Induction of mitochondrial proteins in MA-10 Leydig tumour cells with human choriogonadotropin

Douglas M. STOCCO and Michael W. KILGORE

Department of Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX 79430, U.S.A.

The side-chain cleavage of cholesterol is the rate-limiting enzymic step in steroidogenesis and occurs in the mitochondria of steroid-producing tissues. In these studies, the effect of acute stimulation of MA-10 Leydig-tumour cells on the synthesis of mitochondrial proteins was investigated. Cells were incubated in the presence of stimulating levels of human choriogonadotropin (hCG) and [³⁵S]methionine for 2 h periods. Mitochondria were isolated and their proteins analysed by two-dimensional polyacrylamide-gel electrophoresis and fluorography. At least three mitochondrial-specific proteins were found in cells exposed to gonadotropin, and these proteins were also found in cells treated with dibutyryl cyclic AMP (db cyclic AMP). The appearance of these proteins was prevented by the addition of cycloheximide to the cells, as was the production of progesterone, the major steroid produced in MA-10 cells. In addition, mitochondria isolated from cells stimulated with db cyclic AMP produced progesterone at a rate 3-fold greater than mitochondria isolated from control cells during 3 h of incubation. Lastly, mixing experiments demonstrated that sonicated mitochondria isolated from db cyclic AMP-treated cells stimulated progesterone production in control mitochondria 6-fold. These studies show that hCG and db cyclic AMP stimulation of MA-10 cells results in the rapid induction of cycloheximide-sensitive proteins located in the mitochondria which may be instrumental in the acute regulation of steroidogenesis.

INTRODUCTION

The rate-limiting enzymic step in steroid synthesis in steroidogenic tissues is the side-chain cleavage of cholesterol to yield pregnenolone and isohexanoic acid [1–3]. This reaction is catalysed by a cholesterol side-chain-cleavage complex (CSCC) [4–6], which is located in the mitochondrial inner membrane and is regulated by tropic hormone in all steroidogenic tissues studied [7–11]. In addition to the CSCC, other factors have been postulated to play important roles in the stimulation of steroid synthesis in response to tropic hormone. These include, but may not be limited to, cyclic AMP-mediated phosphorylation–dephosphorylation processes [12], an increase in the availability of cholesterol due to the action of cholesterol ester hydrolases [13,14], increased transport of cholesterol to the mitochondria [15,16], the transport of free cholesterol into the mitochondria [17–20], access of this cholesterol to the CSCC [21–23] and direct activation of the CSCC by tropic hormone [16].

Regulation of steroid production is generally thought to include both acute (those occurring within minutes) and chronic (those occurring on the order of hours to days) events. Acute stimulation appears to be mediated by the rapid synthesis of new proteins, which are instrumental in the delivery of cholesterol to the mitochondria and may also function in the transfer of cholesterol from the outside of the mitochondria to the active site of the CSCC [24,25]. Chronic regulation is thought to result in the increased synthesis and subsequent accumulation of the CSCC components as well as other enzymes active in the steroidogenic pathway [26–28].

Acute stimulation of both rat adrenal and corpus-luteum cells with tropic hormone has been shown to result in the rapid synthesis of a 28 000-*M_r* protein which was speculated to play a role in the regulation of steroidogenesis [29–31]. The synthesis of this protein, as well as the production of steroid in these cells, was shown to be cycloheximide-sensitive. In addition, a cycloheximide-sensitive CSCC activator protein has been isolated from the adrenal cells of rats stimulated with corticotropin [32], and both an activator and an inhibitor protein have been purified from bovine adrenal glands [33]. Lastly, several studies have demonstrated that stimulation of Leydig cells with gonadotropin results in the stimulation of specific proteins which may have a role in the regulation of testosterone production [34,35]. In summary, it appears that tropic-hormone stimulation of steroidogenic tissues evokes the rapid synthesis of protein(s) that increase the production of steroids by as-yet-unidentified mechanisms.

Since the acute synthesis of steroids in steroidogenic tissue is known to require protein synthesis *de novo*, and since the rate-limiting step occurs in the mitochondria, we investigated the effects of acute gonadotropin stimulation of Leydig tumour cells on the synthesis of mitochondrial proteins. Previous studies have shown that stimulation of adrenal tumour cells [36], rat adrenal cells [37] and ovarian follicles [38] result in the synthesis of proteins located in the mitochondria. The present results demonstrate that the acute stimulation of a Leydig-tumour-cell line results in the induction of at least three proteins which are specifically located in the mitochondria. Some of the characteristics of these proteins will be discussed.

