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Glucocorticoids Enhance the Cholesterol Side-chain Cleavage Activity of Ovine Adrenocortical Mitochondria

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We have shown previously that a chronic treatment with glucocorticoids enhances cAMP- or ACTH-induced steroidogenesis of cultured ovine adrenocortical cells. This effect appears to involve a greater amount of cholesterol in mitochondria. Hence, the present study aimed to define the role of glucocorticoids in cholesterol metabolism by these cells. 2-day-old cultures were exposed to different hormones or inhibitors (10^{-6} M ACTH, 10^{-5} M metyrapone) for 28–48 h. At the end of the treatment period, the cells were stimulated for 2 h with 10^{-3} M 8Br-cAMP, in the presence of 10^{-3} M aminoglutethimide (in order to load mitochondria with cholesterol). Mitochondria were then isolated and incubated without or with 100 μM cholesterol either in the presence or absence of 10^{-3} M $CaCl_2$, or with 25 μM 22R-hydroxycholesterol. Mitochondria isolated from dexamethasone-treated cells produced consistently more pregnenolone than mitochondria from control cells, suggesting that at least part of the additional cholesterol present in these mitochondria was available for steroidogenesis. However, similar differences were obtained when mitochondria were incubated in the presence of exogenous cholesterol, both with or without calcium, or in the presence of 22R-hydroxycholesterol. Pregnenolone production under these latter conditions was much higher than when endogenous cholesterol was the only substrate. Conversely, metyrapone treatment of the cells resulted in lower production of pregnenolone from 22R-hydroxycholesterol by their mitochondria. Likewise ACTH treatment enhanced pregnenolone production by isolated mitochondria irrespective of the incubation conditions. These effects of dexamethasone and ACTH were not related to higher amounts of adrenodoxin, adrenodoxin reductase or cytochrome *P*450scc. These results indicate that exposure of ovine adrenocortical cells to glucocorticoids or ACTH enhances their steroidogenic potency not only by increasing the amount of cholesterol available for steroidogenesis but also by enhancing some step(s) involved in the transformation of cholesterol into pregnenolone.

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INTRODUCTION

Many *in vivo* and *in vitro* studies have shown that ACTH plays a key role in the maintenance and regulation of adrenal steroidogenesis [1]. The acute effect of ACTH is to increase cholesterol mobilization and transport to the cytochrome *P*450 side chain cleavage (*P*450scc) located on the inner mitochondrial membrane, where cholesterol is metabolized to pregnenolone [2]. The long-term action of ACTH is to increase the number of ACTH receptors [3], the amount of the Gsα subunit [4] and the level of several genes encoding key enzymes of the steroidogenic pathway, namely *P*450scc [5–7], *P*450 C11 [5], *P*450 C21, *P*450 C17 [5, 7], 3β-HSD [8] and the electron transport proteins adrenodoxin and adrenodoxin reductase [5, 6].

However, other hormones and growth factors are able to regulate in either a positive or a negative manner the number of ACTH receptors and the level and/or the activity of some enzymes of the steroidogenic pathway in the adrenal gland. Among them are angiotensin-II, IGF-I, FGF and TGFβ [9–16]. Moreover, glucocorticoids themselves appear to be involved in these regulations. Studies in our laboratory have shown that a chronic treatment of cultured ovine fetal or adult adrenocortical cells with dexamethasone

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enhances their steroidogenic response to ACTH or 8Br-cAMP [17, 18]. This action is time- and dose-dependent, specific to glucocorticoids and blocked by pretreatment of the cells with the antagonist RU486. In addition, we have shown that dexamethasone exerts its effect at least at two different levels in the cell. On the adenylate cyclase system glucocorticoids increase specifically the number of ACTH receptors; beyond cAMP formation, they enhance the amount of cholesterol present in mitochondria [19]. This latter result, however, does not imply that this higher amount of cholesterol is indeed available for steroidogenesis and that this is the only intramitochondrial step which is enhanced by dexamethasone treatment.

Hence, the present studies were designed to better understand the effect of dexamethasone on the metabolism of cholesterol by mitochrondria from ovine adrenocortical cells. The data reported here indicate that isolated mitochondria from dexamethasone-treated cells produce more pregnenolone *in vitro* than do control mitochondria. This enhanced capacity appears to involve not only a higher amount of cholesterol available for steroidogenesis, but also some step(s) which limit(s) the transformation of cholesterol into pregnenolone.

MATERIALS AND METHODS

Materials

$ACTH_{1-24}$ (Synacthen) was obtained from Ciba (Rueil-Malmaison, France); 8Br-cAMP, aminoglutethimide, 22R-hydroxycholesterol, cholesterol, progesterone, corticosterone, 17β-estradiol, β-NADPH, DL-isocitrate, and malate were purchased from Sigma (La Verpillière, France); dexamethasone was from UVA (Paris, France). WIN 24540 (4α,5-epoxy-17β-hydroxy-5α-androstane) was from Winthrop laboratories (Paris, France) and SU 10603 [7-chloro-3,4-dihydro-2-(3-pyridyl)-1-(24)-naphtalenone] and metyrapone from Ciba-Geigy Corporation (Summit, NJ, U.S.A.). R5020 was kindly provided by Roussel-Uclaf (Romainville, France). Anti-*P*450scc was a gift from Dr E. Chambaz (Grenoble, France) and anti-adrenodoxin and anti-adrenodoxin reductase were kindly provided by Dr D.B. Hales (Ann Arbor, MI, U.S.A.). [^{3}H]corticosterone and [^{3}H]pregnenolone were purchased from NEN (France).

