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STEROID BIOSYNTHESIS BY ZONA GLOMERULOSA-FASCICULATA CELLS IN PRIMARY CULTURE OF GUINEA-PIG ADRENALS

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Summary—Steroidogenesis was studied in guinea-pig glomerulosa-fasciculata cells maintained in primary culture for up to 7 days. The basal secretion which remained stable for the first 2 days in culture rapidly rose to reach a plateau on day 4 at levels 6–7-fold higher than those observed during the first 2 days of culture while the maximal response to ACTH in terms of cortisol and androstenedione secretion was fairly stable throughout the 7-day period. Exposure of glomerulosa-fasciculata cells to ACTH caused a stimulation of pregnenolone, 17-hydroxypregnenolone, progesterone, 17-hydroxyprogesterone, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol, cortisol, dehydroepiandrosterone, androstenedione, 11β-hydroxyandrostenedione and aldosterone while, after 48 h of incubation, a marked accumulation of end-products, namely cortisol and 11β-hydroxyandrostenedione, was observed. The half-maximal steroidogenic response to ACTH occurred at concentrations varying between 1.7×10^{-11} and 1.1×10^{-10} mol/l for the 12 steroids examined. Addition of 8-bromoadenosine 3′, 5′-cyclic monophosphate stimulated steroid secretion in a dose-dependent manner. Maximal response to 8-bromoadenosine 3′, 5′-cyclic monophosphate was obtained at 1 mmol/l, and no further rise of steroid secretion was observed after addition of ACTH. Incubation of glomerulosa-fasciculata cells with labeled corticosterone, cortisol and androstenedione indicates that only androstenedione can be converted into 11β-hydroxyandrostenedione, thus suggesting that this end-product is a good parameter of the C-19 steroid production by guinea-pig glomerulosa-fasciculata cells in primary culture. The present data confirm that guinea-pig glomerulosa-fasciculata cells in primary culture provide an interesting model for the study of the regulation of C-19 steroid formation by the adrenals.

INTRODUCTION

Human adrenal glands, which secrete low quantities of testosterone and dihydrotestosterone, are known to be responsible for high plasma levels of C-19 steroids, namely dehydroepiandrosterone, its sulfate, androst-5-ene-3β,17β-diol and androstenedione. From *in vitro* experiments, it was shown that skin, prostate as well as breast tissues, are capable of converting these adrenal C-19 steroids into testosterone and its metabolites, and these observations were further supported by clinical evidence in both women and men [1–4]. Furthermore, androst-5-ene-3β,17β-diol, which shows estrogenic activity, is also secreted by the adrenal glands and, with its plasma level of approximately of 1–5 nmol/l, likely contributes to a significant extent to the estrogenic action of these endogenous steroids [5, 6]. Considering the role of these steroids in a large number of pathologies, particularly prostate and breast cancer, and their abundance in plasma, it becomes of great importance to better understand the mechanisms which control C-19 steroid secretion by the adrenals.

ACTH, while exerting the major influence on cortisol secretion, is the most generally accepted modulator of C-19 steroid secretion. ACTH in fact stimulates the secretion of C-19 steroids and, in most *in vivo* and *in vitro* studies reported, it is almost impossible to dissociate the stimulatory effect of ACTH on glucocorticoids from that on C-19 steroids [7–10]. However, while ACTH can clearly influence adrenal C-19 steroid secretion, there are many physiological and pathological situations where a divergence in cortisol and C-19 steroid formation occurs [7]. Current hypotheses on the control of C-19 steroid secretion have also suggested a role for prolactin, a specific pituitary factor named cortical androgen-stimulating hormone [11] and finally, for steroids themselves [12].

The increase in dehydroepiandrosterone, its sulfate and androstenedione observed in the human at adrenarche is found exclusively in the human while

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such a process does not occur in currently used animal models, namely the rat, guinea-pig, bovine and dog. Despite the absence of adrenarche and the lack of androgenic activity of steroids from adrenal origin in the guinea-pig plasma, it is known that guinea-pig adrenals contain immunologically measurable C-19 steroids [13]. In agreement with these data, the steroidogenesis of freshly isolated guinea-pig glomerulosa-fasciculata cells have been extensively studied, indicating that both cortisol and C-19 steroids are secreted after addition of ACTH [14–16]; moreover, Black *et al.*[17] demonstrated the viability of these cells in culture and their capability, in those conditions, to produce steroids. Since guinea-pig adrenal cells in primary culture provide a useful experimental model for conducting steroid biosynthesis studies, the purpose of the present work was to characterize the response of steroids to ACTH stimulation of glomerulosa-fasciculata cells in primary culture and to further examine the production of 11β-hydroxyandrostenedione, the major C-19 steroid secreted by the guinea-pig adrenal.