Abbreviations used: hCG, human choriogonadotropin; db cyclic AMP, dibutyryl cyclic AMP; CSCC, cholesterol side-chain-cleavage complex; PBS, phosphate-buffered saline (0.8% NaCl/0.02% KCl/0.115% Na₂HPO₄/0.02% KH₂PO₄, pH 7.3); DMEM, Dulbecco's minimal essential medium; r.i.a., radioimmunoassay; i.e.f., isoelectric focusing; LH, lutropin (luteinizing hormone); BSA, bovine serum albumin.

MATERIALS AND METHODS

Cell culture

The MA-10 cells used in these experiments were generously given by Dr. Mario Ascoli of The Population Council, Rockefeller University, and originally cloned from the solid M5480P mouse Leydig-cell tumour [39]. The cells were maintained in 75 cm² plastic flasks (Corning) in modified Waymouths MB752/1 medium containing 15% (v/v) horse serum and subcultured using standard techniques as described in [39]. For most experiments, cells were subcultured into 60 mm² plastic culture dishes (Corning) and grown for an additional 24 h in medium plus serum. At this time the cells were washed twice with PBS and the medium was replaced with medium containing 1 mg of BSA/ml in which the experiments were conducted. The culture dishes contained approx. 3×10^6 cells (approx. 800 μ g of protein) at the beginning of the experiments. Cells were maintained in a humidified atmosphere of air/CO₂ (19:1) and a temperature of 37 °C.

Leydig cells were freshly isolated from the testes of Sprague-Dawley rats weighing approx. 150 g (Charles River, Wilmington, MA, U.S.A.) employing Percoll gradients [40,41]. These cells were grown in DMEM (Gibco) for 24 h before the beginning of experiments and then in DMEM containing 1 mg of BSA/ml for the experimental period. After the various experiments (described in the Figure legends), both MA-10 and Leydig cells were washed several times with PBS and harvested from the dishes by scraping with a rubber policeman.

Total cellular protein synthesis

Incorporation of [³⁵S]methionine (1100 Ci/mmol; New England Nuclear Corp.) into acid-precipitable material was determined by adding the isotope (10 μ Ci/ml) to cells and, at specific time intervals, removing the medium, washing the cells with PBS and precipitating the contents of the dishes with cold 10% (w/v) trichloroacetic acid. Precipitates were then collected on Millipore filters, washed with several volumes of cold 5% (w/v) trichloroacetic acid, dried, and assayed for radioactivity using Omnifluor (New England Nuclear Corp.) scintillation fluid.

Progesterone production in intact cells

MA-10 cells were grown in culture dishes and stimulated with either hCG or db cyclic AMP. At specific time intervals, samples of the medium were removed, centrifuged at 500 g for 10 min, and assayed for progesterone content by r.i.a.

Two-dimensional polyacrylamide-gel electrophoresis of proteins

For these studies, maximally stimulating doses of hCG (100 ng/ml) or db cyclic AMP (1 mM) (Sigma) were added to the cultures. Immediately after the addition of hormone, [³⁵S]methionine (50–100 μ Ci/ml depending on the experiment) was also added to the dishes. Incubation of the cells was continued for an additional 2 h at 37 °C. Cells were then washed with PBS and harvested by scraping them off. Cells were homogenized in 2 ml of 0.25 M-sucrose/0.01 M-Tris/0.1 mM-EDTA, pH 7.4, using a Potter-Elvehjem motor-driven glass homogenizer with a serrated Teflon pestle. Homogenates were centrifuged

twice at 600 g for 15 min, the supernatant being retained on each occasion. The supernatant was then centrifuged at 13000 g for 15 min to pellet the mitochondrial fraction. The postmitochondrial supernatant was retained, and small portions were removed, precipitated in 10% (w/v) trichloroacetic acid, and assayed for radioactivity and protein [42], with BSA as a standard. The supernatants were then treated with an equal volume of 2 $\times$ i.e.f. buffer [43]. Mitochondrial pellets were resuspended in sucrose buffer, and portions of the mitochondrial suspension were also removed, precipitated in trichloroacetic acid and analysed for protein content and radioactivity. Mitochondrial suspensions were centrifuged again and the pellets solubilized in i.e.f. buffer. Two-dimensional gel electrophoresis was performed on mitochondrial and postmitochondrial supernatant samples containing equal amounts of [³⁵S]methionine-labelled proteins using standard techniques [43,44], with the exception that i.e.f. gels were equilibrated with SDS-containing buffer while they were attached to the top of the second-dimension gel. The total radioactivity and protein applied to the i.e.f. gels varied from experiment to experiment, but were in the range of $(2-5) \times 10^5$ d.p.m. and 75–100 μ g of mitochondrial protein respectively. At the conclusion of the electrophoresis, gels were either silver-stained [45] or processed for fluorography [46] using En³Hance (New England Nuclear Corp). Gels were silver-stained with Gelcode (Health Products, South Haven, MI, U.S.A.) to provide a means of checking the protein profiles to ensure consistency as well as to provide a series of M_r markers for the fluorograms. Gels treated with En³Hance were dried with heat under vacuum and exposed to X-ray film at –70 °C for the appropriate length of time. The X-ray films were developed and photographed.

