J. Steroid Biochem. Molec. Biol. Vol. 43, No. 4, pp. 319–333, 1992
Printed in Great Britain.

0960-0760/92 $5.00 + 0.00

FURTHER EVIDENCE THAT THE MITOCHONDRIAL PROTEINS INDUCED BY HORMONE STIMULATION IN MA-10 MOUSE LEYDIG TUMOR CELLS ARE INVOLVED IN THE ACUTE REGULATION OF STEROIDOGENESIS

DOUGLAS M. STOCCO
Department of Biochemistry and Molecular Biology, Texas Tech University Health Sciences Center, Lubbock, TX 79430, U.S.A.

(Received 30 March 1992)

Summary—In previous studies we and others have described several mitochondrial proteins which are synthesized in response to acute hormone stimulation in several steroidogenic tissues. In both MA-10 mouse Leydig tumor cells and primary cultures of rat adrenal cortex cells, these proteins consist of a family of 37 kilodalton (kDa) and 32 kDa precursor forms and fully processed forms which are 30 kDa in molecular weight. The nature of the appearance of these proteins and their subcellular localization to the mitochondria, the site of the rate limiting step in steroidogenesis, has led to the speculation that they may be involved in the acute regulation of steroidogenesis. In the present study we have taken advantage of another steroidogenic cell, the R_2C rat Leydig tumor cell, to perform studies which further indicate that these mitochondrial proteins are involved in the regulation of steroidogenesis. Unlike the MA-10 cell which requires hormone stimulation for steroid production, the R_2C cell is a constitutive progesterone producer whose steroid production cannot be further increased with hormone stimulation. We have shown that the R_2C cell line is less sensitive to the inhibition of steroid production by the metal chelator orthophenanthroline (OP) than is the MA-10 cell. We have demonstrated that progesterone production and the 30 kDa mitochondrial proteins remain present in the R_2C cells at a concentration of OP which completely inhibits progesterone production and totally eliminates the 30 kDa proteins in MA-10 cells. As further evidence for the role of these proteins in steroidogenic regulation, we have isolated several revertants of the R_2C parent (P) cell line which have lost the ability to synthesize progesterone constitutively, but which can be stimulated to synthesize this steroid by trophic hormone and cAMP analog. In these revertants, designated (R), the normally constitutively present 30 kDa proteins are greatly decreased compared to controls, but reappear in large amounts following hormone stimulation. Taken together, these data provide further evidence that the 30 kDa mitochondrial proteins are involved in the acute regulation of steroidogenesis in Leydig cells.

INTRODUCTION

Acute steroidogenesis in steroidogenic tissue is regulated by the conversion of cholesterol to pregnenolone by the cholesterol side-chain cleavage complex (CSCC) which resides in the inner mitochondrial membrane [1–7]. More specifically, the activity of CSCC is determined by the availability of the substrate cholesterol to this enzyme complex [8–15]. Thus, the transfer of cholesterol from cellular stores as well as the outer mitochondrial membrane to the inner mitochondrial membrane represents the true rate limiting step in steroidogenesis. This step has been shown to require *de novo* protein synthesis in all steroidogenic tissues studied [16–19]. In previous studies we have shown that hormone stimulation of MA-10 mouse Leydig tumor cells results in the induction of a series of 30 kDa proteins which were found to be associated with the mitochondria [20–22]. In a subsequent report we demonstrated that the 30 kDa mitochondrial proteins were synthesized in a dose-responsive manner to both hCG and cAMP analog and also that they arose from two larger precursors [23]. Also, we have demonstrated that in MA-10 cells two of these proteins are modified by phosphorylation [24], a process thought to be indispensable for the acute regulation of steroidogenesis [25]. In addition, we were able to show that these proteins were present constitutively in the R_2C rat Leydig tumor cell line which synthesizes large amounts of steroid in the absence of hormone stimulation [22]. Our belief that these proteins may be involved in the acute regulation of steroidogenesis is further

strengthened by the findings of Orme-Johnson and co-workers who have detected and characterized similar proteins in the mitochondrial fractions of adrenal, luteal and primary cultures of Leydig cells in response to the appropriate hormone stimulation [26–34]. Yet another study has described the induction of mitochondrial proteins similar to these in response to tropic hormone stimulation of small luteal cells [35].

In the present study we have taken advantage of the unique characteristics of the R_2C cell line in an attempt to demonstrate that the mitochondrial proteins induced in MA-10 cells are involved in the acute regulation of steroidogenesis. First, we have exploited the fact that the R_2C cell line is less sensitive to the antisteroidogenic effects of the metal chelator orthophenanthroline (OP) than is the MA-10 cell line. Second, we have isolated a revertant of the R_2C cell line which no longer synthesizes high levels of steroid constitutively as does the parent, but which does so in the presence of LH/CG or cAMP analog. Using differential OP sensitivity in MA-10 and R_2C(P) cells and the R_2C(R) cells we have performed experiments in which the production of steroids were compared quantitatively to the presence of the 30 kDa mitochondrial proteins. In these studies, we have demonstrated that the synthesis of these mitochondrial proteins and steroid production are concomitant events and are highly correlative. These findings offer further support for our [20–24], and others [26–34] contention that these proteins are involved in the acute regulation of steroidogenesis.

EXPERIMENTAL

Chemicals

Waymouth's MB 752/1 medium, horse serum, phosphate buffered saline containing no Ca^{2+} or Mg^{2+} (PBS^-), lyophilized trypsin-EDTA, and tissue culture grade sodium bicarbonate were purchased from Gibco (Grand Island, NY). Phosphate buffered saline containing Ca^{2+} and Mg^{2+} (PBS^+) was obtained from Oxoid Ltd (Basingstoke, England). LH and hCG were obtained from the National Hormone and Pituitary Program NIDDK (Bethesda, MD). N^6, 2′-*O*-dibutyryladenosine-3′:5′-cyclic monophosphate (Bt_2cAMP), 3-[(3-cholamidopropyl)dimethylammonio]1-propanesulfonate (CHAPS), gentamycin sulfate, progesterone and 1,10-phenanthroline or OP were purchased from Sigma Chemical Co. (St Louis, MO). [^{35}S]Methionine-cysteine (Trans^{35}S-Label; approx. 1000 Ci/mmol) was obtained from ICN Biomedicals Inc. (Irvine, CA). [1,2,6,7-^{3}H]-(N)Progesterone (sp. act. 115 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Ampholines were obtained from Pharmacia-LKB Biotech Inc. (Piscataway, NJ). Other chemicals used in the preparation of isoelectric focusing (IEF) gels were obtained from BioRad (Richmond, CA). 10–20% gradient gels were purchased pre-cast from Integrated Separation Systems (Hyde Park, MA). Dextran T-70 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Decolorizing charcoal (Norit) was obtained from Fisher Scientific Co. (Fairlawn, NJ).

Cell culture

The MA-10 cell line used in these experiments was a generous gift from Dr Mario Ascoli of The University of Iowa and was maintained using standard techniques [36]. R_2C(P) cells were purchased from ATCC and were adapted for growth in Waymouth's medium plus horse serum also as described previously [37]. The R_2C(R) subclones were isolated by plating an average of one cell for every two wells in several 96 well plates (Corning, Corning, NY). The plates were inspected at regular intervals and only those wells having one clone were marked for further observation. Clones arising from single cells were then transferred to larger flasks for growth. A number of clones were tested for constitutive steroid production as described below and compared to the R_2C(P) cell line. Clones displaying greatly reduced progesterone production were utilized in further experiments. The results obtained from one of these subclones are shown in the present study, but are representative of the results found with several of the subclones.

For experiments in which progesterone production was measured, 50,000 cells were plated in each well of 96 well plates and grown for 24 h in medium plus 15% horse serum. In experiments designed to study the synthesis of mitochondrial proteins, 1.5×10^6 cells were plated into each well of 12 well Corning dishes and grown for 24 h. After this time the medium was removed, cells washed twice with PBS^+ and Waymouth's medium lacking serum placed back on the cells. Stimulation and radiolabeling of the cells was performed as described below. In the OP dose–response experiments, 100 μl of Waymouth's medium containing maximally

stimulating doses of Bt_2cAMP and the appropriate dose of OP was placed directly on the cells for the incubation periods described in the legend for Fig. 1 at 37°C in a humidified atmosphere containing 5% CO_2. At the end of the treatment period, the medium was removed and frozen at −20°C until radioimmunoassay (RIA) for progesterone could be performed. In experiments measuring progesterone production, the cells in the wells were solubilized with 0.01% SDS and analyzed for protein content.