Methods

Adrenal cell isolation and culture. Adult ovine adrenal cells were isolated and cultured as previously described [18], except that cells were cultured at a density of about 65,000 cells/cm^2. Treatment of cells with hormones or inhibitors of steroidogenesis began on day 2 (48 h after seeding) and lasted for 28–48 h.

For preparation of mitochondrial fractions, at the end of the treatment period, cells were stimulated for 2 h with 10^{-3} M 8Br-cAMP in the presence of 10^{-3} M aminoglutethimide (an inhibitor of *P*450scc), in order to load mitochondria with cholesterol [19, 20]. Then, the cells were washed three times and scraped in ice-cold physiological saline.

In other experiments, cells were cultured and treated as above. At the end of the treatment period, media from the last 24 h of culture were saved and stored at -20°C until assayed for glucocortical contents. Cells were then stimulated for 2 h with 10^{-3} M 8Br-cAMP, in the presence or in the absence of the enzymatic inhibitors, WIN 24540 (4 μM) (a blocker of the 3β-hydroxysteroid dehydrogenase) and SU 10603 (5 μM) (a blocker of 17α-hydroxylase activity) [19]. Media were then withdrawn and stored at -20°C until assayed for pregnenolone or glucocorticoids (mainly corticosterone and cortisol) contents [18] by RIA. For determination of intracellular levels of corticosteroids, at the end of the treatment period, cells were washed three times and scraped in ice-cold physiological saline. These cellular suspensions were added to 6 ml ice-cold ethyl acetate and 2000 cpm [^{3}H]corticosterone were added for recovery purposes. The ethyl acetate extracts were evaporated to dryness. The residues were taken up in the assay buffer for direct measurements of glucocorticoids [18].

Preparation of mitochondrial fractions. The cells were sedimented at 70 ***g*** for 10 min. The pellets were resuspended in hypotonic buffer (10 mM Tris–HCl, 10 mM KCl, 0.5 mM EDTA, pH 7.4) and the cells were centrifuged again at 70 ***g*** for 10 min. Cell pellets were then resuspended in 20 mM Tris–HCl, 250 mM sucrose, pH 7.4 and broken with 10 passes in a Teflon–glass homogenizer. The homogenate was centrifuged for 15 min at 600***g*** to remove cellular debris. The same operation was repeated with the sediment. The combined supernatants were centrifuged for 30 min at 5200***g*** to obtain a mitochondrial pellet which was washed twice with the Tris–sucrose buffer. The mitochondrial pellets were resuspended in buffer consisting of 36 mM Tris, 10.4 mM sodium phosphate, 5.2 mM magnesium chloride, and 0.25 M sucrose, pH 7.5 [21]. All steps were performed with subcellular fractions kept at 0–4°C. These mitochondrial fractions were used for pregnenolone production analysis and for western blotting.

Incubation of mitochondria. Mitochondrial proteins were assayed by the Bradford method, using bovine serum albumin as a standard [22]. Incubations were performed according to Vahouny *et al.*, [21] at 37°C in a final volume of 2 ml containing 50–100 μg of protein. Mitochondria were preincubated for 10 min in the presence of 10 μM WIN 24540, without or with 100 μM cholesterol either in the presence or absence of 1 mM $CaCl_2$, or with 25 μM 22R-hydroxycholesterol. Pregnenolone formation was initiated by adding DL-isocitrate and malate, to give a final concentration of 10 mM. 200 μM NADP was added when incubation of mitochondria was performed in the presence of 1 mM

$CaCl_2$. 500 μl of the mitochondrial suspensions were removed after 0, 2, 4 or 8 min of incubation and added to 5 ml ice-cold ethyl acetate. 2000 cpm of [^{3}H]pregnenolone were added for recovery purposes. The ethyl acetate extracts were evaporated to dryness. The residues were taken up in assay buffer for direct measurements of pregnenolone by RIA [18].

Immunoblotting. Mitochondrial proteins were separated by SDS–PAGE as described by Laemmli [23] using a Bio-Rad apparatus. 1 and 2 μg of protein (in order to improve the accuracy of the quantification) were submitted to 15% SDS polyacrylamide gel electrophoresis under reducing conditions. The separated proteins were electroblotted onto nitrocellulose filters (0.2 μm pore size). After transfer and preincubation, the nitrocellulose filters were incubated as described by Naaman-Reperant *et al.* [24] with the antisera at the following dilutions: 1/10,000 for cytochrome *P*450scc and 1/5000 for adrenodoxin and adrenodoxin reductase. ECL Western blot reagents (Amersham, U.K.) were used for detection of signals. An image analysis system (Samba 2005, Alcatel TITN, Meylan, France) was used for densitometric analysis.