Glucose-6-phosphatase and 5'-nucleotidase enzyme assays

Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) was measured to give an indication of the amount of microsomal material in the various fractions [47], whereas 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) was assayed in order to determine the distribution of plasma-membrane fragments [48]. Enzyme activities were calculated as P_i liberated/30 min (nM) and expressed as a percentage of the total activity.

Progesterone production in isolated mitochondria

Cells were grown in 150 cm² culture flasks, and half of the cultures were stimulated with 1 mM db cyclic AMP for 3 h. At this time mitochondria were isolated from both stimulated and control cultures and resuspended in 2 ml of a buffer consisting of 200 mM-sucrose, 25 mM-Tris/HCl, 10 mM-potassium phosphate, 20 mM-KCl, 0.2 mM-EDTA, 1% BSA and 5 mM-sodium citrate. Mitochondria were incubated at 37 °C with shaking, and 0.5 ml aliquots were removed at 1 h intervals. Samples were centrifuged at 10000 g for 15 min; the supernatant was retained and its progesterone content measured by r.i.a.

Reconstitution experiments

MA-10 cells were treated with 1 mM-db cyclic AMP for 3 h, and mitochondria from both stimulated and control cultures were isolated. Mitochondrial pellets were resuspended in 1.0 ml of 0.01 M-Tris/0.25 M-

Fig. 1. $[^{35}\text{S}]$ Methionine-labelling of acid-precipitable material

Cells were grown in medium plus serum for 24 h and shifted to medium containing BSA (1 mg/ml). Cycloheximide was added to half the dishes and, after 10 min, hCG (100 ng/ml) was added to selected dishes and 1 mM-db cyclic AMP to others. $[^{35}\text{S}]$ Methionine (10 $\mu\text{Ci}/\text{ml}$) was then added to all the dishes, the incubation continued at 37 °C, samples taken at specific time intervals and the acid-insoluble fraction assayed for radioactivity. Each time point represents the mean for triplicate dishes $\pm$ S.D. $\circ$, Control; $\bullet$, control + cycloheximide; $\square$, hCG (100 ng/ml); $\blacksquare$, hCG (100 ng/ml) + cycloheximide; $\triangle$, 1 mM-db cyclic AMP; $\blacktriangle$, 1 mM-db cyclic AMP + cycloheximide.

sucrose/0.1 M-EDTA buffer, pH 7.4 and sonicated with a Teckmar sonic disruptor using a microtip. For these experiments, the 13000 g supernatants were also retained. The sonicated mitochondrial preparations and the 13000 g supernatants were treated twice with activated charcoal (50 mg/ml) to remove any steroids that might have been present. These fractions were then added to freshly isolated mitochondria, which were resuspended in the buffer described in the above subsection. The mixtures were incubated for 3 h at 37 °C with constant shaking and centrifuged at 20000 g for 15 min. The supernatants were retained and their progesterone contents analysed by r.i.a. Identical experiments were performed in the absence of sodium citrate. Also, sonicated mitochondria and postmitochondrial supernatants were incubated separately in complete incubation mixture, centrifuged, and the supernatants assayed for progesterone.

R.i.a.

R.i.a. of progesterone was performed as described in [49] directly on aliquots of the medium of control,

Fig. 2. Progesterone production in MA-10 cells

Half of the cell cultures were treated with cycloheximide for 10 min. At this time hCG (100 ng/ml) was added to some of the dishes and 1 mM-db cyclic AMP to others and the incubation continued at 37 °C. Aliquots of the medium were removed from all the dishes at the time intervals indicated and assayed for progesterone by r.i.a. Points shown are the means $\pm$ S.D. for triplicate dishes. $\circ$, Control; $\bullet$, control + cycloheximide; $\square$, hCG (100 ng/ml); $\blacksquare$, hCG (100 ng/ml) + cycloheximide; $\triangle$, 1 mM-db cyclic AMP; $\blacktriangle$, 1 mM-db cyclic AMP + cycloheximide.

hormone-treated and inhibitor-treated cells, as well as isolated mitochondria. Antibody to progesterone was obtained from Holly Hill Biologicals (Hillsboro, OR, U.S.A.). Analysis of the r.i.a. data was performed by using a program kindly provided by Dr. Reid Norman of the Department of Cell Biology and Anatomy of this Center.