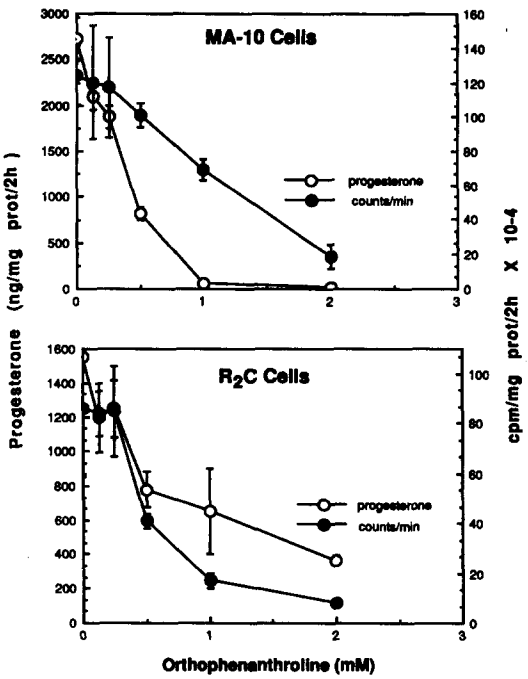

Fig. 1. Effect of OP on progesterone production and protein synthesis in MA-10 and $R_2C(P)$ cells. Cells were grown as described in Experimental. In experiments designed to measure progesterone production, MA-10 cells were subjected to maximally stimulating doses of Bt_2cAMP (1 mM) while $R_2C(P)$ cells were unstimulated. In each case, varying concentrations of OP were also placed on the cells and following a 2 h incubation period the medium was collected and subjected to RIA for progesterone while the cells in the wells were solubilized and assayed for protein content. Each point represents the mean of 4 observations within an experiment performed 4 times. Standard deviations (SD) are depicted by the vertical bars. In experiments designed to determine the effects of OP on protein synthesis in whole cells, Waymouth's medium minus serum containing 25 μCi/ml [^{35}S]methionine-cysteine was placed on the cells in the presence of the appropriate doses of OP for a 2 h period. At the end of this time, the cells were washed twice with PBS^+ followed by the addition of 100 μl of cold 10% TCA to the wells. After at least 1 h at 4°C, the cells were scraped from the wells and washed onto glass fiber filters with several volumes of 5% TCA. The filters were dried and assayed for radioactivity in a liquid scintillation counter. Each point represents the mean of 4 observations within an experiment which was performed in duplicate. SD are shown by the vertical bars at each point.

RIA

Quantitation of progesterone was performed by RIA as described previously [38], directly on aliquots of the medium from control and treated cells. Antibodies to progesterone were obtained from Holly Hills Biological (Hillsboro, OR). Analysis of the RIA data was performed using a computer program specifically designed for this purpose. The data were expressed as ng progesterone per mg protein per unit time.

Radiolabeling of cells

In experiments to determine the effects of OP on protein synthesis in whole cells, Waymouth's medium minus serum containing 25 μCi/ml [^{35}S]methionine-cysteine was placed on 50,000 cells in separate wells of 96 well plates in the presence of the appropriate doses of OP for a 2 h period. At the end of this time, the cells were washed twice with PBS^+ followed by the addition of 100 μl of 10% cold TCA to the wells. After at least 1 h at 4°C, the cells were scraped from the wells and washed onto glass fiber filters with several volumes of 5% TCA. The filters were dried and assayed for radioactivity in a liquid scintillation counter. In experiments in which mitochondrial proteins were analyzed, radiolabeling of proteins was accomplished by covering the cells with Waymouth's medium containing the appropriate level of hormone as well as [^{35}S]methionine-cysteine at concentrations given in the figure legends. At the end of the labeling period, the medium was removed and the cells washed 3 times with PBS^+ prior to further processing.

Preparation of protein samples for electrophoresis

For isolation of mitochondria, cells were washed with PBS^+ and scraped from dishes in a buffer consisting of 0.25 M sucrose/0.01 M Tris/0.01 M EDTA, pH 7.4 and homogenized using a Potter–Elvejhelm motor-driven glass homogenizer with a Teflon pestle. Homogenates were centrifuged at 600 *g* for 15 min and the supernatant was collected and centrifuged at 13,000 *g* for 15 min. The mitochondrial pellet was washed once in sucrose–Tris–EDTA buffer and again collected by centrifugation. Mitochondrial pellets were then solubilized in a small volume of IEF lysis buffer (IEF lysis buffer; 9.8 M urea; 2% CHAPS; 2% ampholines and 100 mM dithiothreitol). Following centrifugation in an Eppendorf centrifuge, aliquots of

the solubilized mitochondrial proteins were assessed for radioactivity by liquid scintillation counting. Previous studies in our laboratory have indicated that mitochondria prepared using this method have approx. 5% contamination with plasma membranes and 23% contamination with cytosol [20].

Two-dimensional gel electrophoresis (2-D PAGE)

Equal cpm from each sample were then applied to the IEF gels and 2-D PAGE was performed on the mitochondrial proteins as described previously [20–24, 39, 40]. At the conclusion of electrophoresis, gels were prepared for fluorography [41] using Resolution (E.M. Corp., Chestnut Hill, MA), dried under moderate heat and vacuum and exposed to X-ray film at −80°C for the appropriate length of time. Radioactive molecular weight protein standards for the second dimension were included in each series of experiments. pH gradients of the IEF gels were determined using a surface electrode.

Quantitation of proteins on gels

Proteins of interest on 2-D gels were quantitated using a BioImage Visage 2000 (BioImage, Ann Arbor, MI) computer assisted image analysis system as described previously [21–24]. Briefly, X-ray images of 2-D PAGE protein profiles were scanned, captured and spotlists of the proteins automatically generated. The images were then adjusted for differences in the amount of radioactivity loaded onto the gels by selecting a number of identical proteins in each gel which did not change with hormone treatment and determining the ratios of the total integrated intensities of these proteins in each gel in an experimental series using the Visage 2000. Proteins on the 2-D gels described in this study were then quantitated using a comparative log software program and the results reported as total corrected integrated intensity in each spot.

RESULTS

Effects of OP on progesterone production and protein synthesis in MA-10 and $R_2C(P)$ cells

MA-10 and $R_2C(P)$ cells were incubated in the presence of various concentrations of OP for a period of 2 h. OP is a metal ion chelator and as such inhibits the activity of both the mitochondrial matrix protease as well as the inner membrane protease I. Thus, the signal and targeting sequences present at the N terminal of the mitochondrial precursor proteins cannot be removed and the precursor proteins are unable to be inserted into the mitochondria [42]. We have previously demonstrated that treatment of MA-10 cells with OP results in complete inhibition of the processing of the 37 kDa precursor protein into the 32 and 30 kDa forms [23].

In the case of the MA-10 cells, maximally stimulating concentrations of Bt2cAMP were included while no stimulation of the $R_2C(P)$ cells was required to obtain maximum progesterone production. In a separate series of experiments treatment of the cells was identical to that described above with the exception that 25 μCi/ml [^{35}S]methionine-cysteine was also included in the incubation mixture. As shown in Fig. 1, OP results in a rapid decrease in progesterone production in MA-10 cells, reaching zero at a concentration of 1 mM. In contrast, the $R_2C(P)$ cell line is less sensitive to the effects of OP on steroidogenic activity and at 1 mM approx. 50% of the progesterone producing capacity remains. Also as depicted in this figure, OP has significant effects on protein synthesis in both the MA-10 and $R_2C(P)$ cell lines. At 1 mM, protein synthesis is decreased by approx. 40% in MA-10 cells and 75% in $R_2C(P)$ cells. The implications of this will be discussed later.

Effects of OP on the synthesis of the hormone induced mitochondrial proteins in MA-10 and $R_2C(P)$ cells

Shown in the electrophoretograms in Fig. 2 are the effects of OP on the synthesis of the 30 kDa mitochondrial proteins in Bt_2cAMP stimulated MA-10 cells and non-stimulated $R_2C(P)$ cells. In MA-10 cells it can clearly be seen that with increasing concentrations of OP, the 30 kDa mitochondrial proteins decrease rapidly and are totally absent at 1 mM. In contrast, in $R_2C(P)$ cells the 30 kDa mitochondrial proteins persisted at all concentrations of OP employed in the incubations. It should be noted that the pattern of 30 kDa mitochondrial proteins is somewhat different in the two cell types with proteins 1, 2 and 3 in MA-10 cells having the same isoelectric points as proteins 1, 2a and 3 in $R_2C(P)$ cells. Also 30 kDa protein 2 in $R_2C(P)$ cells is absent in MA-10 cells while 30 kDa protein 4 in MA-10 cells is absent in $R_2C(P)$ cells. This has been previously described and is a result of post-translational modification of the proteins since it has been demonstrated