RNA extraction and Northern blot analysis. At the end of dexamethasone or ACTH treatments, total RNA was extracted from cells by the method of Chomczynski and Sacchi [25] modified by Xie and Rothblum [26]. Samples (15 μg per lane) were separated by electrophoresis through a 1.5% agarose/formaldehyde gel and transferred to a Hybond-N membrane (Amersham, U.K.). Prehybridation was performed at 42°C for 3 h in 5 × SSC, pH 7, containing 50% formamide, 1 × Denhardt's reagent, 20 mM Hepes, 1% SDS and 200 μg/ml heat-denatured salmon sperm DNA. Hybridization was then performed by adding the probe to the prehybridization solution and incubation was continued for 18 h. The *P*450scc, adrenodoxin and adrenodoxin reductase probes were generously provided by Dr E. R. Simpson, M. R. Waterman and Y. Sagara, respectively. These probes were labeled with a Megaprime DNA-labeling system (Amersham, U.K.) to a specific activity of 10^9 dpm/μg and used in hybridization reactions at a concentration of 10^6 dpm/ml. All the blots were first washed twice in 2 × SSC containing 0.1% SDS at 42°C for 20 min.

The blots hybridized with the *P*450scc probe were then washed three times in 2 × SSC containing 0.1% SDS for 20 min at 50°C and finally in 0.5 × SSC with 0.1% SDS for 5 min at room temperature.

The blots hybridized with the adrenodoxin probe were then washed twice in 1 × SSC containing 0.1% SDS for 20 min at 42°C, once in 0.5 × SSC with 0.1% SDS for 20 min at 42°C, and finally in 0.2 × SSC with 0.1% SDS for 15 min at 42°C.

The blots hybridized with the adrenodoxin reductase probe were then washed twice in 1 × SSC with 0.1% SDS for 15 min at 42°C, and then in 0.5 × SSC with 0.1% SDS for 10 min at 42, 50 and 60°C.

The blots were autoradiographed at −80°C with intensifying screens. The intensities of the autoradiographic bands were quantified by image analysis. Equal loading of RNA samples was confirmed by scanning the 18S negatives.

Analysis of results. All experimental data have been expressed as the mean ± SEM. Since the net amount of pregnenolone produced by isolated mitochondria, under similar experimental conditions varied from one culture to another, two-way analysis of variance (two-way super ANOVA) was used to compare the pregnenolone production of mitochondria from control and treated cells or that of mitochondria incubated under different conditions (1st way), for different repeated experiments, each performed in triplicate. This two-way analysis of variance was used at 2, 4 or 8 min. Two-way analysis of variance was also used to compare the pregnenolone production of control and treated cells (1st way), for different repeated experiments, each performed in triplicate (2nd way). The significance of the effect of treatments was statistically tested by the *F*-test.

Table 1. Effect of different steriods and analogs on pregnenolone and corticosteroid productions of cultured adrenocortical cells

	Stimulation 8Br-cAMP		Medium from last 24 h
Treatment	Pregnenolone (ng ml)	Corticosteroids (ng/ml)	Corticosteroids (ng/ml)
Control	80.8 ± 12.4	72.7 ± 1.9	3.1 ± 0.9
Dexamethasone 10^{-6} M	114.4 ± 25.1*	117.5 ± 3.8	2.6 ± 0.8
Corticosterone 10^{-6} M	100.3 ± 16.7*	91.3 ± 4.4	224.5 ± 44.3
Progesterone 10^{-6} M	114.5 ± 17.5*	106.7 ± 2.8	122.6 ± 6.4
R5020 10^{-6} M	91.8 ± 12.1*	80.8 ± 5.1	3.4 ± 1.4
Estradiol 17β 10^{-6} M	65.2 ± 3.8*	59.6 ± 1.7	1.25 ± 0.3

Adrenocortical cells were cultured in the absence (control) or presence of different steroids or analogs. At the end of the culture period, cells were stimulated for 2 h with 10^{-3} M 8Br-cAMP (in the absence or presence of WIN 24540 and SU 10603, two inhibitors of pregnenolone metabolism) and their productions of pregnenolone and corticosteroids were determined. The amounts of glucocorticosteroids accumulated in the medium during the last 24 h of the culture were also assessed. For pregnenolone concentrations, values represent the mean ± SEM of 2–4 experiments each performed in triplicate. For corticosteroids concentrations, values are the mean ± SEM of one to 3 different experiments each performed in triplicate. $^{**}P < 0.01$, $^{*}P < 0.001$ vs control.