RESULTS

MA-10-cell culture and stimulation

The MA-10 cell line used in the present studies has been shown to possess functional LH/hCG receptors and produces progesterone as the major steroid in response to gonadotropin stimulation [39]. The generation time of this cell line is approx. 18.5 h under the growth conditions employed in the present studies (results not shown). Dose-response curves were produced for both hCG and db cyclic AMP and indicated that maximal stimulation of progesterone production could be obtained at 100 ng of hCG/ml and 1 mM-db cyclic AMP (results not shown), and therefore these doses were used

Table 1. Distribution of 5'-nucleotidase and glucose-6-phosphatase in subcellular fractions

Fractionation of MA-10 cells was performed as described in the text and portions of each fraction were assayed for 5'-nucleotidase ($n = 4$) or glucose-6-phosphatase ($n = 2$). Enzyme activities were calculated as nm-P_i liberated/30 min and expressed as a percentage of the total activity. Abbreviation: N.D., not determined.

Enzyme	Fraction...	Activity (% of total)		
		600 g pellet	13000 g pellet	13000 g supernatant
5'-Nucleotidase		33.6	4.9	61.5
Glucose-6-phosphatase		N.D.	23.1	76.9

in all the experiments described. In addition, the progesterone production obtained with an hCG concentration of 100 ng/ml is identical with that obtained with the same dose of rat LH (NIADDK-rLH-1-6).

Protein synthesis and inhibition with cycloheximide

Incorporation of [³⁵S]methionine into total cellular protein demonstrated that there were no differences in protein synthesis in control, hCG- or db cyclic AMP-treated cells over a 90 min period (Fig. 1). It can also be seen that 0.2 mM-cycloheximide resulted in the inhibition of 95% of protein synthesis. Longer periods of hCG and db cyclic AMP stimulation of up to 24 h also failed to demonstrate a change in protein synthesis in subsequent pulses with [³⁵S]methionine (results not shown).

Progesterone production

Treatment of MA-10 cells with either hCG or db cyclic AMP resulted in the rapid and significant increase in progesterone production (Fig. 2). Incubation of the cells in cycloheximide for 10 min before the addition of hormone resulted in the total inhibition of progesterone production in these cultures.

Marker-enzyme studies

The results of the distribution of marker enzymes are shown in Table 1. It can be seen that the twice-washed mitochondrial pellet used in the present studies contains approx. 5% of the 5'-nucleotidase activity, indicating a relatively small contamination with plasma-membrane material and approx. 23% of the glucose-6-phosphatase activity, which indicates the presence of a significant amount of microsomal material.

Induction of mitochondrial proteins in MA-10 cells with hCG and db cyclic AMP

Two-dimensional polyacrylamide-gel electrophoresis profiles of mitochondrial proteins from control and cultures treated with 100 ng of hCG/ml for 2 h are shown in Fig. 3. The fluorograms clearly show the presence of at least three proteins in hCG-treated cells not found in control cultures. These proteins have estimated M_r values of between 25000 and 27000 and pI values between 5.8 and 6.0. These proteins were also shown to be synthesized in response to db cyclic AMP (Fig. 4). Fig. 5 illustrates that the appearance of these

Fig. 3. Induction of specific mitochondrial proteins with hCG

Some cultures were stimulated with hCG (100 ng/ml) and others were used as controls. [³⁵S]Methionine (100 μ Ci/ml) was added to all the dishes, which were incubated for a further 2 h at 37 °C. At the end of this time the cells were harvested and the mitochondria were isolated. Mitochondrial proteins were separated by two-dimensional gel electrophoresis and the gels were prepared for fluorography. After appropriate exposure times, X-ray films were developed and photographed. (a) Control; (b) hCG (100 ng/ml).

proteins is blocked by cycloheximide, indicating the involvement of protein synthesis *de novo* in this process. In Fig. 6 it can be seen that these induced proteins were not detected in the same region of the gels when samples of the postmitochondrial supernatant were subjected to two-dimensional gel electrophoresis. However, an increase in the level of several proteins with similar M_r values but different pI values was observed in the unstimulated postmitochondrial supernatant fraction.

Induction of mitochondrial proteins in rat Leydig cells with hCG

In another experiment, mitochondria were isolated from 24 h cultured rat Leydig cells after 2 h of concomitant hCG stimulation and [³⁵S]methionine labeling. Two-dimensional gel electrophoresis of the mitochondrial proteins resulted in the fluorograms shown in Fig. 7. Once again, the presence of mitochondrial proteins in the stimulated cells that are not present in the control cells can be clearly seen. These proteins are in the same

Fig. 4. Induction of specific mitochondrial proteins with db cyclic AMP

The conditions for this experiments were identical with those of Fig. 3 with the exception that 1 mM-db cyclic AMP, rather than hCG, was added to half the dishes. (a) Control; (b) 1 mM-db cyclic AMP. In this and succeeding two-dimensional electrophoretograms the layout is similar to Fig. 3 and the scales of pI and M_r are not repeated.