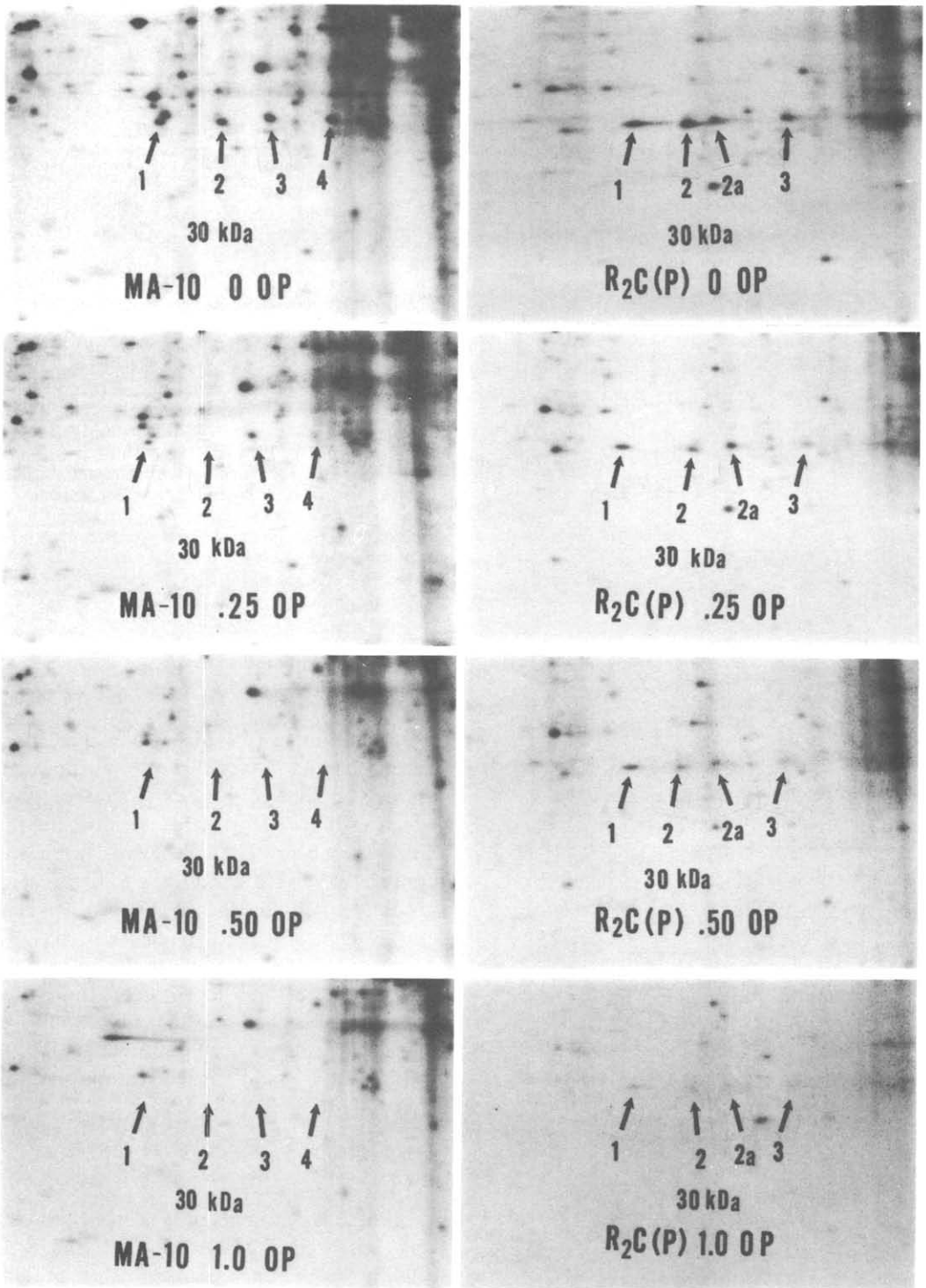

Fig. 2. Effects of OP on the synthesis of the 30 kDa mitochondrial proteins in MA-10 and $R_2C(P)$ cells. For these experiments, cells growing as described in Experimental were harvested and 1.5×10^6 cells were plated into each well of 12 well Corning dishes. The cells were grown for 24 h, washed twice with PBS^+ and covered with Waymouth's medium minus serum containing varying concentrations of OP. Once again, the MA-10 cells also received 1 mM Bt_2cAMP while the $R_2C(P)$ cells were left unstimulated. All cells received 1.0 mCi/ml [^{35}S]methionine-cysteine. Incubations were continued for a 6 h period at 37°C after which time the cells were washed, harvested and mitochondria isolated as described. Mitochondrial pellets were solubilized and subjected to 2-D PAGE as described. The 30 kDa mitochondrial proteins are pointed to by arrows in each of the photographs shown in this figure, while the cell type and concentration of OP are also shown on the photographs. These results are typical of an experiment performed in triplicate.

by tryptic mapping that the 30 kDa proteins are identical to each other [22]. In Fig. 3, the relationship between the production of progesterone and the quantity of the 30 kDa mitochondrial proteins synthesized is shown. In MA-10 cells, there is a concomitant decrease in the ability of the cells to produce progesterone in response to Bt_2cAMP and the presence of the 30 kDa proteins, with both steroid production and the proteins being completely absent at 1 mM. In $R_2C(P)$ cells, however, while progesterone production does decrease significantly with increasing concentrations of OP, at 1 mM OP approx. 50% of the steroid production observed in control cells remains. In contrast to the MA-10 cells, the 30 kDa mitochondrial proteins remain present in $R_2C(P)$ cells at all concentrations of OP. It is important to point out

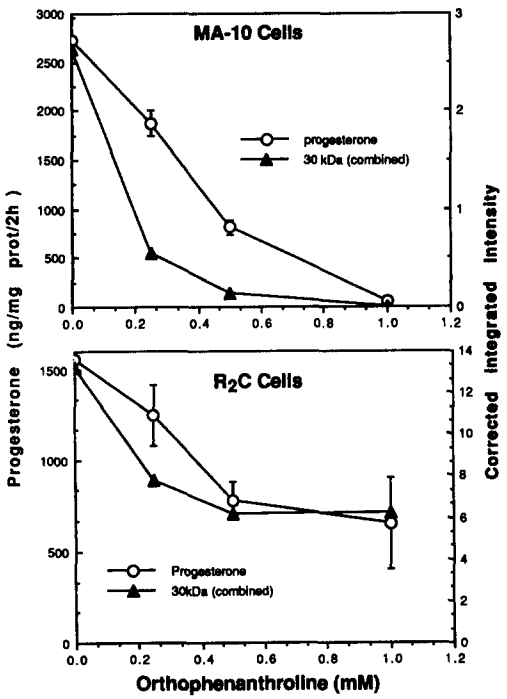

Fig. 3. Comparison of progesterone production and the quantity of the 30 kDa mitochondrial proteins in MA-10 and $R_2C(P)$ cells. This figure is a summary of the data shown in Figs 1 and 2. The 30 kDa mitochondrial proteins shown in Fig. 2 were quantitated using the Visage 2000 image analysis system as described in Experimental. The values given for corrected integrated intensity represent the sum of the total integrated intensity of the 30 kDa mitochondrial proteins following correction of the gels for protein loading differences which may have occurred. Thus, the values given represent the integrated intensity of the 30 kDa proteins in relation to several mitochondrial marker proteins which were determined to remain unchanged in the presence of tropic hormone or Bt_2cAMP. This calculation was critical since OP was shown to have a significant effect on overall protein synthesis in these cells as noted in Fig. 1. The data shown here are from a representative experiment which was performed in triplicate.

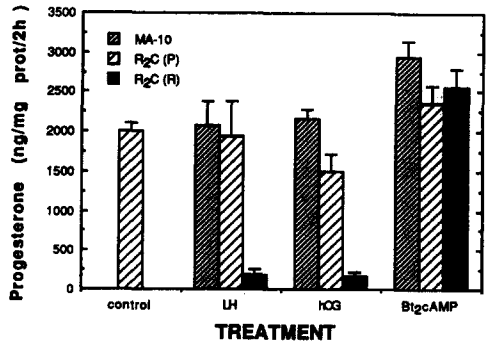

Fig. 4. Progesterone production in MA-10, $R_2C(P)$ and $R_2C(R)$ cells. Cells were grown as described and 50,000 cells of each type were plated in each well of 96 well Corning dishes. After 24 h the cells were washed and the appropriate treatments were placed on the cells. The doses of the hormones used were maximal in all cases being 50 ng/ml for both LH and hCG and 1 mM for Bt_2cAMP. Incubations were carried out for 2 h at 37°C after which time the medium was collected, assayed for progesterone content by RIA and the cells were solubilized and assayed for protein content. The data shown are the means of 4 observations within an experiment performed in triplicate. Progesterone production in unstimulated MA-10(P) and $R_2C(R)$ cells are 7.0 ± 0.7 and 9.5 ± 2.0, respectively, and as such cannot be seen on the graph.

at this time that the units used to express the content of the 30 kDa proteins are corrected integrated intensity. This is calculated by comparing the quantity of these proteins present on the gels to that of a series of marker proteins which do not change with hormone treatment. Since overall protein synthesis is decreased by approx. 75% at 1 mM OP in $R_2C(P)$ cells, the absolute amount of the 30 kDa proteins is actually lower in OP treated cells. However, when compared to other proteins in the mitochondria, they remain at high levels, decreasing by only approx. 50%. The important point, however, is that these proteins are still present at all concentrations of OP and can be readily quantitated from 2-D gels with the Visage 2000.

Progesterone production in MA-10, $R_2C(P)$ and $R_2C(R)$ cells

In these experiments progesterone production was compared in MA-10, $R_2C(P)$ and $R_2C(R)$ cells in the presence of maximally stimulating doses of LH, hCG and dbcAMP. It should be noted that the R_2C subclone is referred to as a revertant subclone in that it appears to have reverted back to the normal Leydig cell phenotype of low basal steroid production and high stimulated steroid production, but this designation is merely descriptive. As shown in Fig. 4, hormone stimulation of MA-10 cells results in very large increases in progesterone production.