Table 2. Effect of metyrapone and dexamethasone on pregnenolone and corticosteroids productions of cultured adrenocortical cells

	Stimulation 8Br-cAMP	
Treatment	Pregnenolone ng/ml	Corticosteroids ng/ml
Control	61.3 ± 1.2	73.7 ± 2.2
Metyrapone 10^{-7} M	57.3 ± 2.4	67.8 ± 3.7
Metyrapone 10^{-6} M	53.5 ± 1.9	65.0 ± 3.2
Metyrapone 10^{-5} M	48.1 ± 8.7*	41.9 ± 1.7
Metyrapone 10^{-4} M	38.1 ± 0.1	10.1 ± 0.8
Met. 10^{-5} M + Dex 10^{-6} M	82.1 ± 1.4*†	80.9 ± 6.9
Dexamethasone 10^{-6} M	95.6 ± 9.2*	117.5 ± 3.8

Adrenocortical cells were cultured in the presence of different concentrations of metyrapone or 10^{-6} M dexamethasone. At the end of the culture period, cells were stimulated for 2 h with 10^{-3} M 8Br-cAMP (in the absence or presence of inhibitor of pregnenolone metabolism) and their productions of pregnenolone and glucocorticosteroids were determined. Each value is the mean ± SEM of 6 different experiments each performed in triplicate, except for corticosteroids determinations and for the following conditions: metyrapone 10^{-7}, 10^{-6} and 10^{-4} M, where the values were obtained from triplicate wells in one experiment.

*$P < 0.001$ vs control. †$P < 0.001$ vs metyrapone 10^{-5} M. The significance of the dose–effect relationship of metyrapone was determined by testing the significance of the linearity of the regression line obtained by relating the corticosteroids or pregnenolone productions to the doses of metyrapone ($P < 0.05$ in both cases).

For the relative quantification of *P*450scc, adrenodoxin and adrenodoxin reductase protein and mRNA, *t*-paired analyses were performed to compare treated and control groups.

RESULTS

In the first series of experiments, cells were cultured for 48 h in the presence of different hormones or analogs, or of inhibitors of corticosteroidogenesis. Then, the media were withdrawn and the acute response to 8Br-cAMP was assessed in terms of both corticosteroids and P5 productions. The results are presented in Tables 1 and 2. Dexamethasone and corticosterone treatments enhanced the 8Br-cAMP-induced production of pregnenolone ($P < 0.001$) or that of glucocorticoids as expected [17, 18], whereas 17β-estradiol did not. Progesterone enhanced both responses (pregnenolone production, $P < 0.001$) and these effects were associated with a high amount of glucocorticosteroids in the culture medium, indicating that this corticosteroid precursor was transformed into glucocorticoids during the culture. R5020, an agonist of the progesterone receptor not metabolized into glucocorticoids was much less active. Conversely, metyrapone (an inhibitor of 11β-hydroxylation) decreased 8Br-cAMP induced production of both P5 ($P < 0.001$ at 10^{-5} M metyrapone) and corticosterone in a dose-dependent manner ($P < 0.05$) and dexamethasone could prevent those effects ($P < 0.001$ when compared to control or metyrapone alone). These data confirm and extend our previous results on the specificity of the effect of glucocorticoids on adrenal steroidogenesis and on the determination of the step of the steroidogenic pathway involved in this effect [18, 19].

Hence, to better understand the effect of corticosteroids on P5 production, the next set of experiments aimed to test whether isolated mitochondria from dexa-

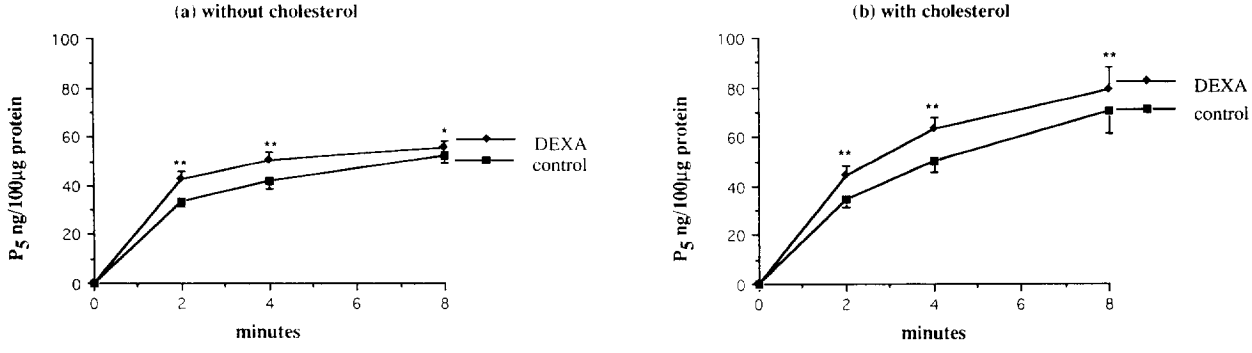

Fig. 1. Pregnenolone production by isolated mitochondria from cells cultured in the absence (■) or presence (◆) of 10^{-6} M dexamethasone, incubated in the absence (a) or presence (b) of 100 µM cholesterol. Each point is the mean ± SEM of 6 experiments, each performed in triplicate. *$P < 0.05$ vs control. **$P < 0.001$ vs control.