M_r and pI range as those found in MA-10 cells. However, it appears that there may be as many as six such proteins in the rat Leydig cells, whereas usually only three were found in MA-10 cells.

Steroid production in isolated MA-10-cell mitochondria

Fig. 8 illustrates that mitochondria isolated from MA-10 cells which had been stimulated with db cyclic AMP for 3 h were able to produce progesterone at a rate three times that of control mitochondria. The production of progesterone was found to be linear for approx. 2 h, slowing significantly after this time. The production of progesterone in isolated mitochondria was found to be dependent on the inclusion of sodium citrate or some other source of reducing equivalents in the incubation. It should be pointed out at this time that the measurement of progesterone in isolated mitochondria constitutes a valid measurement of steroid production in these organelles, since additional experiments (results not shown) have indicated that negligible amounts of pregnenolone accumulate under these conditions. This is in all probability due to the contamination with microsomal material (and thus the enzyme 3 β -hydroxy-

Fig. 5. Effect of cycloheximide on the synthesis of the induced mitochondrial proteins

Cells were radiolabelled with 100 μ Ci of [35 S]methionine/ml for 1 h. Cycloheximide was added to several dishes for 10 min and 1 mM-db cyclic AMP was added to cultures containing cycloheximide as well as several cultures not containing the inhibitor. Incubation was continued for an additional 2 h at 37 °C. Mitochondria were isolated and the proteins were analysed as described for Fig. 3. Only those sections of the gels containing the induced proteins are shown. (a) Control; (b) 1 mM-db cyclic AMP; (c) 1 mM-db cyclic AMP + cycloheximide.

steroid dehydrogenase) that occurs during the isolation procedure.

Reconstitution experiments

Reconstitution experiments indicated that progesterone production in control mitochondria was increased only in the presence of sonicated mitochondria isolated from db cyclic AMP-stimulated cells (Fig. 9). Sonicated mitochondria isolated from control cells or postmitochondrial supernatants from control or db cyclic AMP-treated cells had no stimulatory effect on progesterone production. Incubations in the absence of sodium citrate resulted in the production of negligible amounts of progesterone. Incubation of sonicated mitochondria and postmitochondrial supernatants separately resulted in no production of progesterone (results not

Fig. 6. Two-dimensional gel electrophoresis of postmitochondrial supernatant

Cells were radiolabelled with 100 μ Ci of [35 S]methionine/ml for 2 h at 37 $^{\circ}$ C in the presence or absence of hCG. Cells were fractionated as described in the text, and the postmitochondrial supernatant was retained, adjusted with i.e.f. buffer and samples containing equivalent radioactivities were analysed by two-dimensional electrophoresis. The arrows numbered 1–3 indicate the positions on the gels where the induced mitochondrial proteins appear, whereas those numbered 4–6 indicate proteins which appear to be present in larger amounts in control postmitochondrial supernatants. (a) Control; (b) hCG.

shown), indicating that the increase observed in the incubation containing sonicated mitochondria from db cyclic AMP-treated cells was not due to the sonicated preparation itself.

DISCUSSION

It has been proposed that, in all steroid-producing tissues, acute stimulation with tropic hormone results in the rapid synthesis of a protein or proteins which are involved in the stimulation of steroidogenesis. In addition to the requirement for rapid synthesis, it is also believed that this protein(s) has a short half-life. The evidence for this is derived mainly from protein-synthesis-inhibitor experiments employing cycloheximide [21,24,25,32,50] as

Fig. 7. Induction of specific mitochondrial proteins in rat Leydig cells with hCG

Rat Leydig cells were isolated on Percoll gradients and cultured for 24 h. Half the cultures were treated with hCG (100 ng/ml) and all the cultures received [35 S]methionine (100 μ Ci/ml). Incubation was continued for 2 h, at which time mitochondrial proteins were analysed as described for Fig. 3. (a) Control; (b) hCG (100 ng/ml).