In contrast, the constitutive steroid producing $R_2C(P)$ cells produce large amounts of progesterone in the absence of hormone stimulation and this level is not further increased by any of the hormones employed. The $R_2C(R)$ displayed yet another pattern of progesterone production following hormone stimulation. Stimulation with both LH and hCG resulted in significant increases in progesterone production over that seen in controls, but was much lower than that seen in MA-10 cells. Treatment with dbcAMP, however, resulted in the production

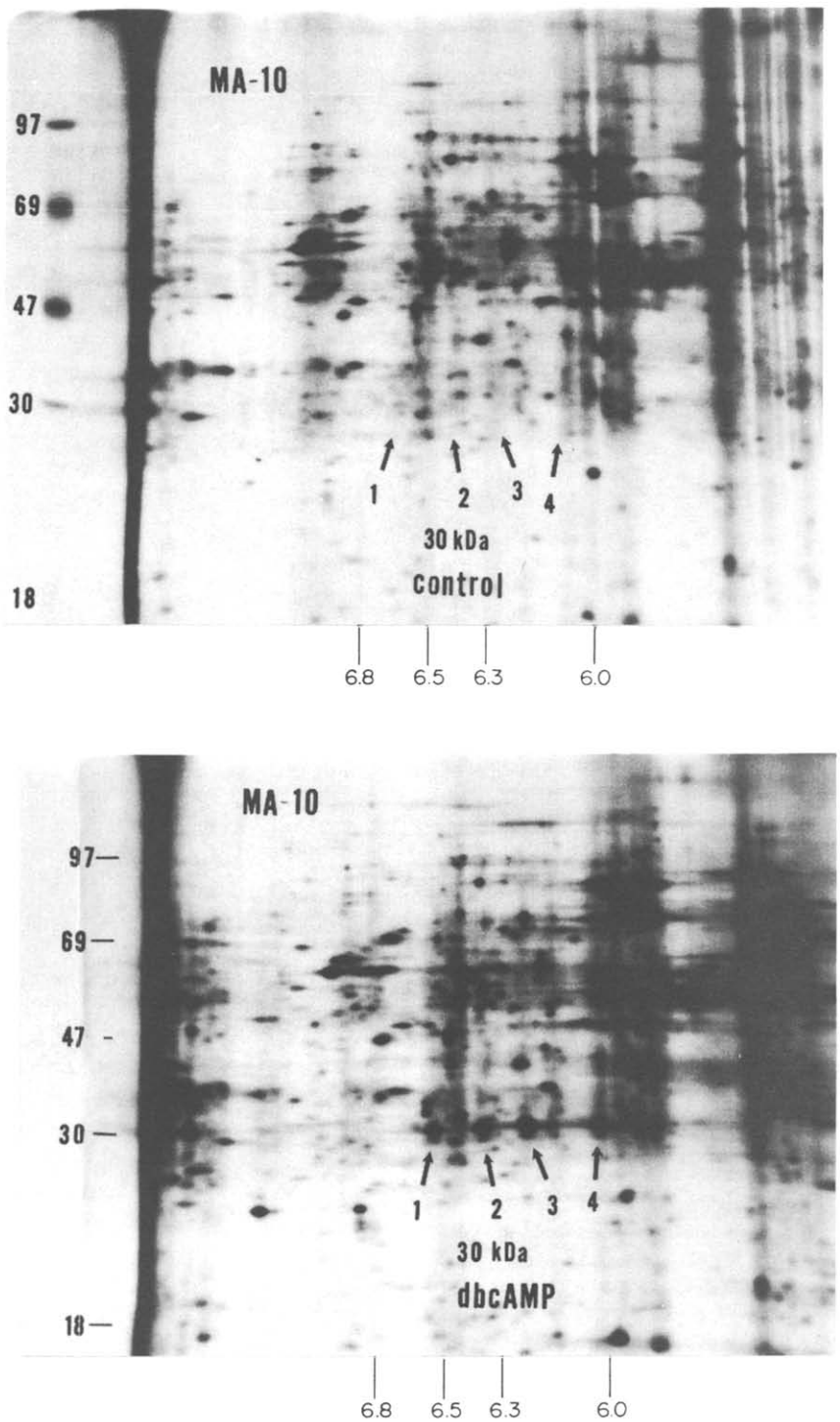

Fig. 5(A)—*legend on p. 327*

of progesterone at levels observed in both stimulated MA-10 cells and stimulated or non-stimulated non-stimulated $R_2C(P)$ cells. These observations indicate that the $R_2C(R)$ cells, while having the enzymatic machinery necessary for the production of progesterone, appear to have LH/CG receptors which are either fewer in number or less functional than those in the MA-10 cells and is in agreement with earlier observations on these cells [37].

Synthesis of the 30 kDa mitochondrial proteins in MA-10, $R_2C(P)$ and $R_2C(R)$ cells

Figure 5 depicts a series of photographs of 2-D PAGE mitochondrial protein profiles of MA-10, $R_2C(P)$ and $R_2C(R)$ cells. It can clearly