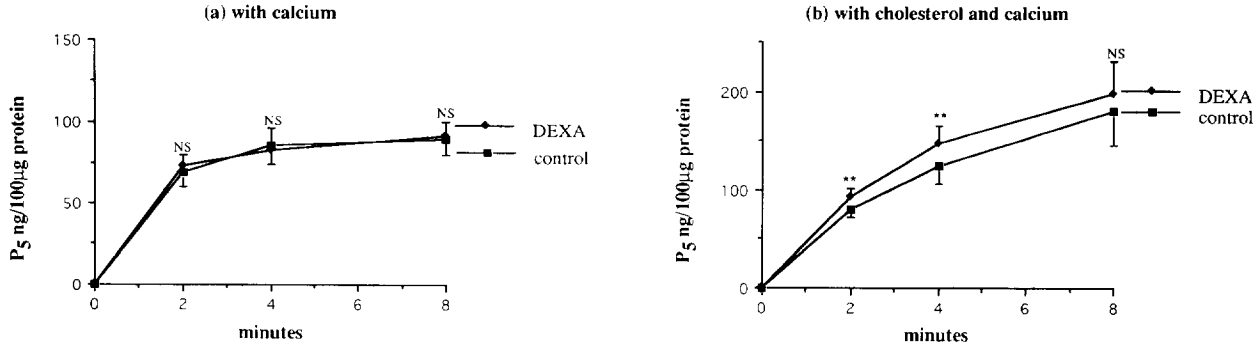

Fig. 2. Pregnenolone production by isolated mitochondria from cells cultured in the absence (■) or presence (◆) of 10^{-6} M dexamethasone, incubated in presence of 1 mM $CaCl_2$ without (a) or with 100 μ–M cholesterol (b). Each point is the mean ± SEM of 6 experiments, each performed in triplicate. **$P < 0.001$ vs control. NS, non significant.

methasone-treated adrenocortical cells were able to produce more pregnenolone from endogenous cholesterol when incubated *in vitro*. Cells were cultured either in the absence or presence of 10^{-6} M dexamethasone, then incubated for 2 h in the presence of both aminoglutethimide and 8Br-cAMP in order to load mitochondria with cholesterol [19, 20]. The kinetic studies presented in Fig. 1(a) show that mitochondria prepared from dexamethasone-treated cells produced consistently more pregnenolone *in vitro* than mitochondria from control cells ($P < 0.05$–$P < 0.001$). In an attempt to decide if this result was due solely to the higher loading with cholesterol of mitochondria from treated cells [19] or if other step(s) was (were) also involved, in the subsequent experiments isolated mitochondria were preincubated for 15 min with 100 μM cholesterol, following which, their pregnenolone production was assessed as above. Cholesterol enhanced the amount of pregnenolone synthesized by mitochondria from both control and dexamethasone-treated cells at 4 and 8 min ($P < 0.001$). Once again, this production was higher for mitochondria from dexamethasone-treated cells than from control cells ($P < 0.001$ at every time) [Fig. 1(b)]. This suggests that in addition to the loading within cholesterol other intramitochondrial step(s) might be enhanced by glucocorticoids. In order to delve further into this phenomenon, mitochondria were incubated in the presence of 10^{-3} M calcium which facilitates cholesterol transport across mitochondrial membranes [27]. Under these conditions the production of pregnenolone was both accelerated and enhanced [compare Fig. 2(a) to Fig. 1(a)]. For control mitochondria, maximal production was already attained after 2 min of incubation and the value reached (68.5 ± 8.6 ng pregnenolone) was significantly higher ($P < 0.001$) than that obtained when the incubation was performed without $CaCl_2$ (33.3 ± 2.2 ng). A difference between control and dexamethasone-treated mitochondria was no longer observed under these conditions, but it appeared that cholesterol availability became rate-limiting early during the incubation. Therefore in subsequent experiments, mitochondria were incubated in the presence of both $CaCl_2$ and exogenous cholesterol [Fig. 2(b)]. Under these conditions of incubation, the production of pregnenolone was dramatically enhanced when compared to all the other experimental conditions [compare Fig. 2(b) to 2(a), 1(a) and 1(b)] (all $P < 0.001$). Moreover, the amount of pregnenolone synthesized by mitochondria harvested from dexamethasone-treated cells was higher again than that produced by control mitochondria ($P < 0.001$), but at 8 min of incubation, the difference was not significant. The enhancing effect of dexamethasone treatment on the capacity of mitochondria to metabolize cholesterol was further substantiated by the next experiments in which the production of pregnenolone from 25 μM 22R-hydroxycholesterol was studied (Fig. 3). The amount of pregnenolone in the media increased almost linearly between 2 and 8 min of incubation for both control and dexamethasone-treated mitochondria. However, the production of pregnenolone by mitochondria from treated cells was higher (all $P < 0.001$) than that of mitochondria from control cells. Conversely, mitochondria from cells cultured in the presence of 10^{-5} M metyrapone (an inhibitor

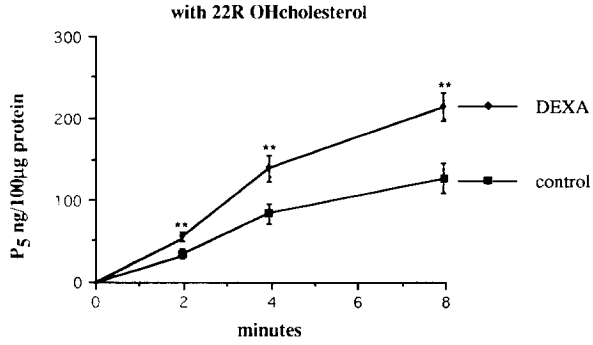

Fig. 3. Pregnenolone production by isolated mitochondria from cells cultured in the absence (■) or presence (◆) of 10^{-6} M dexamethasone, incubated in the presence of 25 μM 22R-hydroxycholesterol. Each point is the mean ± SEM of 7 experiments, each performed in triplicate. **$P < 0.001$ vs control.