well as reports describing the isolation of such proteins [32,33]. In the present paper we report the rapid synthesis of at least three proteins in MA-10 Leydig tumour cells in response to both gonadotropin and db cyclic AMP treatment. Their induction by db cyclic AMP would appear to indicate that the regulation of their synthesis occurs via classical phosphorylation pathways. A similar group of proteins was also found to be synthesized in freshly isolated cultured rat Leydig cells. Of particular interest is the finding that these proteins have been shown to be associated with the mitochondrial fraction of the cells. Since the marker-enzyme analyses indicated significant levels of microsomal contamination, it was necessary to analyse the postmitochondrial supernatant for the presence of these proteins. These analyses failed to show the presence of the induced proteins in a fraction containing three times the amount of microsomal material as found in the mitochondrial pellet. Our observation that postmitochondrial-supernatant proteins with similar M_r values but different pI values were found in larger amounts in unstimulated cultures, as shown in Fig. 6, cannot be explained at this time. It is possible that a relationship exists between these proteins and those shown to be induced by hormone stimulation, a situation that is similar to that described for adrenal and luteal cells [29–31]. In this regard it will be interesting to

Fig. 8. Progesterone production in isolated mitochondria

Cell cultures were stimulated with 1 mM-db cyclic AMP for 3 h. Mitochondria were isolated, resuspended in the buffer described in the Materials and methods section and incubated at 37 °C with shaking. Aliquots were taken at hourly intervals for a 3 h time period and centrifuged at 10000 *g* for 15 min. Supernatants were retained and analysed for progesterone by r.i.a. ●, Mitochondria isolated from control cells; Δ, mitochondria isolated from db cyclic AMP-treated cells.

determine if the induced proteins described here are phosphorylated.

The synthesis of the mitochondrial proteins described here, as well as the production of steroids, has been demonstrated to be completely sensitive to cycloheximide and is in complete agreement with observations in other steroidogenic cells [19,22,24,25,29–31,50]. Although the synthesis of these proteins in response to both hCG and db cyclic AMP was shown to occur within 2 h of stimulation, it is very possible that their synthesis begins more rapidly after stimulation, and shorter labelling periods with increased levels of isotope should aid in determining the time course of the appearance of these proteins.

The use of the MA-10-Leydig-tumour-cell line in these studies would seem justified by our findings that Leydig cells from intact animals also demonstrate the induction of similar mitochondrial-specific proteins in response to gonadotropin treatment. One of the major differences in this cell line appears to be the accumulation of progesterone rather than testosterone in response to hormone stimulation [39]. This difference would not appear to present any problems in the studies on the mechanism of regulation of cholesterol side-chain cleavage described here.

Fig. 9. Stimulation of progesterone production in control mitochondria

Mitochondria were isolated from control and 3 h db cyclic AMP-stimulated cells and sonicated. In addition, the postmitochondrial supernatant was also retained. The mitochondria were sonicated and all the fractions extracted with charcoal. Aliquots of sonicated mitochondria and supernatants were mixed with mitochondria that had been isolated from non-stimulated cells. Incubation of the mixtures was performed for 3 h at 37 °C with shaking, at which time the mitochondria were pelleted by centrifugation at 20000 *g* for 15 min and the supernatant assayed for progesterone content by r.i.a. Abbreviations used: 12K SUP, postmitochondrial supernatant; cAMP, dibutyryl cyclic AMP-treated cells; Mt. SON, sonicated mitochondrial preparation. ■, + citrate; □, – citrate.

The functions of the induced proteins described here are not yet known. It is tempting to speculate that they may have an integral role in the observed increase in progesterone production in MA-10 cells. The observation that mitochondria isolated from db cyclic AMP-treated cells synthesize progesterone at three times the rate that mitochondria isolated from control cells do is consistent with this contention. The results from the reconstitution experiments in which sonicated preparations of mitochondria isolated from db cyclic AMP-treated cells stimulated progesterone production 6-fold over those from unstimulated MA-10-cell mitochondria are also consistent with this idea. Nevertheless, it is clear that experiments employing these proteins in a more purified form will be necessary to prove their direct involvement in CSCC regulation. The stimulation of CSCC activity in isolated mitochondria by subcellular fractions isolated

from hormone-treated cells has previously been reported. In one of these studies, the stimulating factor appears to be cytosolic in origin [15], whereas in another an activator of adrenal CSCC activity, which appears to be mitochondrial in origin, has been implicated [52].

The proteins observed in the present study have characteristics which are very similar to proteins recently described in both the adrenals and the corpus luteum [29–31]. These proteins, termed 'i proteins', are found to be induced within minutes of ACTH and LH stimulation respectively, have an M_r value of 28000 and pI 6.5. In addition, the synthesis of protein i as well as steroid production in both of these tissues is completely sensitive to cycloheximide, which is in agreement with our present findings. Recent studies [31] have further characterized these proteins and indicate that they may arise from a tropic-hormone-induced co-translational phosphorylation of a similar protein in the cell (protein p_i). The cellular location of protein i was not determined in those studies, and it is possible that it may be mitochondrial in origin.