MATERIALS AND METHODS

Chemicals

Trypsin inhibitor (from soybean), trypsin, deoxyribonuclease I (from bovine pancreas) and collegenase (from clostridium histolyticum) were purchased from Boehringer Mannhiem (Indianapolis, Ind.). Eagle's Minimum Essential Medium (MEM), Hank's balanced salt solution and HEPES (*N*-2-hydroxyethylpiperazine *N'*2-ethanesulfonic acid) were obtained from Gibco (Grand Island, N.Y.). Bovine serum albumin (BSA) (fraction V), Ficoll-Histopaque solution (Histopaque-1077) and 8-bromoadenosine 3', 5'-cyclic monophosphate (8 Br-cAMP) were purchased from Sigma Chemical Co. (St Louis, Mo.). Fetal calf serum was obtained from Hyclone Laboratories (Logan, Ut.) and 24-well culture plates from Flow Laboratories (McLean, Va.). ACTH as the synthetic tetracosapeptide (Cortrosyn) was supplied by Organon Inc. (West Orange, N.J.). [1,2,6,7-^{3}H]cortisol (94 Ci/mmol), [1,2,6,7-^{3}H]corticosterone (81 Ci/mmol), [1,2-^{3}H]deoxycorticosterone (36 Ci/mmol) and [1,2,6,7-^{3}H]androstenedione (80 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, England).

Preparation of dispersed guinea-pig adrenal glomerulosa-fasciculata cells

Adult male guinea-pigs (Hartley) weighing approximately 750 g obtained from Charles River Canada Inc. (St Constant, Quebec, Canada) were used for the preparation of primary culture of glomerulosa-fasciculata cells. Animals were decapitated and adrenals were quickly removed using sterile instruments, trimmed of fat and placed in cold Hank's solution. The glomerulosa-fasciculata cells were isolated using an adaptation of the procedure of Black *et al.*[17]. In brief, the adrenals were placed in a plastic Petri dish containing a small volume of Hank's solution and were cut in half length-wise with a surgical blade, and the inner brown core was separated from the yellow outer rim. The zona reticularis is easily distinguishable by its brown colour and its fragile gelatinous texture. The zona glomerulosa-fasciculata is yellow and consists of tougher tissue. No attempt was made to separate the zona glomerulosa from the zona fasciculata. The zona glomerulosa-fasciculata tissue was minced with scissors, placed in MEM and the tissue fragments from 6 adrenals incubated in 25 ml MEM with trypsin (25 mg), collagenase (62.5 mg) and BSA (375 mg) for 15 min, deoxyribonuclease (50 μg) was then added for 1 min. The tissue fragments were then incubated in 25 ml of MEM with trypsin inhibitor (25 mg) and BSA (375 mg) for 5 min. Then the tissue pieces were incubated in Hanks solution with BSA (650 mg) for 15 min. All incubations were carried out in 50 ml conical test tube at 37°C in a shaker bath oscillating at 65–70 strokes per min and between each incubation, the fragments were allowed to settle and the supernatant was replaced. After the last incubation, the pieces were mechanically dispersed into cells by repeating pipetting through a 5 ml pipette and then through a Pasteur pipette. The total isolate contained zona glomerulosa cells, zona fasciculata cells, erythrocytes, connective tissue elements and cell debris. Erythrocytes and cell debris were discarded by centrifugation on a Ficoll–Histopaque solution. Briefly, the total isolate was centrifuged at 200 *g* and resuspended in 6 ml Hank's solution and layered onto 6 ml of a Ficoll–Histopaque solution consisting of Ficoll [Type 400] (5.7 g/100 ml) and sodium diatrizoate (9 g/100 ml) adjusted to a density of 1.007 $\pm$ 0.001. After centrifugation at 400 *g* for 30 min at 20°C, glomerulosa-fasciculata cells were accumulated at the interface between the Hank's solution and Ficoll–Histopaque solution. The cells were then recovered, resuspended and washed twice with MEM supplemented with 12% dextran-coated charcoal-treated fetal calf serum, 10 U/mol penicillin, 50 μg/ml streptomycin and $NaHCO_3$ (2 mg/ml) (MEMS). Cells recovered were counted, and the viability assessed by trypan blue exclusion. Cells were plated in 24-well Petri dishes in 1 ml MEMS at a density of 2.5×10^5 glomerulosa-fasciculata cells/ml under 5% carbon dioxide in humidified air at 37°C; the medium was replaced every third day of culture when appropriate. Before ACTH stimulation, medium was removed, cells were washed twice with MEM and the incubation was started by the addition of MEMS in presence or absence of ACTH or 8 Br-cAMP.