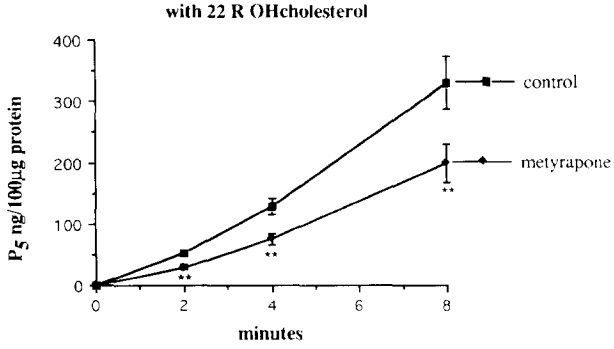

Fig. 4. Pregnenolone production by isolated mitochondria from cells cultured in the absence (■) or presence (◆) of 10^{-5} M metyrapone, incubated in the presence of 25 μM 22R-hydroxycholesterol. Each point is the mean ± SEM of 4 experiments performed each in triplicate. $^{**}P < 0.001$ vs control.

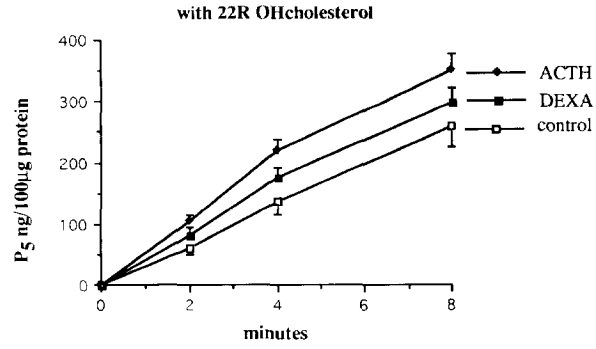

Fig. 5. Pregnenolone production by isolated mitochondria from cells cultured in the absence (□) or presence of 10^{-6} M dexamethasone (■) or 10^{-9} M ACTH (◆), incubated in the presence of 25 μM 22R-hydroxycholesterol. Each point is the mean ± SEM of 3 experiments, each performed in triplicate. Differences between ACTH-treated cells or dexamethasone-treated cells and control cells were significant at $P < 0.001$ at every time tested. Differences between ACTH- and dexamethasone-treated cells were significant at $P < 0.01$ at every time tested.

of 11β-hydroxylation) [28] synthesized less pregnenolone from 22R-hydroxycholesterol than control mitochondria (all $P < 0.001$) (Fig. 4).

Next we investigated whether ACTH could also induce a similar effect on the cholesterol side-chain cleavage activity of isolated mitochondria. Adrenocortical cells were cultured in the presence of 10^{-9} M ACTH, then mitochondrial fractions were prepared and incubated as described above. ACTH treatment of cells resulted in concentrations of glucocorticoids of $5.7 \pm 0.4 \times 10^{-6}$ M in the culture medium and 35.1 ± 1 pmol/10^6 cells in the cell pellet. These concentrations were about 700- and 40-fold higher than in control media and cell pellets, respectively. The production of pregnenolone from endogenous cholesterol