It is unlikely that the proteins described in the present study are the same as several other proteins which have been reported to result from tropic-hormone stimulation of steroidogenic cells. For example, sterol-carrier protein (SCP₂) has an M_r of 13500 [53], the activator protein described by Pederson & Brownie [32] has an M_r of only 2200, that described by Warne *et al.* [33] is less than 15000, cholesterol ester hydrolase has an M_r of 41000 [54], calmodulin has an M_r of 16790 [55] and, lastly, the components of the side-chain cleavage complex in the adrenal have M_r values of 49000 for the flavoprotein [56], 13000 for the iron-sulphur protein [57] and 48000 for the cytochrome *P*-450_{sec} [58]. A sterol-carrier protein with M_r 26500 and pI 5.6 has been described in rat liver [59], and its physical properties appear to be similar to those of the proteins reported here. At this time we have no evidence that any of the induced proteins observed in the present study have the capacity to bind cholesterol, and therefore a comparison with this protein is not yet possible.

Since earlier studies have indicated that the cholesterol content of the mitochondrial membrane can increase in response to hormone stimulation, even in the presence of cycloheximide [17,19,23], and that calcium-swollen adrenal mitochondria do not appear to require protein synthesis *de novo* for the stimulation of steroid production [50], it may be argued that the role of the induced protein(s) in steroidogenic cells is to bring about the delivery of cholesterol from the mitochondrial membranes to the active site of the cytochrome *P*-450_{sec}. If this is the case, one would clearly expect that such proteins would be associated in some way with the mitochondria. The data presented here are consistent with this model.

We acknowledge the technical assistance of Ms. Mary Shepherd Bowen. Thanks are due to Dr. C. W. Garner and Dr. L. R. Chaudhary for their helpful comments, and Mr. Harvey Olney for drawing the Figures. The support of National Institutes of Health grants HD 17481 and HD 00685 in these studies is also acknowledged.

REFERENCES

1. Stone, D. & Hechter, O. (1954) *Arch. Biochem. Biophys.* **51**, 457–469
2. Karaboyas, G. C. & Koritz, S. B. (1965) *Biochemistry* **4**, 462–468
3. Garren, L. D., Gill, G. N., Masui, H. & Walton, G. M. (1971) *Rec. Prog. Horm. Res.* **27**, 433–478
4. Simpson, E. R. (1979) *Mol. Cell. Endocrinol.* **13**, 213–217
5. Churchill, P. F. & Kimura, T. (1979) *J. Biol. Chem.* **254**, 10443–10448
6. Yago, N. & Ichii, S. (1969) *J. Biochem. (Tokyo)* **65**, 215–224
7. Haynes, R. C., Koritz, S. B. & Person, F. G. (1959) *J. Biol. Chem.* **234**, 1421–1423
8. Grahame-Smith, D. G., Butcher, R. W., Ney, R. L. & Sutherland, E. W. (1967) *J. Biol. Chem.* **242**, 5535–5541
9. Davis, W. W. & Garren, L. D. (1968) *J. Biol. Chem.* **243**, 5153–5157
10. Marsh, J. M. (1975) *Adv. Cyclic Nucleotide Res.* **6**, 137–199
11. Catt, K. J. & Dufau, M. L. (1978) *Receptors Horm. Action* **3**, 291–339
12. Koroscil, T. M. & Gallant, S. (1980) *J. Biol. Chem.* **255**, 6276–6283
13. Moyle, W. R., Jungas, R. L. & Greep, R. O. (1973) *Biochem. J.* **134**, 407–414
14. Moyle, W. R., Jungas, R. L. & Greep, R. O. (1973) *Biochem. J.* **134**, 415–424
15. Crivello, J. F. & Jefcoate, C. R. (1980) *J. Biol. Chem.* **255**, 8144–8151
16. Mori, M. & Marsh, J. M. (1982) *J. Biol. Chem.* **257**, 6178–6183
17. Vahouny, G. V., Dennis, P., Chanderbhan, R., Fiskum, G., Noland, B. J. & Scallen, T. J. (1984) *Biochem. Biophys. Res. Commun.* **122**, 509–515
18. Chanderbhan, R., Noland, B. J., Scallen, T. J. & Vahouny, G. V. (1982) *J. Biol. Chem.* **257**, 8928–8934
19. Privalle, C. T., Crivello, J. F. & Jefcoate, C. R. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 702–706
20. Coneely, O. M., Headon, D. R., Olson, C. D., Ungar, F. & Dempsey, M. E. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2970–2974
21. Mason, J. I., Arthur, J. R. & Boyd, G. S. (1978) *Biochem. J.* **173**, 1045–1051
22. Toaff, M. E., Strauss, J. F., Flickinger, G. L. & Shattil, S. J. (1979) *J. Biol. Chem.* **254**, 3977–3982
23. Vahouny, G. V., Chanderbhan, R., Noland, B. J., Irwin, D., Dennis, P., Lambeth, J. D. & Scallen, T. J. (1983) *J. Biol. Chem.* **258**, 11731–11737
24. Mendelson, C., Dufau, M. & Catt, K. (1975) *Biochim. Biophys. Acta* **411**, 222–230
25. Cooke, B. A., Janszen, F. H. A., Clotscher, W. F. & van der Molen, H. J. (1975) *Biochem. J.* **150**, 413–418
26. Kramer, R. E., Anderson, C. A., McCarthy, J. L., Simpson, E. R. & Waterman, M. R. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 1298
27. Kramer, R. E., Anderson, C. M., Peterson, J. A., Simpson, E. R. & Waterman, M. R. (1982) *J. Biol. Chem.* **257**, 14921–14925
28. Funkenstein, B., McCarthy, J. L., Dus, K. M., Simpson, E. R. & Waterman, M. R. (1982) *J. Biol. Chem.* **258**, 9398–9405
29. Krueger, R. J. & Roberts Orme-Johnson, N. (1983) *J. Biol. Chem.* **258**, 10159–10167
30. Pon, L. A. & Roberts Orme-Johnson, N. (1986) *J. Biol. Chem.* **261**, 6594–6599
31. Pon, L. A., Hartigan, J. A. & Roberts Orme-Johnson, N. (1986) *J. Biol. Chem.* **261**, 13309–13316
32. Pederson, R. J. & Brownie, A. C. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1882–1886
33. Warne, P. A., Greenfield, N. J. & Lieberman, S. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1877–1881