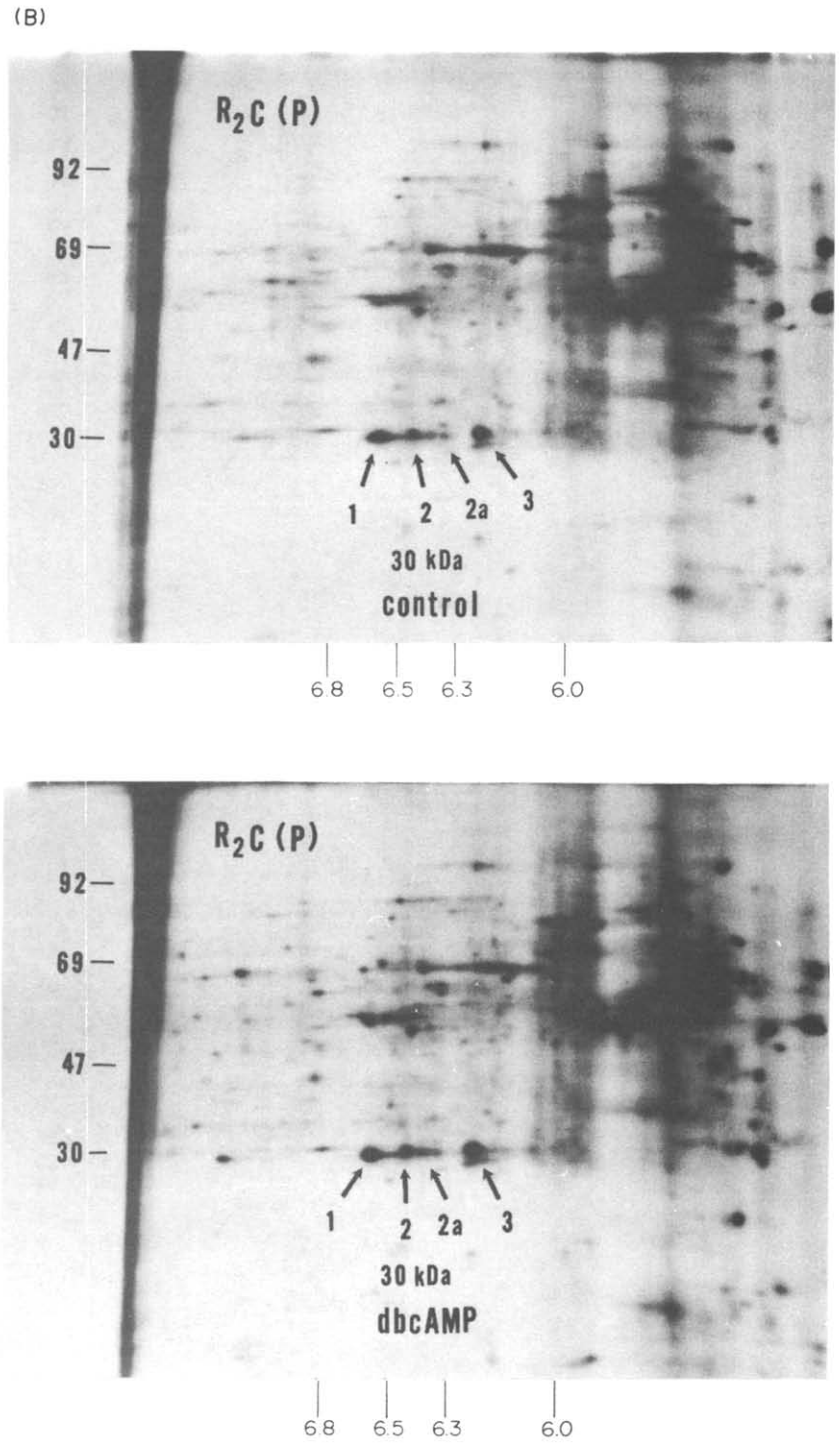

Fig. 5(B)—*legend opposite*

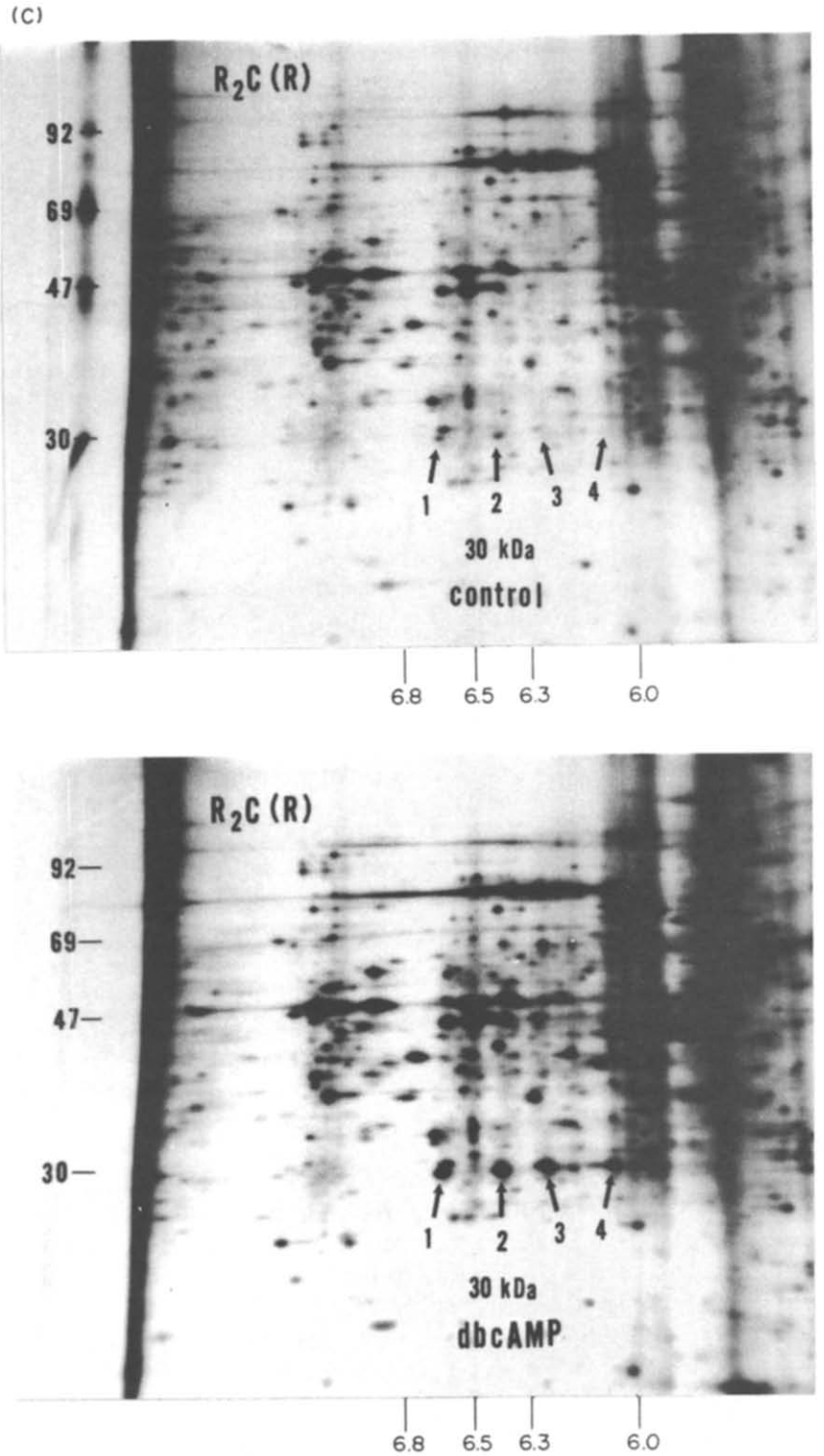

Fig. 5(C)

Fig. 5. Comparison of the synthesis of the mitochondrial 30 kDa proteins in MA-10 (A), $R_2C(P)$ (B) and $R_2C(R)$ (C) cells. Photographs of the 2-D PAGE protein profiles of mitochondria from control and Bt_2cAMP stimulated cells are shown in this figure. In each case, 1.5×10^6 cells were plated into each well of 12 well Corning dishes and grown for 24 h as described. The cells were washed with PBS^+ and covered with Waymouth's medium minus serum containing 1.0 mCi/ml [^{35}S]methionine-cysteine. In half of the cells 1 mM Bt_2cAMP was also included in the incubation. Incubations were for 6 h at 37°C at which time the cells were washed once again and the cells harvested and mitochondria isolated as described. Mitochondrial pellets were solubilized and subjected to 2-D PAGE and subsequent analyses also as described. The cell type and treatment are given on the photographs while the molecular weight values are depicted on the *y* axis and the isoelectric points on the *x* axis. Arrows point to the 30 kDa mitochondrial proteins. This experiment is representative of an experiment performed in triplicate.

be seen that Bt_2cAMP stimulation of MA-10 cells results in the synthesis of a series of 30 kDa mitochondrial proteins which we have described previously [20–24]. In the $R_2C(P)$ cells, the pattern is quite different. In these cells, we have previously shown that proteins identical to the 30 kDa proteins induced in MA-10 cells are present constitutively in the $R_2C(P)$ and cannot be further induced with Bt_2cAMP [22]. Experiments performed here and shown in Fig. 5 are consistent with those earlier studies. The $R_2C(R)$ cells, however, which produce much less progesterone constitutively have also lost most of the constitutive presence of the 30 kDa proteins. Stimulation of the revertant cells with Bt_2cAMP not only results in the production of large amounts of progesterone as seen in Fig. 4, but also results in a very large increase in the synthesis of the 30 kDa mitochondrial proteins. Quantitation of the mitochondrial proteins with the Visage 2000 in all three cell types in the presence and absence of hormone stimulation is shown in Table 1.

Effects of LH, hCG and Bt_2cAMP on the synthesis of the 30 kDa mitochondrial proteins in $R_2C(R)$ cells

The photographs shown in Fig. 6 illustrate 2-D PAGE mitochondrial protein profiles from $R_2C(R)$ cells treated with different stimuli. While very small amounts of the 30 kDa proteins remain present in unstimulated $R_2C(R)$ cells, this amount is many fold smaller than that found in $R_2C(P)$ cells. As shown in this figure, stimulation with LH, hCG and Bt_2cAMP all result in significant increases in the amount of these proteins present in the mitochondrial protein fraction. Stimulation with Bt_2cAMP, however, results in a much larger increase in the total amount of the 30 kDa proteins synthesized during the same incubation period than either LH or hCG. Quantitation of the 30 kDa proteins shown in Fig. 6 are shown in Table 2. It can readily be seen that while stimulation of the $R_2C(R)$ cells with LH and hCG results in 5- to 6-fold increases in the synthesis of these proteins, stimulation with Bt_2cAMP results in a 25-fold increase, a relationship which closely parallels that seen in progesterone production in response to these stimuli.

DISCUSSION

Acute stimulation of steroidogenesis by tropic hormones in steroidogenic tissues requires *de novo* protein synthesis [16–19]. This observation was first made almost three decades ago and many studies have been performed in the hope of unequivocally characterizing the nature and mechanism of action of this protein(s). It is now generally believed that the role of the newly synthesized protein(s) is to aid in the delivery of cellular stores of cholesterol to the inner mitochondrial membrane where cleavage of this substrate to pregnenolone occurs [7–15]. A number of interesting candidates for this cycloheximide sensitive, "labile protein" have arisen over the years, with some of the most promising being Steroidogenesis Activator Polypeptide (SAP) [43–45], an 8.2 kDa steroidogenic stimulatory protein which is virtually identical to endozepine [46–48] and the mitochondrial (peripheral type) benzodiazepine receptor (MBR) [49–53].

In addition, our laboratory and that of Orme-Johnson have proposed that several mitochondrial proteins are intimately involved in the acute regulation of steroidogenesis in Leydig, luteal and adrenal cells [20–24, 25–34]. These proteins are rapidly synthesized in response to tropic hormone or cAMP analog and recent studies indicate that they are part of a family of proteins in which 37 and 32 kDa proteins are precursors which are processed by the removal of a signal and a targeting sequence to 30 kDa proteins prior to being directed to the inner

Table 1. Quantitation of the 30 kDa mitochondrial proteins in control and Bt_2cAMP stimulated MA-10, $R_2C(P)$ and $R_2C(R)$ cells

	Corrected integrated intensities					
Treatment	30 kDa1	30 kDa2	30 kDa2a	30 kDa3	30 kDa4	Totals
MA-10 control	0.00	0.00		0.00	0.00	0.00
MA-10 + Bt_2cAMP	8.66	8.58		11.14	9.97	38.35
$R_2C(P)$ control	7.35	3.98	0.78	5.92		18.03
$R_2C(P)$ + Bt_2cAMP	6.62	4.89	0.94	8.35		20.80
$R_2C(R)$ control	0.86	0.96		0.21	0.00	2.03
$R_2C(R)$ + Bt_2cAMP	3.90	7.11		4.48	4.81	20.30

This table represents the data obtained from the quantitation of the 30 kDa mitochondrial proteins shown in Fig. 5. The numbers given are in units of total integrated intensity which have been corrected as described in Experimental for differences in loading of protein which may have occurred.

