western blot. Steroid production was measured in the peripheral blood in order to determine the efficiency of ACTH treatment (Neher et al. 1982).

Cell preparation and stimulation

Adrenal ZF cells were obtained by trypsin digestion, following published procedures (Solano et al. 1988). ZF cells were suspended in Krebs-Ringer-Bicarbonate buffer, pH 7·4, containing 10 mM glucose and 0·5% BSA at a final concentration of 10^5 cells/ml and they were maintained throughout under carbogen (95% O_2; 5% CO_2) atmosphere. Adrenal ZF cells were incubated with 1 mM 8Br-cAMP for 15, 30 and 60 min at 37°C. Following dexamethasone treatment, animals were killed by cervical dislocation and adrenal glands were excised and kept on ice. The samples were then homogenized in a buffer consisting of 0·4 ml in the following buffer 10 mM Tris–HCl pH 7·4, 5 mM EDTA, 5 mM MgCl_2, 200 mM sucrose, 0·5% Triton-X 100 and the cocktail of inhibitors described above. The suspension was incubated on ice for 10 min, vortexed for 1 min and centrifuged at 11 000 g for 4 min. The supernatant (total lysate) was kept to analyze cellular proteins by Western blot.

35S-Methionine incorporation and ACS4 immunoprecipitation

Y1 cell cultures were incubated with the inclusion of 400 µCi/ml 35S-methionine (specific activity 1175 Ci/mmol; New England Nuclear, Perkin-Elmer, Boston, MA, USA) for 45 min. Following this pre-incubation period, the cells were washed with PBS and scraped into a buffer containing 35 mM Tris pH 7·4, 5 mM EDTA, 5 mM MgCl_2, 200 mM sucrose, 0·5% Triton-X 100 and the cocktail of inhibitors described above. The suspension was incubated on ice for 10 min, vortexed for 1 min and centrifuged at 12 000 g for 4 min. The supematant (total lysate) was kept to analyze cellular proteins by Western blot.

Western blot analysis

Equal amounts of protein were separated by SDS-PAGE (10% acrylamide), and dried gels exposed to autoradiography films to detect 35S-methionine incorporated into newly synthesized ACS4.
were visualized by enhanced chemiluminescence. In all Western blots, detection of β-tubulin was used as loading control.

Northern blot analysis

For Northern blot analysis, total RNA was isolated from Y1 and MA-10 cells by the guanidinium isothiocyanate method using TriZol reagent, according to the manufacturer protocol. Total RNA (24 µg) was separated by electrophoresis on 1.5% agarose gels and blotted onto Hybond N+ membranes. ACS4 mRNA was detected using a specific 32P-labeled cDNA probe (Kang et al. 1997). The levels of 28S RNA were also detected and used as loading control.

siRNA transfection

siRNAs targeting ACS4 and MTE-I coding sequences were custom-designed by Dharmacon (Lafayette, CO, USA). One day before transfection, Y1 cells (5 × 10⁵ cells/well) were grown up to 80% confluency onto 24-well plates. Transfection was performed using siRNA (530 nM) with Opti-MEM medium and 2 µl Lipofectamine 2000 reagent according to the instructions of the manufacturer. Cells were placed into normal
culture medium for 6 h after transfection and further grown for 48 h. Y1 cells were stimulated with 5 mIU/ml ACTH in culture medium containing 0-1% fatty acid-free BSA, in the presence or absence of 0-3 mM AA. Following treatments, media was kept to determine steroid production by radioimmunoassay.

**Protein determination**

Protein concentrations were determined by Bradford assay, using BSA as standard (Bradford 1976).

**Statistics**

Results are shown as the mean ± S.D. Unless otherwise indicated, statistical significance was evaluated using ANOVA followed by Tukey test. P<0.05 was considered significant.

**Results**

Given that ACTH regulates intracellular concentration of AA in adrenal cells and that ACS4 and MTE-I are enzymes involved in AA release, the first experiments were performed in order to determine whether ACTH has any effect on ACS4 and MTE-I protein levels. With this purpose, we analyzed the levels of both proteins in adrenal glands from animals stimulated with a single dose of ACTH for 30 min. As shown in Fig. 1, Western blot analysis revealed an increase in ACS4 protein levels in both ZG (Fig. 1, panels A and D) and ZF (Fig. 1, panels B and D). In addition, when isolated rat adrenal ZF cells were stimulated _in vitro_ with 8Br-cAMP (15–60 min), we also observed an increase in the protein (Fig. 1, panels C and D). In contrast, _in vivo_ ACTH treatment nor _in vitro_ 8Br-cAMP incubation modifies the levels of MTE-I (Fig. 1).