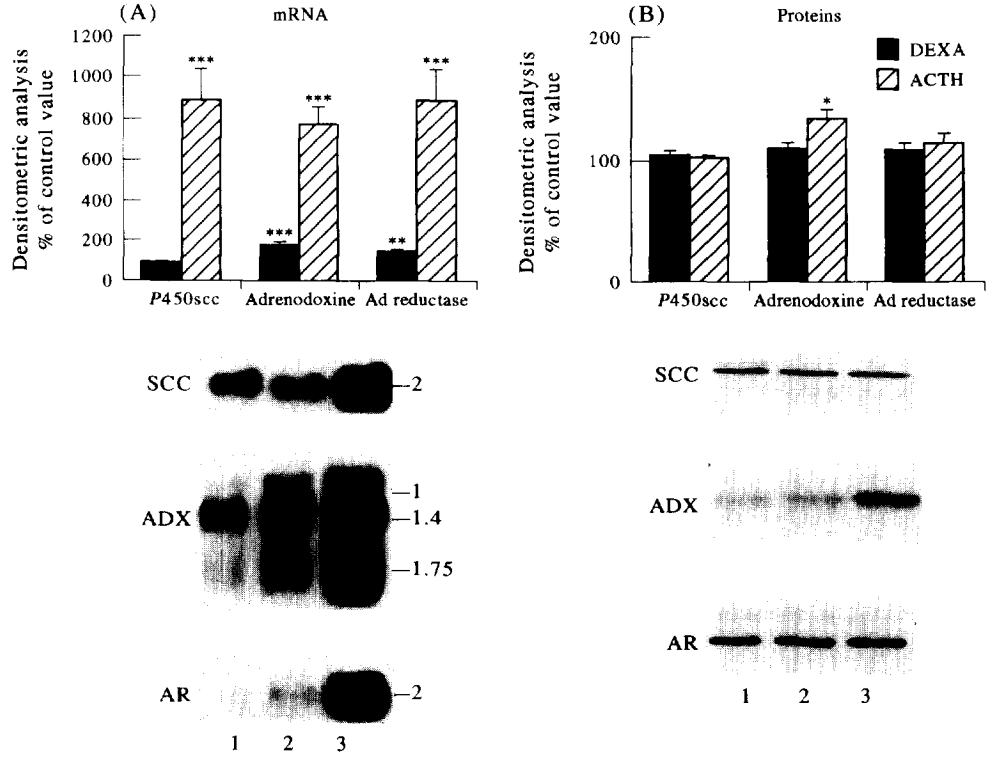

Fig. 6. Comparative quantification of *P*450scc, adrenodoxin and adrenodoxin reductase mRNA (A) and proteins (B) from cells cultured in the absence or presence of 10^{-6} M dexamethasone (▨) or 10^{-9} M ACTH (■). Densitometric values were calculated relatively to the value obtained with the control cells which was considered as equal to 100%. Each point is the mean ± SEM of 4–7 experiments. $^{*}P < 0.05$, $^{**}P < 0.02$, $^{***}P < 0.01$, when compared to control cells (*t*-paired analysis). Lower panels: representative Northern blots (A) and Western blots (B) for *P*450scc (SCC), adrenodoxin (ADX) and adrenodoxin reductase (AR). 1 = control, 2 = 10^{-6} M dexamethasone, 3 = 10^{-9} M ACTH.

by mitochondria from cells treated with ACTH was higher than that of control cells, as expected [29, data not shown]. Moreover, treatment of the cells with the hormone resulted in an enhancement of the capacity of their mitochondria to produce pregnenolone from 22R-hydroxycholesterol when compared to that of mitochondria from control cells (all $P < 0.001$), or even to that of mitochondria from dexamethasone-treated cells (all $P < 0.001$) (Fig. 5).

In the last experiment we attempted to relate this increase in the ability of mitochondria from dexamethasone- or ACTH-treated cells to metabolize 22R-hydroxycholesterol to changes in those enzymes known to be involved in the cholesterol side-chain cleavage system. Therefore, we evaluated the amounts of the mRNAs and the proteins for *P*450scc, adrenodoxin and adrenodoxin reductase in both control and treated cells.

Dexamethasone treatment enhanced the accumulation of adrenodoxin ($P < 0.001$) and adrenodoxin reductase ($P < 0.02$) mRNAs but did not change the amount of *P*450scc mRNAs (Fig. 6), while ACTH enhanced dramatically the accumulation of the mRNAs for all 3 enzymes ($P < 0.001$).

Conversely, dexamethasone was unable to enhance the mitochondrial amounts of *P*450scc, adrenodoxin and adrenodoxin reductase. As for ACTH, it increased slightly only the amount of adrenodoxin ($P < 0.05$).

DISCUSSION

The present results reinforce the view that both exogenous and endogenous glucocorticoids are able to regulate the productions of both pregnenolone and glucocorticosteroids by ovine adrenocortical cells. Only these steroids possessing glucocorticoid activity, or able to be metabolized into glucocorticoids by adrenal cells, increased the production of pregnenolone (and corticosterone) by adrenocortical cells stimulated with 8Br-cAMP. The slight increase induced by R5020 likely resulted from a small binding of the compound to the glucocorticoid receptor [30, 31]. Conversely a treatment of the cells with metyrapone (an inhibitor of 11β-hydroxylation) decreased concomitantly the secretions of pregnenolone and glucocorticosteroids stimulated by 8Br-cAMP.

More importantly, our data indicate that glucocorticoids are able to regulate the production of pregnenolone by mitochondria of the ovine adrenal gland, not only by increasing the amount of cholesterol present in the mitochondria [19] but also by enhancing their cholesterol side-chain cleavage activity. Isolated mitochondria from dexamethasone-treated adrenal cells produced more pregnenolone than mitochondria from control cells both in the absence and presence of exogenous cholesterol. Moreover, when calcium was added to cholesterol loaded incubation media, in order to facilitate cholesterol transport across mitochondrial membranes [27], the effect of dexamethasone was observed again. Lastly, when the incubations were performed in the presence of 22R-hydroxycholesterol, a permeating cholesterol derivative [32] (or even with 20α-hydroxycholesterol, N. Picard-Hagen and P. Durand, unpublished) similar results were obtained. This effect of glucocorticosteroids on the mitochondrial cholesterol side-chain cleavage activity might seem unexpected. Indeed we did not observe an effect of dexamethasone treatment on the metabolism of 22R-hydroxycholesterol by cultured (intact) adrenocortical cells, whether the cells were stimulated for 2 h with 8Br-cAMP in the presence of aminoglutethimide, washed, and then incubated in the presence of 22R-hydroxycholesterol (our unpublished results) or not stimulated before testing for 22R-hydroxycholesterol metabolization [19]. However, it should be underlined that under the conditions of incubation of mitochondria, energetic substrates and cofactors required by the reaction were employed at optimal concentrations. Moreover recent studies have shown that the access of hydroxylated cholesterol derivatives to the *P*450scc system of intact steroidogenic cells may not be simple [33]. Indeed, the presence of HDL in the incubation medium enhances the delivery of 22R-hydroxycholesterol to ovine luteal cells. Therefore, it may be difficult to compare the two systems.