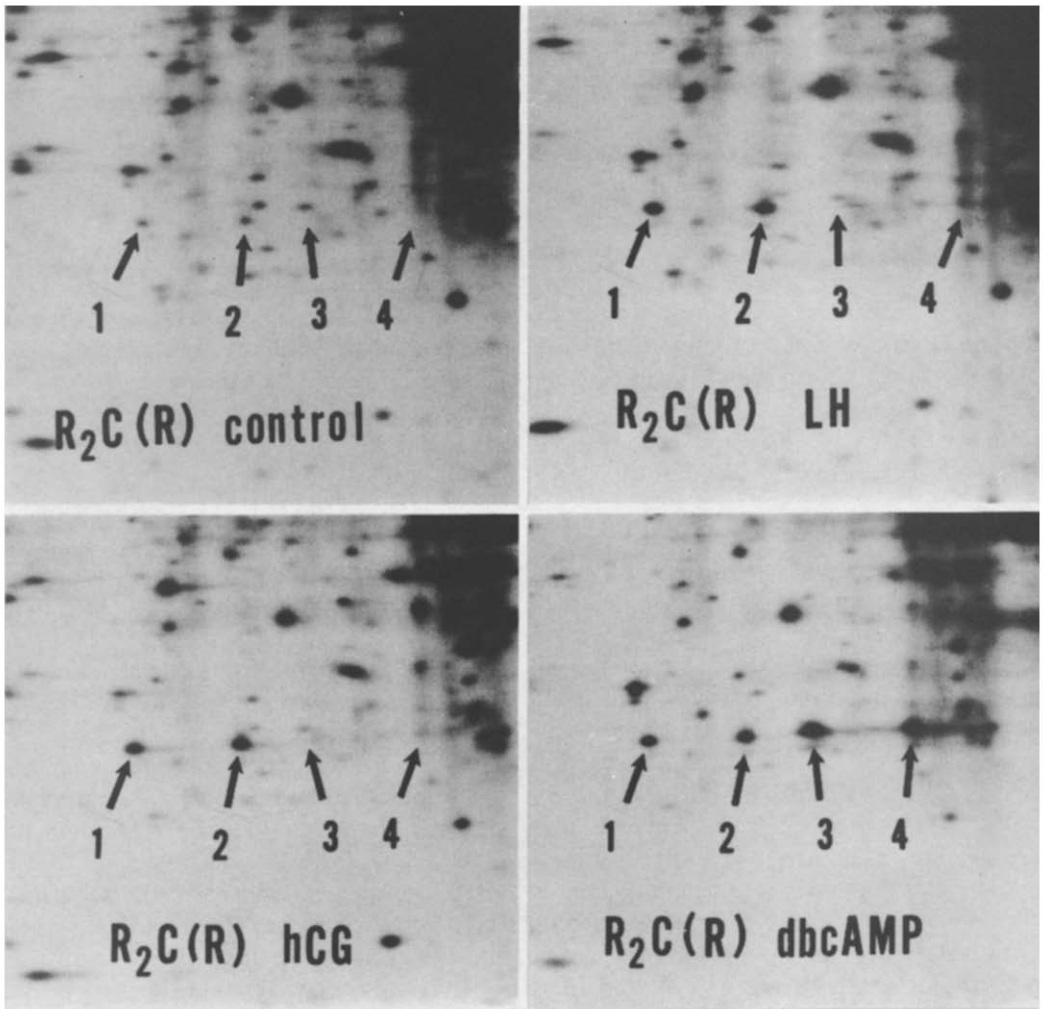

Fig. 6. Effects of LH, hCG and Bt_2cAMP on the synthesis of the 30 kDa mitochondrial proteins in $R_2C(R)$ cells. For these experiments, cells were grown as described and 1.0×10^6 cells were plated into each well of 12 well Corning dishes. After 24 h, cells were washed and covered with Waymouth's medium containing no treatment, 50 ng/ml LH, 50 ng/ml hCG or 1 mM Bt_2cAMP. Each group of cells also received 1.0 mCi/ml [^{35}S]methionine-cysteine. Incubation was for 6 h at which time the cells were harvested, mitochondria isolated and mitochondrial pellets solubilized. Proteins were separated by 2-D PAGE, prepared for fluorography and analyzed as described. The arrows on the gels point to the 30 kDa mitochondrial proteins. Only those areas of the gels containing the 30 kDa proteins are shown for the sake of clarity. This experiment is representative of an experiment performed in triplicate.

mitochondrial membrane [23, 34]. It has been well established that processing and insertion of precursor proteins into the mitochondria during the process of mitochondrial assembly occurs at points of very close contact between the outer and inner mitochondrial membranes referred to as contact sites [54–63]. We and Orme-Johnson's group have suggested that when such processing occurs the contact points thus formed may allow for the transfer of cholesterol from the outer to the inner mitochondrial membrane by an as yet unknown mechanism. Such a relationship has been demonstrated for phospholipids [64, 65] and has been suggested as a possible mechanism for the transfer of sterols between membranes [65].

Thus, the present studies were performed to determine if stronger correlations could be

Table 2. Quantitation of the effects of LH, hCG and Bt_2cAMP on the 30 kDa mitochondrial proteins in $R_2C(R)$ cells

	Corrected integrated intensities				
Treatment	30 kDa1	30 kDa2	30 kDa3	30 kDa4	Totals
Control	0.25	0.35	0.32	0.00	0.92
LH	1.53	1.96	0.19	0.90	4.58
hCG	1.96	2.60	0.19	0.51	5.26
Bt_2cAMP	2.61	3.78	8.87	8.22	23.48

This table represents the data obtained from the quantitation of the 30 kDa mitochondrial proteins shown in Fig. 6. The numbers given are in units of total integrated intensity which have been corrected as described in Experimental for differences in loading of protein which may have occurred.

made for the appearance and the quantity of these proteins present in the mitochondria and the acute synthesis of steroids. We have attempted to correlate progesterone production in both the MA-10 and R_2C cells with the total production of the 30 kDa mitochondrial proteins. In these studies we have assumed that the total of the 30 kDa proteins represent the sum of the product of the processing of the 37 and 32 kDa precursors and thus give an accurate indication of the amount of precursor which has been processed in a given period of time. This strategy was necessitated in part by our earlier observations that we were unable to demonstrate the existence of the 32 kDa mitochondrial precursor proteins in $R_2C(P)$ cells on 2-D gels [22]. The reasons for this are unclear, but since the nature of the lesion whereby the $R_2C(P)$ cells constitutively produce progesterone is not known, it is not possible to speculate on this at the present time. It is also interesting to note that the 2-D PAGE mitochondrial protein profiles obtained from the $R_2C(R)$ cells are somewhat different than those seen in the $R_2C(P)$ cells. They are also different than those seen in the MA-10 cells. The reasons for these differences are not understood but may be related to the fact that steroidogenic mitochondria are morphologically and functionally different from non-steroidogenic mitochondria and the change from a constitutively steroid producing mitochondria to a non-constitutively steroid producing mitochondria could result in a change in many of the proteins.

During the course of previous studies [23] we observed that progesterone production in MA-10 mouse Leydig tumor cells was more sensitive to the effects of OP than it was in the R_2C rat tumor Leydig cells. Our current observation that the quantity of the 30 kDa mitochondrial proteins was highly correlated with progesterone production in these two cell types at different concentrations of OP lends strong support to our belief that they are involved in the acute regulation of steroid production. This is all the more striking when one considers that OP has a greater effect on protein synthesis in R_2C cells than in MA-10 cells. Thus, in R_2C cells in which 75% inhibition of total cellular protein synthesis has occurred, the 30 kDa mitochondrial proteins continue to be synthesized and progesterone continues to be produced. This is in sharp contrast to the MA-10 cells in which protein synthesis is inhibited by only 40%, but the 30 kDa proteins are completely absent and progesterone production is completely inhibited. While we are aware that inhibition of protein synthesis itself results in a loss of steroidogenic capacity, it is convincing that when hormone stimulated proteins no longer appear in the mitochondria of MA-10 cells progesterone production ceases, while in R_2C cells, progesterone production remains at a high level and these proteins are present, albeit at reduced levels, in the mitochondria. This observation is in keeping with our hypothesis that it is during the insertion and processing of the precursor proteins at sites of specific contact between the inner and outer mitochondrial membrane that cholesterol transfer may occur.

Isolation of the R_2C revertant subclones also provided another means to explore the correlation between the quantity of progesterone produced and the amount of the 30 kDa mitochondrial proteins present in the cells. That the R_2C cells would "revert" from constitutive steroid producers to a non-constitutive producing phenotype is of great interest. This phenomenon has been observed not only in our laboratory but in others as well (unpublished observations) and may be a result of the adaptation of the R_2C cells at low cell densities for growth from F-10 medium containing 15% horse serum and 2.5% fetal bovine serum to Waymouth's medium containing 15% horse serum as described previously [37]. The $R_2C(R)$ cells thus isolated displayed greatly diminished constitutive production of progesterone and a corresponding decrease in the amount of the 30 kDa proteins present in the mitochondria. Stimulation with gonadotropin resulted in a modest increase in both progesterone production and the synthesis of the 30 kDa proteins whereas stimulation with Bt_2cAMP resulted in stimulation of progesterone synthesis comparable to that seen in the $R_2C(P)$ or MA-10 cells and a correspondingly large increase in the quantity of the 30 kDa proteins. Once again, in this series of experiments, the correlation between progesterone production and the quantity of these proteins found in the mitochondria was very high. That gonadotropin stimulation of the $R_2C(R)$ cells was lower than that seen with Bt_2cAMP is indicative of the lack of functional LH/CG receptors as was indicated in a previous study with R_2C cells [37]. This fact, however, has allowed us to even more convincingly show the relationship between the 30 kDa proteins and progesterone production as shown in the data presented in Fig. 4 and Table 2. Analysis of

these data indicated that the correlation coefficient between progesterone production and the total integrated intensity of the 30 kDa proteins following LH, hCG and Bt_2cAMP stimulation was 0.992.