In order to further characterize the effect of hormone action on ACS4 levels, we used two well recognized steroidogenic cell lines, adrenocortical Y1 and Leydig MA10 cells. The levels of ACS4 and MTE-I were analyzed in Y1 cells stimulated with different concentrations of ACTH for different periods of time. None of the concentrations or times tested (1–10 mIU/ml, 15–45 min) proved to modify the intracellular content of MTE-I (data not shown). However, ACTH produces an

---

**Figure 2** Effect of ACTH stimulation of Y1 cells on ACS4 and MTE-I levels. Adrenocortical Y1 cells were stimulated with ACTH (doses and times stated in the figure). Cellular proteins were subjected to SDS-PAGE and Western blot analysis was performed using anti-ACS4 antibodies. Specific bands were detected by enhanced chemiluminescence. (A) Western blot representative of three independent experiments. (B) Quantitative representation of three independent Western blots. Intensity of the specific bands was quantified and results are expressed in arbitrary units as mean±S.D. *P<0.1 and ***P<0.001 vs non treated cells. Inset, representative autoradiography of 35S-Methionine incorporated into ACS4 by Y1 cells. After 35S-Methionine incorporation and ACTH stimulation, ACS4 protein was immunoprecipitated using a specific antibody and the presence of 35S-Methionine-labeled ACS4 was analyzed by SDS-PAGE and autoradiography, as described in Materials and methods.
increase of ACS4 levels evaluated by Western blot. Analysis of the intensity of ACS4 signal demonstrated that ACTH (10 mIU/ml) induces ACS4 levels to rise after 15 min (Fig. 2) and 5 mIU/ml of ACTH is able to induce ACS4 protein after 45 min (Fig. 2). Maximal induction by ACTH (approximately 3-fold) was reached using 10 mIU/ml during 30 min and remained constant thereafter. The observed effect of the hormone on ACS4 protein levels could be due to an action on the rate of synthesis of the protein or on the rate of its degradation. Then, the effect of ACTH on ACS4 induction was also evaluated by 35S-Met incorporation and immunoprecipitation. As shown in the inset of Fig. 2, ACTH increases the incorporation of the radioactive amino acid into ACS4. To confirm this result, we next studied the effect of CHX on ACS4 levels. For this purpose, Y1 adrenal cells were incubated with or without 10 µg/ml of CHX during 30 min before ACTH stimulus (5–60 min). Fig. 3, panel A shows that ACTH induces ACS4 levels as early as 5 min, while there is no effect on MTE-I protein levels. CHX treatment reduces ACTH-induced ACS4 protein levels at all times studied (Fig. 3, panels A and B). Notwithstanding a possible effect of the hormone on protein degradation, these results indicate that ACTH affects, at least in part, ACS4 synthesis. Basal ACS4 levels decreased rapidly after serum deprivation and this decrease is more evident after CHX treatment (Fig. 3, panels C and D).

The next series of experiments was carried out in order to analyze whether the hormonal regulation of ACS4 protein levels is also observed with 8Br-cAMP in Y1 and in MA-10 Leydig cells, since in MA-10 cells the participation of AA as modulator of the hormone- and cAMP-induced StAR protein levels and steroidogenesis is well recognized (Wang et al. 2000). The results shown in Fig. 4 indicate that hormone-mediated induction of ACS4 is a cAMP-dependent process, since in both cell lines the effect is reproduced by 8Br-cAMP, a permeable analog of the second messenger cAMP. As expected, StAR protein is induced by 8Br-cAMP treatment (Fig. 4, panel A), while there is no action of the second messenger on MTE-I levels (Fig. 4, panel C).

In order to investigate whether the acute effect of hormone on ACS4 protein levels is due to an increase in protein or in RNA synthesis, we studied the effect of hormone treatment on ACS4 RNA abundance in Y1 (Fig. 5, panel A) and MA-10 cells (Fig. 5, panel B). In contrast with the effect observed on protein levels, stimulation of both cell lines did not change ACS4 RNA levels during the first hour of treatment. The effect on mRNA levels could only be observed after 1·5 and 6 h of
treatment in Y1 and MA-10 cells respectively, indicating that short term effect of hormone treatment on protein levels is due to an increase in mRNA translation and not in mRNA synthesis.