Steroid determination

At the end of an incubation, the medium was recovered and centrifuged at 200 *g* for 5 min at 4°C and the supernatant frozen until assayed. Steroid levels were determined by procedures originally

Table 1. Percentage cross-reaction of various steroids with antisera raised against 11-deoxycorticosterone-3-*O*-carboxymethyloxime-BSA (DOC-3CMO), corticosterone-3-*O*-carboxymethyloxime-BSA (CORTICO-3CMO), 11-deoxycortisol-3-*O*-carboxymethyloxime-BSA (11-DOC-3CMO) and cortisol-3-*O*-carboxymethyloxime-BSA (CORT-3CMO)

Steroids	DOC-3CMO	CORTICO-3CMO	11-DOC-3CMO	CORT-3CMO
Deoxycorticosterone	**100.0**	14.6	26.6	ND
Corticosterone	0.9	**100.0**	0.3	2.1
11-Deoxycortisol	0.5	0.9	**100.0**	17.6
Cortisol	<0.1	1.9	1.4	**100.0**
17-Hydroxypregnenolone	<0.1	<0.1	ND	<0.1
17-Hydroxyprogesterone	0.4	ND	ND	0.2
11β-hydroxyandrostenedione	<0.1	2.3	<0.1	<0.1
Aldosterone	ND	1.7	<0.1	<0.1
Androst-5-ene-3β,17β-diol	<0.1	<0.1	<0.1	ND
Testosterone	0.4	ND	<0.1	<0.1
Androstane-3α17β-diol	<0.1	ND	ND	ND
Androstane-3β,17β-diol	<0.1	ND	ND	ND
Estrone	<0.1	<0.1	<0.1	ND
Estradiol	ND	<0.1	<0.1	<0.1

ND: not determined.

devised for the analysis of steroids in plasma [18]. In brief, the medium was extracted with ethyl ether and the extract was chromatographed on a LH-20 column. Pregnenolone, 17-hydroxypregnenolone, progesterone, 17-hydroxyprogesterone, dehydroepiandrosterone, and androstenedione were determined as previously described [18]. Fractions containing deoxycorticosterone, corticosterone, 11-deoxycortisol, cortisol, 11β-hydroxyandrostenedione and aldosterone were also collected from the LH-20 column and analyzed by RIA. Table 1 illustrates the specificity of antisera against deoxycorticosterone-3-*O*-carboxymethyloxime-BSA, corticosterone-3-*O*-carboxymethyloxime-BSA, 11-deoxycortisol-3-*O*-carboxymethyloxime-BSA, cortisol-3-*O*-carboxymethyloxime-BSA used for the determination of deoxycorticosterone, corticosterone, 11-deoxycortisol and cortisol, respectively. Specificity of the 11β-hydroxyandrostenedione-3-*O*-carboxymethyloxime-BSA and aldosterone-3-*O*-carboxymethyloxime-BSA antisera used to measure 11β-hydroxyandrostenedione and aldosterone was comparable to data previously reported [19, 20]. Intra-assay variation for steroid analysis was less than 16%; all culture medium samples from the same experiment were assayed simultaneously to avoid inter-assay variation.