While we are aware that the data presented in this study do not constitute absolute proof of the involvement of these mitochondrial proteins in the acute regulation of steroidogenesis, the high degree of correlation between their presence and progesterone production make them excellent candidates for such a role. This may be all the more relevant in view of recent studies which have shown the activity of SAP to be marginal in stimulating steroid production in isolated rat adrenal mitochondria [67]. Also, while a recent report [53] has indicated that tropic hormone stimulated steroid production in Y-1 adrenal cells and MA-10 Leydig tumor cells could be inhibited by flunitrazepam, a benzodiazepine which binds with high affinity to the mitochondrial benzodiazepine receptor, this inhibition was only 30–60%, leaving large amounts of steroid still being synthesized, especially in the case of the MA-10 cells where inhibition was only 30–40%. Thus, while it is clear that proteins such as SAP and the MBR may play some role in the local regulation of steroidogenesis these roles appear to be minor in view of the low level of stimulation observed or the incomplete inhibition of steroidogenesis caused by interfering with their activity.

The results reported here further indicate a strong correlation between the appearance and quantity of the 30 kDa mitochondrial proteins and progesterone production in MA-10 and R_2C cells. Studies currently underway in our laboratory also point to a strong correlation between progesterone production and the quantity of the 30 kDa mitochondrial proteins in two different MA-10 cell constructs. One construct was made through transfection of the wild type with a mutant gene of the type 1 regulatory subunit of the cAMP-dependent protein kinase while the second construct results in the overproduction of a cAMP phosphodiesterase. While additional studies will be required to unequivocally establish if the 37, 32 and 30 kDa mitochondrial proteins function in the acute regulation of steroidogenesis during their processing and insertion into the mitochondria, correlative evidence available at this time would seem to make them strong candidates for this role.

Acknowledgements—The author would like to acknowledge the technical assistance of Deborah Alberts and Jeffrey Wells as well as the support of NIH Grants HD 17481, HD 00685 and RR 03948.

REFERENCES

1. Stone D. and Hechter O.: Studies on ACTH action in perfused bovine adrenals: the site of action of ACTH in corticosteroidogenesis. *Archs Biochem. Biophys.* **51** (1954) 457–469.
2. Karaboyas G. C. and Koritz S. B.: Identity of the site of action of 3′,5′-adenosine monophosphate and adrenocorticotropic hormone in corticosteroidogenesis in rat adrenal and beef adrenal cortex slices. *Biochemistry* **4** (1965) 462–468.
3. Garren L. D., Gill G. N., Masui H. and Walton G. M.: On the mechanism of action of ACTH. *Recent Prog. Horm. Res.* **27** (1971) 433–478.
4. Simpson E. R.: Cholesterol side-chain cleavage, cytochrome *P*450, and the control of steroidogenesis. *Molec. Cell. Endocr.* **13** (1979) 213–217.
5. Churchill, P. F. and Kimura T.: Topological studies of cytochromes $P\text{-}450_{scc}$ and $P\text{-}45011\beta$ in bovine adrenocortical inner mitochondrial membranes. *J. Biol. Chem.* **254** (1979) 10443–10448.
6. Yago N. and Ichii S.: Submitochondrial distribution of components of the steroid 11β-hydroxylase and cholesterol side chain-cleavage enzyme systems in hog adrenal cortex. *J. Biochem. (Toyko)* **65** (1969) 215–224.
7. Lambeth J. D. and Stevens V. L.: Cytochrome $P\text{-}450_{scc}$: enzymology and the regulation of intramitochondrial cholesterol delivery to the enzyme. *Endocrine Res.* **10** (1985) 283–309.
8. Hall P. F., Charpponnier C., Nakamura M. and Gabbiani G.: The role of microfilaments in the response of adrenal tumor cells to adrenocorticotropic hormone. *J. Biol. Chem.* **254** (1979) 9080–9084.
9. Crivello J. C. F. and Jefcoate C. R.: Intramitochondrial movement of cholesterol in rat adrenal cells. *J. Biol. Chem.* **255** (1980) 8144–8151.
10. Iida S., Papadopoulos V. and Hall P. F.: The influence of exogenous free cholesterol on steroid synthesis in cultured adrenal cells. *Endocrinology* **124** (1989) 2619–2624.
11. Jefcoate C. R., DiBartolomeos M. J., Williams C. A. and McNamara B. C.: ACTH regulation of cholesterol movement in isolated adrenal cells. *J. Steroid Biochem.* **27** (1987) 721–729.
12. Privalle C. T., Crivello J. F. and Jefcoate C. R.: Regulation of intramitochondrial cholesterol transfer to side-chain cleavage cytochrome *P*450 in rat adrenal gland. *Proc. Natn. Acad. Sci. U.S.A.* **80** (1983) 702–706.
13. Privalle C. T., McNamara, B. C., Dhariwal M. S. and Jefcoate C. R.: ACTH control of cholesterol side-chain cleavage at adrenal mitochondrial cytochrome $P450_{scc}$. Regulation of intramitochondrial cholesterol transfer. *Molec. Cell. Endocr.* **53** (1983) 87–101.
14. Lambeth J. D., Xu X. X. and Glover M.: Cholesterol sulfate inhibits adrenal mitochondrial cholesterol side-chain cleavage at a site distinct from cytochrome $P450_{scc}$. Evidence for an intramitochondrial cholesterol translocator. *J. Biol. Chem.* **262** (1987) 9181–9188.
15. Stevens V. L., Aw T. Y., Jones D. P. and Lambeth J. D.: Oxygen dependence of adrenal cortex cholesterol side chain cleavage. Implications in the rate-limiting steps in steroidogenesis *J. Biol. Chem.* **259** (1984) 1174–1179.
16. Fergusoni J. J.: Protein synthesis and adrenocorticotropin responsiveness. *J. Biol. Chem.* **238** (1963) 2754–2759.