Previously, we have demonstrated the role of ACS4 in steroidogenesis showing that inhibition of ACS4 activity using triacsin C results in an inhibition of steroidogenesis. Triacsin C acts as an inhibitor of Acyl-CoA synthetases, with a preferential effect on arachidonoyl-CoA synthetases in intact cells (Tomoda et al. 1987, Hartman et al. 1989). We also showed that the hormonal regulation of steroid synthesis needs the concerted action of ACS4 and MTE-I (Maloberti et al. 2002). In addition, it is also known that AA regulates StAR induction and steroidogenesis (Wang et al. 2000). Thus, in order to confirm that the inhibition of ACS4 activity leads to a reduction of the substrate of MTE-I and therefore to the inhibition of AA release, StAR induction and steroidogenesis, we next studied the ability of exogenous AA to overcome the inhibition exerted by triacsin C. For that purpose, MA10 cells were stimulated with 8Br-cAMP in the presence and absence of triacsin C and AA. As shown in Fig. 6A, triacsin C decreases progesterone production stimulated by 8Br-cAMP. The addition of AA into the culture medium reverses the inhibitory effect of triacsin C (Fig. 6, panel A). Moreover, triacsin C reduces StAR protein induction by 8Br-cAMP, an effect that is also overcome by the addition of AA (Fig. 6, panel B). As expected triacsin C treatment does not affect ACS4 protein levels (Fig. 6, panel B). Even when ACS4 is present, its action is inhibited by this compound. Then, StAR protein is not induced and steroidogenesis is reduced. Regarding the effect of AA on CHX action, exogenous AA was not capable of rescuing steroid biosynthesis inhibited by CHX (8Br-cAMP, 8·5 ± 0·8; 8Br-cAMP+CHX, 0·6 ± 0·1; 8Br-cAMP+CHX+AA, 1·3 ± 0·2 ng progesterone/ml), expectable result given that protein synthesis is needed for StAR protein induction. Similar results were obtained when Y1 adrenocortical cells were used as a steroidogenic system (data not shown).

In order to confirm the obligatory role of ACS4 on steroidogenesis, we knocked out its expression in Y1 cells by using a siRNA approach. Transfection with siRNA against ACS4 produced a marked reduction of acyl-CoA synthetase levels as assayed by Western blot (Fig. 7, panel A). The reduction of ACS4 levels by siRNA transfection concomitantly reduced the levels of ACTH-induced StAR protein levels (Fig. 7, panel A). These results are similar to those obtained by the

![Figure 4 Effect of 8Br-cAMP on ACS4 protein levels. Y1 cells (A and B) and MA-10 cells (C and D) stimulated with 1 mM 8Br-cAMP during 60 min. Cellular proteins were subjected to SDS-PAGE and Western blot analysis using an anti-ACS4 antibody. After stripping, the membranes were reprobed using an antibody against STAR (A) or MTE-I (C). Specific bands were detected by enhanced chemiluminescence. (A and C) Representative Western blot of three independent experiments. (B and D) Densitometry quantitation of three independent Western blots. Intensity of ACS4 specific bands was quantified and results are expressed in arbitrary units as mean±S.D. a, P<0·001 vs non-treated cells by Student’s t-test.](www.endocrinology-journals.org)
inhibition of ACS4 activity by triacsin C (Fig. 6). Also, progesterone production by Y1 cells transfected with siRNA against ACS4 was inhibited when compared with control cells (Fig. 6, panel B). Based on the fact that ACS4 and MTE-I act together on AA release, silencing the expression of MTE-I should also result in inhibition of hormone-induced steroid synthesis. siRNA duplexes directed against MTE-I were also produced for transfection of Y1 cells. siRNA transfection resulted in a marked reduction of MTE-I and StAR protein levels and steroidogenesis as detected by Western blot and RIA respectively (Fig. 7, panels A and B). The concerted action of ACS4 and MTE-I on AA release is firmly supported by the experiments showing that addition of exogenous AA blunted the inhibition of progesterone production produced by both siRNAs (Fig. 7, panel B). As expected, since AA facilitates cholesterol accessibility to the inner mitochondrial membrane, steroid synthesis was not affected by ACS4 or MTE-I siRNA transfection when stimulated by 22(R)-OH-cholesterol, a permeable analog of cholesterol (Fig. 7, panel B inset).