34. Cooke, B. A., Lindh, L. M. & van der Molen, H. J. (1979) *Biochem. J.* **184**, 33–38
35. Janszen, F. H. A., Cooke, B. A., Driel, M. J. A. & van der Molen, H. J. (1976) *FEBS Lett.* **71**, 269–272
36. Ray, D. B., Horst, I. A. & Kowal, J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4648–4652
37. Dazord, A., Gallet de Santerre, D. & Saez, J. M. (1981) *Biochem. Biophys. Res. Commun.* **98**, 885–891
38. Rowe, M. J., Bieszczad, R. R., Neymark, M. A. & Dimino, M. J. (1981) *Endocrinology (Baltimore)* **108**, 127–132
39. Ascoli, M. (1981) *Endocrinology (Baltimore)* **108**, 88–95
40. Browning, J. Y., D'Agata, R. & Grotjan, H. E. (1981) *Endocrinology (Baltimore)* **109**, 667–669
41. Browning, J. Y., Heindel, J. J. & Grotjan, H. E. (1983) *Endocrinology (Baltimore)* **112**, 543–549
42. Sedmark, J. J. & Grossberg, S. E. (1977) *Anal. Biochem.* **79**, 544–552
43. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
44. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–688
45. Sammons, D. W., Adams, L. D. & Nishizawa, E. E. (1981) *Electrophoresis* **2**, 135–141
46. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
47. Zakim, D. & Vessey, D. A. (1973) *Methods Biochem. Anal.* **21**, 1–37
48. Aronson, N. & Touster, O. (1974) *Methods Enzymol.* **31**, 90–102
49. Resko, J. A., Norman, R. L., Niswender, G. D. & Spies, H. G. (1974) *Endocrinology (Baltimore)* **94**, 128–135
50. Farese, R. V. & Prudente, W. J. (1977) *Biochim. Biophys. Acta* **496**, 567–570
51. Ray, P. & Strott, C. A. (1981) *Life Sci.* **28**, 1529–1534
52. Kan, K. W. & Ungar, F. (1973) *J. Biol. Chem.* **248**, 2868–2875
53. Noland, B. J., Arebalo, R. E., Hansbury, E. & Scallen, T. J. (1980) *J. Biol. Chem.* **255**, 4282–4289
54. Beckett, G. J. & Boyd, G. S. (1977) *Eur. J. Biochem.* **72**, 223–233
55. Klee, C. B., Crouch, T. H., & Richman, P. G. (1980) *Annu. Rev. Biochem.* **49**, 489–515
56. Hiwatashi, A., Ichikawa, Y. & Yamano, T. (1977) *FEBS Lett.* **82**, 201–205
57. Suhara, K., Takemori, S. & Katagiri, M. (1971) *Biochim. Biophys. Acta* **263**, 272–278
58. Kashiwagi, K., Dafeldecker, W. P. & Salhanick, H. A. (1980) *J. Biol. Chem.* **255**, 2606–2611
59. Erickson, S. K., Meyer, D. J. & Gould, R. G. (1978) *J. Biol. Chem.* **253**, 1817–1826

Received 23 March 1987/14 July 1987; accepted 18 September 1987