17. Garren L. D., Ney R. L. and Davis W. W.: Studies on the role of protein synthesis in the regulation of corticosterone production by ACTH *in vivo*. *Proc. Natn. Acad. Sci. U.S.A.* **53** (1965) 1443–1450.
18. Mendelson C., Dufau M. and Catt K.: Dependence of gonadotropin induced steroidogenesis upon RNA and protein synthesis in the interstitial cells of the rat testis. *Biochim. Biophys. Acta* **411** (1975) 222–230.
19. Cooke B. A., Janszen F. H. A., Clotscher W. F. and van der Molen H. J.: Effect of protein-synthesis inhibitors on testosterone production in rat testis interstitial tissue and Leydig cell preparations. *Biochem. J.* **150** (1975) 413–418.
20. Stocco D. M. and Kilgore M. W.: Induction of mitochondrial proteins in MA-10 Leydig tumor cells with human choriogonadotropin. *Biochem. J.* **249** (1988) 95–103.
21. Stocco D. M. and Chaudhary L. R.: Evidence for the functional coupling of cAMP in MA-10 mouse Leydig tumor cells. *Cell. Signalling* **2** (1990) 161–170.
22. Stocco D. M. and Chen W.: Presence of identical mitochondrial proteins in unstimulated constitutive steroid-producing R_2C rat Leydig tumor cells and stimulated nonconstitutive steroid-producing MA-10 mouse Leydig tumor cells. *Endocrinology* **128** (1991) 1918–1926.
23. Stocco D. M. and Sodeman T. C.: The 30 kDa mitochondrial proteins induced by hormone stimulation in MA-10 mouse Leydig tumor cells are processed from larger precursors. *J. Biol. Chem.* **266** (1991) 19731–19738.
24. Chaudhary L. R. and Stocco D. M.: Effect of different steroidogenic stimuli on protein phosphorylation in MA-10 mouse Leydig tumor cells. *Biochim. Biophys. Acta* **1094** (1991) 175–184.
25. Griffin Green E. and Orme-Johnson N. R.: Inhibition of steroidogenesis in rat adrenal cortex cells by a threonine analog. *J. Steroid Biochem. Molec. Biol.* **40** (1991) 421–429.
26. Krueger R. J. and Orme-Johnson N. R.: Acute adrenocorticotropin hormone stimulation of adrenal corticosteroidogenesis. *J. Biol. Chem.* **258** (1983) 10153–10167.
27. Pon L. A. and Orme-Johnson N. R.: Acute stimulation of steroidogenesis in corpus luteum and adrenal cortex by peptide hormones. *J. Biol. Chem.* **261** (1986) 6594–6599.
28. Pon L. A., Epstein L. F. and Orme-Johnson N. R.: Acute cAMP stimulation in Leydig cells: rapid accumulation of a protein similar to that detected in adrenal cortex and corpus luteum. *Endocrine Res.* **12** (1986) 429–446.
29. Pon L. A., Hartigan J. A. and Orme-Johnson N. R.: Acute ACTH regulation of adrenal corticosteroid biosynthesis: rapid accumulation of a phosphoprotein. *J. Biol. Chem.* **261** (1986) 13309–13316.
30. Krueger R. J. and Orme-Johnson N. R.: Evidence for the involvement of a labile protein in stimulation of adrenal steroidogenesis under conditions not inhibitory to protein synthesis. *Endocrinology* **122** (1988) 1869–1875.
31. Pon L. A. and Orme-Johnson N. R.: Acute stimulation of corpus luteum cells by gonadotropin or adenosine 3′,5′-monophosphate causes accumulation of a phosphoprotein concurrent with acceleration of steroid synthesis. *Endocrinology* **123** (1988) 1942–1948.
32. Epstein L. F., Alberta J. A., Pon L. A. and Orme-Johnson N. R.: Subcellular localization of a protein produced in adrenal cortex cells in response to ACTH. *Endocrine Res.* **15** (1989) 117–127.
33. Alberta J. A., Epstein L. F., Pon L. A. and Orme-Johnson N. R.: Mitochondrial localization of a phosphoprotein that rapidly accumulates in adrenal cortex cells. *J. Biol. Chem.* **264** (1989) 2368–2372.
34. Epstein L. F. and Orme-Johnson N. R.: Regulation of steroid hormone biosynthesis. Identification of precursors of a phosphoprotein targeted to the mitochondrion in stimulated rat adrenal cortex cells. *J. Biol. Chem.* **266** (1991) 19739–19745.
35. Mittre H., Aunai P., Benhaim A. and Leymarie P.: Acute stimulation by lutropin of mitochondrial protein synthesis in small luteal cells. *Eur. J. Biochem.* **187** (1990) 721–726.
36. Ascoli M.: Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses. *Endocrinology* **108** (1981) 88–95.
37. Freeman D. A.: Constitutive steroidogenesis in the R_2C Leydig tumor cell line is maintained by the adenosine 3′,5′-cyclic monophosphate-independent production of a cycloheximide-sensitive factor that enhances mitochondrial pregnenolone biosynthesis. *Endocrinology* **120** (1987) 124–132.
38. Resko J. A., Norman R. L., Niswender G. D. and Spies H. G.: The relationship between progestins and gonadotropins during the late luteal phase of the menstrual cycle in Rhesus monkeys. *Endocrinology* **94** (1974) 128–135.
39. O'Farell P. H.: High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250** (1975) 4007–4021.
40. Laemmli U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** (1970) 680–688.
41. Bonner W. M. and Laskey R. A.: A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46** (1974) 83–88.
42. Reid G. A. and Schatz G.: Import of proteins into mitochondria. Extramitochondrial pools and post-translational import of mitochondrial protein precursors *in vivo*. *J. Biol. Chem.* **257** (1982) 13062–13067.
43. Pedersen R. C. and Brownie A. C.: Cholesterol side-chain cleavage in the rat adrenal cortex: isolation of a cycloheximide-sensitive activator protein. *Proc. Natn. Acad. Sci. U.S.A.* **80** (1983) 1882–1886.
44. Pedersen R. C. and Brownie A. C.: Steroidogenesis-activator polypeptide isolated from a rat Leydig cell tumor. *Science* **236** (1987) 188–190.
45. Frustaci J., Mertz L. M. and Pedersen R. C.: Steroidogenesis activator peptide (SAP) in the guinea pig adrenal cortex. *Molec. Cell. Endocr.* **64** (1989) 137–143.
46. Yanagibashi K., Ohno Y., Kawamura M. and Hall P. F.: The regulation of intracellular transport of cholesterol in bovine adrenal cells: purification of a novel protein. *Endocrinology* **123** (1988) 2075–2082.
47. Besman M. J., Yanagibashi K., Lee T. D., Kawamura M., Hall P. F. and Shively J. E.: Identification of des-(Gly-Ile)-endozepine as an effector of corticotropin-dependent adrenal steroidogenesis: stimulation of cholesterol delivery is mediated by the peripheral benzodiazepine receptor. *Proc. Natn. Acad. Sci. U.S.A.* **86** (1989) 4897–4901.
48. Brown S. H. and Hall P. F.: Stimulation by endozepine of the side-chain cleavage of cholesterol in a reconstituted enzyme system. *Biochem. Biophys. Res. Commun.* **180** (1991) 609–614.
49. Mukhin A. G., Papadopoulos V., Costa E. and Kreuger K. E.: Mitochondrial benzodiazepine receptors regulate steroid biosynthesis. *Proc. Natn. Acad. Sci. U.S.A.* **86** (1989) 9813–9816.
50. Yanagibashi K., Ohno Y., Kakamichi N., Matsui T., Hayashida K., Takamura M., Yamada K., Tou S. and Kawamura M.: Peripheral-type benzodiazepine receptors are involved in the regulation of cholesterol side chain cleavage in adrenocortical mitochondria. *J. Biochem.* **106** (1989) 1026–1029.

51. Papadopoulos V., Mukhin A. G., Costa E. and Kreuger K. F.: The peripheral-type benzodiazepine receptor is functionally linked to Leydig cell steroidogenesis. *J. Biol. Chem.* **265** (1990) 3772–3779.
52. Papadopoulos V., Berkovich A., Kreuger K. F., Costa E. and Guidotti A.: Diazepam binding and its processing products stimulate mitochondrial steroid biosynthesis via an interaction with mitochondrial benzodiazepine receptors. *Endocrinology* **129** (1991) 1481–1488.
53. Papadopoulos V., Nowzari F. B. and Kreuger K. E.: Hormone stimulated steroidogenesis is coupled to mitochondrial benzodiazepine receptors. Tropic hormone action on steroid biosynthesis is inhibited by flunitrazepam. *J. Biol. Chem.* **266** (1991) 3682–3687.
54. Hay R., Bohni P. and Gasser S.: How mitochondria import proteins. *Biochim. Biophys. Acta* **779** (1984) 65–87.
55. Hartl F. U. and Neupert W.: Protein sorting to mitochondria: evolutionary conservations of folding and assembly. *Science* **247** (1990) 930–938.
56. Pfanner N., Rassow J., Wienhues U., Hergersberg C., Sollner T., Becker K. and Neupert W.: Contact sites between inner and outer membranes: structure and role in protein translocation into the mitochondria. *Biochim. Biophys. Acta* **1018** (1990) 239–242.
57. Brdiczka D., Bucheler K., Kottke M., Adams V. and Nalam V. K.: Characterization and metabolic function of mitochondrial contact sites. *Biochim. Biophys. Acta* **1018** (1990) 234–238.
58. Hurt E. C. and van Loon A. P. G. M.: How proteins find mitochondria and intramitochondrial compartments. *Trends Biochem. Sci.* **11** (1986) 204–207.
59. Gasser S. M., Ohashi A., Daum G., Bohni P. C., Gibson J., Reid G. A., Yonetani T. and Schatz G.: Imported mitochondrial proteins cytochrome b_2 and cytochrome c_1 are processed in two steps. *Proc. Natn. Acad. Sci. U.S.A.* **79** (1982) 267–271.
60. Schwaiger M., Herzog V. and Neupert W.: Characterization of translocation contact sites involved in the import of mitochondrial proteins. *J. Cell Biol.* **105** (1987) 235–246.
61. Vestweber D. and Schatz G.: A chimeric mitochondrial precursor protein with internal disulphide bridges blocks import of authentic precursors into mitochondria and allows quantitation of import sites. *J. Cell Biol.* **107** (1988) 2037–2043.
62. Rassow J., Guiard B., Weinhues U., Herzog V., Hartl F. U. and Neupert W.: Translocation arrest by reversible folding of a precursor protein imported into mitochondria. A means to quantitate translocation contact sites. *J. Cell Biol.* **109** (1989) 1421-1428.
63. Pon L., Moll T., Vestweber D., Marshallsay B. and Schatz G.: Protein import into mitochondria: ATP-dependent protein translocation activity in a submitochondrial fraction enriched in membrane contact sites and specific proteins. *J. Cell Biol.* **109** (1989) 2603–2616.
64. Simbeni R., Paltauf F. and Daum G.: Intramitochondrial transfer of phospholipids in the yeast, *Saccharomyces cerevisiae*. *J. Biol. Chem.* **265** (1990) 281–285.
65. Simbeni R., Pon L., Zinser E., Paltauf F. and Daum G.: Mitochondrial membrane contact sites of yeast. Characterization of lipid components and possible involvement in intramitochondrial translocation of phospholipids. *J. Biol. Chem.* **266** (1991) 10047–10049.
66. Reinhart M. P.: Intracellular sterol trafficking. *Experentia* **46** (1990) 599–611.
67. Xu T., Bowman E. P., Glass D. B. and Lambeth J. D.: Stimulation of adrenal mitochondrial cholesterol side-chain cleavage by GTP, steroidogenesis activator polypeptide (SAP), and sterol carrier protein$_2$. *J. Biol. Chem.* **266** (1991) 6801–6807.