Discussion

The inhibitory effect of cycloheximide on the activation of steroidogenesis has been known for many years (Garren et al. 1965, Cooke et al. 1975) and the kinetics of the inhibition has led to the hypothesis that a labile protein is required for hormone action (Garren et al. 1965, 1971).

The rate limiting step in steroidogenesis is the delivery of the substrate cholesterol to the inner mitochondrial membrane where P450 scc is located. A fundamental observation was that this regulation had an absolute requirement for protein synthesis. The role of the newly synthesized protein[s] would be to mediate the transfer of cholesterol from the outer mitochondrial membrane through the aqueous intermediate space.

Recently, we published results supporting the regulation of AA release and hormone-induced steroid synthesis by the concerted action of ACS4 and MTE-I (Maloberti et al. 2002). In the present report we describe the hormonal regulation of ACS4 protein levels. Interestingly, the activity of this protein appears to be
Figure 7 Effect of ACS4 and MTE-I siRNA on protein levels and steroid production. Y1 cells were transfected with siRNA targeted against ACS4, MTE-I or a scramble siRNA, as described in Materials and methods. (A) Representative Western blot of three independent experiments. Cellular proteins were subjected to SDS-PAGE and Western blot analysis using anti-ACS4, MTE-I, StAR and β-tubulin antibodies sequentially. Specific bands were detected by enhanced chemiluminescence. (B) Progesterone production after 60 min of treatment. Y1 cells transfected with scramble (open bars), ACS4 (light grey bars) or MTE-I (dark grey bars) siRNA were incubated in the presence or absence of 5 mIU/ml ACTH and with or without 0.3 mM AA. Inset: Progesterone production by Y1 cells incubated for 60 min in the presence of 5 µM 22R(OH)-cholesterol. Results are expressed as the mean±S.D. of one representative experiment performed in triplicate. a: P<0.01 vs. scramble siRNA-transfected+ACTH; b: P<0.05 vs. ACS4 siRNA-transfected+ACTH; c: P<0.01 vs. MTE-I siRNA-transfected+ACTH.
essential for StAR induction. The present data led us to propose that hormone stimulation of AA release, StAR induction and steroid production through the cAMP-dependent phosphorylation involves the new synthesis of ACS4 as an early step.

The data supporting this hypothesis are: i) the inhibition of protein synthesis decreases basal levels of ACS4 rapidly, ii) ACS4 is rapidly induced by hormones through a cAMP-dependent process in vivo and in vitro (ZF and ZG of adrenal tissue, ZF cells, Y1 adrenal cells and MA10 Leydig cells), iii) the acute increase in protein levels seems to be due to an increase in protein synthesis rather than to a decrease in protein degradation or an increase in mRNA levels, iv) exogenous AA is capable of restoring StAR induction and steroidogenic activity of cells treated with the ACS4 activity inhibitor triacsin C, v) ACS4-targeted siRNA reduces both ACS4 and StAR protein levels, and vi) ACS4-targeted siRNA results also in a decreased steroid production, an effect that is reversed by exogenous arachidonic acid.

According to this model, ACS4 induction should be an early event in hormone action. Here we demonstrate a rapid action (5 min) of hormone treatment on ACS4 induction. This protein is newly synthesized since 30 min of incubation with the protein-synthesis inhibitor prior to hormone treatment is sufficient to abolish the induction of ACS4. These results are confirmed by the 35S-methionine incorporation experiments. Moreover, in the presence of CHX, both basal and stimulated protein levels decrease rapidly, suggesting that ACS4 is a high turnover protein. The acute effect of the hormone is at the level of protein synthesis, since mRNA levels can be stimulated only after a longer time of stimulation.

The results in the present report suggest a hormone regulation of arachidonoyl-CoA (AA-CoA) levels through the control of ACS4 induction. ACS4 characteristically being a protein with a high turnover is in agreement with the fact that fatty acyl-CoA esters need a tight control of their intracellular concentrations since they are important intermediates in lipid metabolism and signal molecules, being strong detergents at the same time.