Products of the metabolism of tritiated steroids were assayed as follows. 1 ml of medium was extracted twice with 5 ml ethyl ether–acetone (1:1, by vol), the extract was evaporated to dryness and the remaining residue was dissolved in a mixture of methanol–water (1:1, by vol) subjected to HPLC analysis using a Waters model 510 chromatograph (Waters Instruments, Milford, Mass) and a C_{18} column (Radial-Pak, Waters Instruments, Milford, Mass). Gradient of methanol–water (1:1, by vol) to 41% tetrahydrofuran–acetonitrile (1:1, by vol) over a 35-min period was used for the analysis of cortisol, corticosterone and 11-deoxycorticosterone metabolism. Products of the metabolism of androstenedione were analyzed using a mixture water–methanol–tetrahydrofuran–acetonitrile (50:35:10:5, by vol). The formation of tritiated metabolites was calculated by dividing the amount of radioactivity in the product peak by the sum of the radioactivity in all of the peaks recovered after chromatography, thus obtaining the percentage conversion to that product. Percentage conversion was expressed in picomoles of product formed from the known amount of precursor added.

Calculations

RIA data were analyzed using a program based on model II of Rodbard and Lewald[21]. Medium steroid levels are shown as the means ± SEM of duplicate determinations of individual samples. Statistical significance was measured according to the multiple-range test of Duncan–Kramer[22]. Dose–response curves as well as ED_{50} values were calculated using a weighted iterative non-linear least square regression [23].

RESULTS

To assess optimal steroidogenic response in glomerulosa-fasciculata cells over the 7-day period of primary culture, glomerulosa-fasciculata cells were incubated for 24 h in presence or absence of ACTH (10 nmol/l) at different period of time after plating; medium was assayed for basal and ACTH-stimulated secretion of cortisol and androstenedione (Table 2). The basal secretion of both steroids remained low for the first 2 days while it rapidly rose to reach a plateau

Table 2. Secretion of cortisol and androstenedione by glomerulosa-fasciculata cells over 24 h under basal conditions and in response to ACTH (10 nmol/l). Incubation begun after 24, 48, 72, 96, 120 and 144 h after plating of glomerulosa-fasciculata cells. Results are expressed in nmol/l and values are means ± SEM for three wells

Time after plating (h)	Cortisol		Androstenedione	
	Basal	ACTH	Basal	ACTH
24	35.5 ± 2.9	1116 ± 121	1.7 ± 0.1	68.9 ± 4.4
48	30.5 ± 2.2	1222 ± 307	2.1 ± 0.2	92.9 ± 4.2
72	142.5 ± 8.6	1005 ± 191	5.3 ± 1.0	84.3 ± 7.9
96	185.5 ± 7.1	1130 ± 105	11.2 ± 3.7	93.4 ± 2.6
120	240.6 ± 9.9	1259 ± 200	15.3 ± 0.9	94.1 ± 4.4
144	201.6 ± 9.6	1289 ± 301	14.5 ± 4.3	83.4 ± 4.4

Table 3. Metabolism of tritiated cortisol (3.6 pmol), corticosterone (CORTICO) (4.7 pmol), deoxycorticosterone (DOC) (6.9 pmol) and androstenedione (Δ4) (3.7 pmol) by glomerulosa-fasciculata cells in primary culture. Cells were incubated with the tritiated marker for 48 h. Analysis of radioactive steroids formed was carried out by HPLC. Results are expressed in picomoles of product formed from the known amount of precursor added

Radiolabeled steroid	Cortisol	CORTICO	DOC	Δ4	11β-OH-Δ4
[^{3}H]Cortisol	3.4	ND	ND	ND	ND
[^{3}H]CORTICO	0.9	3.4	ND	ND	ND
[^{3}H]DOC	1.1	5.1	0.4	ND	ND
[^{3}H]Δ4	ND	ND	ND	0.3	3.0

ND: not detected.

on day 4 at levels 6–7-fold higher than those observed during the first 2 days of culture ($P < 0.01$). It can also be seen in Table 2 that the maximal response of cortisol and androstenedione secretion to ACTH remained fairly stable throughout the 7-day period. A similar pattern of secretion was observed for pregnenolone, 17-hydroxypregnenolone, progesterone, 17-hydroxyprogesterone, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol, dehydroepiandrosterone and 11β-hydroxyandrostenedione (data not shown). Based on these results, a 2-day period of preincubation after plating cells was then allowed systematically before assessment of steroidogenesis.