The phenomenon described here is of rather low magnitude under *in vitro* conditions but highly reproducible as attested by its high degree of statistical significance. Several observations suggest that it should be operative *in vivo* and physiologically relevant: (i) the intra-adrenal concentrations of glucocorticoids are high ($\geq 10^{-5}$ M [34, 35]; (ii) ACTH stimulation of cultured adrenocortical cells resulted in glucocorticoid concentrations in the medium in the micromolar range; (iii) a chronic treatment of cells with an inhibitor of 11β-hydroxylation (metyrapone) resulted in a lower ability of mitochondria from these cells to produce pregnenolone from 22R-hydroxycholesterol, when compared to that of mitochondria from control cells. Hence, glucocorticoids produced by the cells, even under resting conditions, appear able to regulate this step (see Fig. 4). In that situation the concentrations of "endogenous" glucocorticoids in the medium are well below those necessary to observe an effect of "exogenous" glucorticoids. However, we have calculated (details not shown) that the intracellular concentration of glucocorticoids under *in vitro* resting conditions is 0.4 ± 0.1 μM. Indeed, this concentration is large enough to interact with the intra-adrenal corticosteroid receptor, the affinity of which is about 15 nM [36]. Therefore, we can understand the small amplitude of the effect of dexamethasone observed under our *in vitro* conditions: as a matter of fact, this amplitude corresponds to the difference between the effect of dexamethasone-treatment and the effect of endogenous corticosteroids.

It is widely accepted that a chronic treatment of adrenocortical cells with ACTH enhances the cholesterol side-chain cleavage activity of their isolated mitochondria [29]. Our results show that the magnitude of the enhancement of the 22R-hydroxycholesterol side-chain cleavage activity by ACTH or dexamethasone, although significantly different, was rather close. Taken together, these results might indicate that part of the ACTH action on this system is mediated through the corticosteroids produced by the cells in response to ACTH. Moreover they reinforce the view that the effect of glucocorticosteroids, despite being rather small *in vitro*, is physiologically relevant.

The molecular mechanism of this effect of glucocorticoids is not clear at the present time, but cannot be related simply to the higher cholesterol content of mitochondria from dexamethasone-treated cells [19]. Iida *et al.* [37] have shown that the production of pregnenolone by mitochondria from tumor adrenal cells incubated with aminoglutethimide with or without dibutyryl cyclic AMP shows no difference irrespective of the concentration of cholesterol in the inner mitochondrial membrane. Although dexamethasone slightly increased the concentrations of mRNAs encoding for adrenodoxin and adrenodoxin reductase, there was neither an enhancement of their corresponding proteins, nor a higher amount of the *P*450scc. Likewise, ACTH only slightly enhanced the amount of adrenodoxin, but not that of either adrenodoxin reductase or *P*450scc, despite multiplying by 8 or 9 the concentrations of their respective mRNAs. A similar discrepancy between mRNAs and protein levels has been observed by others [6, 7]. Hence, the increased cholesterol side-chain cleavage activity induced by dexamethasone- or ACTH-treatment was not related to a higher amount of these enzymes in the mitochondria. Conflicting results on the mechanism of regulation of the cholesterol side-chain cleavage activity have been reported in the literature. John *et al.* [38] and Naaman-Reperant *et al.* [24] reported roughly similar amounts of *P*450scc in the adrenal glands of newborn lambs and those of 100–120-day-old ovine fetuses, despite a 5-fold higher cholesterol side-chain cleavage activity in the glands from newborns. Likewise, Lambert *et al.* [39] concluded from their studies in the guinea pig that the prolonged stimulatory effect of ACTH on pregnenolone synthesis involves an increase in the synthesis of a protein factor which promotes the availability of cholesterol to the side-chain cleavage system, with no stimulated synthesis of the side-chain cleavage complex itself. Further, Lehoux *et al.* [7], in the hamster, observed that an *in vivo* ACTH treatment for 20 h did enhance the amount of *P*450scc mRNA but not the amount of *P*450scc nor the activity of the enzyme. In contrast, Hanukoglu *et al.* [6] in the bovine species, observed that ACTH enhances the mRNA and protein levels of adrenodoxin, adrenodoxin reductase and cytochrome *P*450scc. Unfortunately, the activity of the enzyme complex was not monitored at the same time. Trzeciak *et al.* [40] reported an inhibitory effect of dexamethasone on the ACTH-induced accumulation of *P*450scc mRNAs at a transcriptional level, but once again, the enzymatic activity was not evaluated.

We conclude from the results we obtained that glucocorticoids can regulate the cholesterol side-chain cleavage activity of ovine adrenocortical cells at a step beyond cholesterol delivery to the mitochondria. This effect does not involve higher amounts of adrenodoxin, adrenodoxin reductase or of cytochrome *P*450scc. Whether post-translational modification of these enzymes or another unidentified protein is involved in the regulation of this step by corticosteroids remains to be determined.

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