On the other hand, the induction of the acyl-CoA thioesterase MTE-I appears not to be controlled by hormone treatment. Previous observations showed that ACTH stimulates MTE-I activity in Y1 cells (Maloberti et al. 2002). The thioesterase activity can be regulated by PKA-dependent phosphorylation (Maloberti et al. 2000), however, it also requires an acyl-CoA pool as a source of AA. Therefore we cannot rule out a possible activation of the enzyme by a hormone-increased availability of its substrate, concept supported by the fact that ACS4 is regulated by hormone treatment. The obligatory role of MTE-I could be demonstrated by the use of siRNA, showing that reduction of the expression of MTE-I is accompanied by reduction in steroid synthesis, an effect that can be reversed by addition of AA.

The role of ACS4 in steroidogenesis was previously demonstrated by the fact that an inhibitor of the enzyme activity reduces steroid production. This is further supported by the results obtained in knock out experiments using siRNA showing that a reduction in the levels of ACS4 reduces steroid synthesis. Moreover, here we also demonstrate that AA can restore steroidogenesis when the activity of ACS4 is inhibited by triacsin C or when ACS4 expression is inhibited by siRNA. The results of the present study indicate that ACS4 induction and AA release are essential for steroid production and StAR protein expression. Since StAR protein was demonstrated to play a critical role in the cholesterol transfer to the mitochondrial inner membrane, our results are in agreement with earlier experiments suggesting that AA regulates steroidogenesis at the rate-limiting step of mitochondrial cholesterol transfer (Wang et al. 2000).

It has been suggested that the intracellular concentration of unbound acyl-CoA esters is tightly controlled by the presence of specific acyl-CoA binding proteins (ACBP) and acyl-CoA thioesterases (Faergeman & Knudsen 1997, Knudsen et al. 2000). In such a cellular environment, transport by diffusion of unbound acyl-CoA esters is very unlikely, suggesting that supply of substrate to acyl-CoA-consuming enzymes depends on direct transfer from acyl-CoA synthetases, or relies on ACBPs or carrier proteins. In this regard, it is known that an acyl-CoA binding protein known also as DBI (diazepam binding inhibitor) is expressed in high concentration in specialized cells such as steroid producing cells of the adrenal cortex and testis (Papadopoulos 1993). Thus it is possible that after the hormone induction of the acyl-CoA synthetase, the AA-CoA binds to DBI, which in turn binds to the peripheral benzodiazepine receptor (PBR) located in the outer mitochondrial membrane (Papadopoulos 1993). This will possibly lead to a facilitated transfer of AA-CoA to the mitochondria, with the consequent availability of the substrate for the thioesterase at its site of action. Although the role of AA in StAR induction and steroidogenesis is well recognized, we cannot rule out a direct effect of AA on cholesterol transfer. Cholesterol binding to P450 scc in lipid vesicles is greatly potentiated when the local membrane is rendered more fluid by the addition of free fatty acids (Dhariwal & Jefcoate 1989). The increase in membrane fluidity in the presence of fatty acids possibly favors the interaction of cholesterol with PBR, StAR or P450 scc (Dhariwal & Jefcoate 1989, Petrescu et al. 2001, Jefcoate 2002). Further studies need to be performed to elucidate this process.

Taken together our current data indicate the presence of a new hormone-dependent labile protein in steroidogenic tissues (ACS4) essential for AA release,
StAR induction and steroidogenesis. This study further supports the new concept in the regulation of intracellular distribution of AA through a mechanism different from the classical phospholipase A2-mediated pathway that involves the activity of a hormone-induced acyl-CoA synthetase and a hormone-regulated acyl-CoA thioesterase.

Acknowledgements

This work was supported by grants from University of Buenos Aires (UBA) to EJP (M064), CP (M059) and FCM (M079) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT) to EJP (PICT 6738). Thanks are also due to Carlos F Méndez for critical reading. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References


Dharwal MS & Jefcoate CR 1989 Cholesterol metabolism by purified cytochrome P-450 scc is highly stimulated by octyl cycloheximide effect on adrenal steroidogenesis. Endocrine Research 22 533–539.


Papadopoulos V 1993 Peripheral-type benzodiazepine/diazepam binding inhibitor receptor biological role in steroidogenic cell function. Endocrine Reviews 14 222–240.


Stocco DM & Clark BJ 1996 Regulation of the acute production of steroids in steroidogenic cells. *Endocrine Reviews* **17** 221–244.


Received 16 March 2005
Accepted 18 March 2005
Made available online as an Accepted Preprint 31 March 2005