Figure 1 shows the effect of a single addition of 10 nmol/l ACTH on the C-19 steroid and C-21 steroid secretion for a period of 48 h. ACTH caused a marked accumulation in the incubation medium of almost all steroids except pregnenolone, 17-hydroxypregnenolone, 17-hydroxyprogesterone and deoxycorticosterone which increased rapidly in the first 12 h of incubation to approximately 5–20 nmol/l; these levels were maintained or even slightly decreased. Although the exact timing of this change in the pattern of secretion varied sometimes slightly for different pool of cells, these four steroids never accumulated in the medium. After 48 h of incubation, cortisol,11β-hydroxyandrostenedione,corticosterone, 11-deoxycortisol, androstenedione and progesterone were the major steroids found in medium at concentrations of 3847 ± 583, 320 ± 57, 272 ± 30, 111 ± 15, 100 ± 4 and 59 ± 5 nmol/l, respectively. Less than 11 nmol/l of aldosterone and dehydroepiandrosterone were recovered.

Figure 2 shows a ACTH concentration-dependent stimulation of all steroids measured. A steroidogenic response was observed with the half-maximal effect occurring at ACTH concentrations varying between 1.7×10^{-11} and 1.1×10^{-10} mol/l for the 12 steroids with no significant difference. Even if the degree of stimulation in steroid production was variable between each experiment, the concentration of ACTH needed to achieve the half-maximal effect on steroid stimulation remained, however, unchanged. Incubation of glomerulosa-fasciculata cells with 8 Br-cAMP increased the secretion of corticosterone, cortisol, androstenedione and 11β-hydroxyandrostenedione in a dose-dependent manner (Fig. 3). As illustrated in Fig. 3, maximal stimulation of cortisol, corticosterone and 11β-hydroxyandrostene-

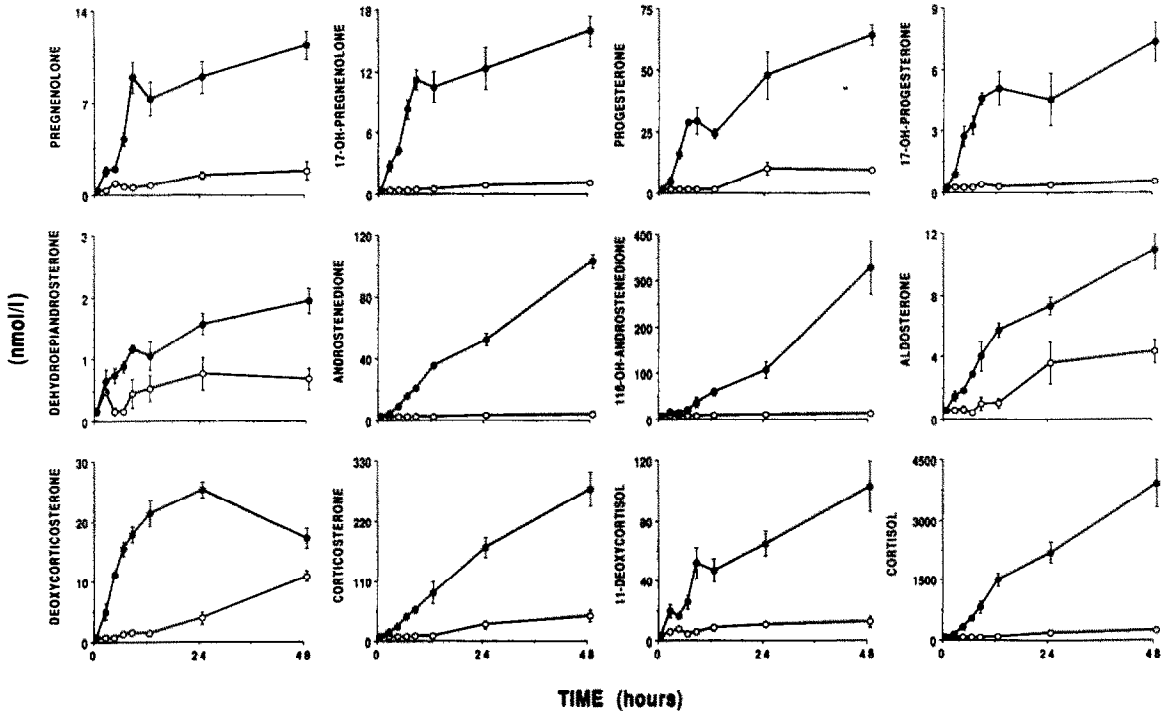

Fig. 1. Secretion of 12 steroids by guinea-pig glomerulosa-fasciculata cells maintained in the absence (○) or the presence (●) of 10 nmol/l ACTH for a period of 48 h. Each data point represents the mean ± SEM of results from triplicate incubations.

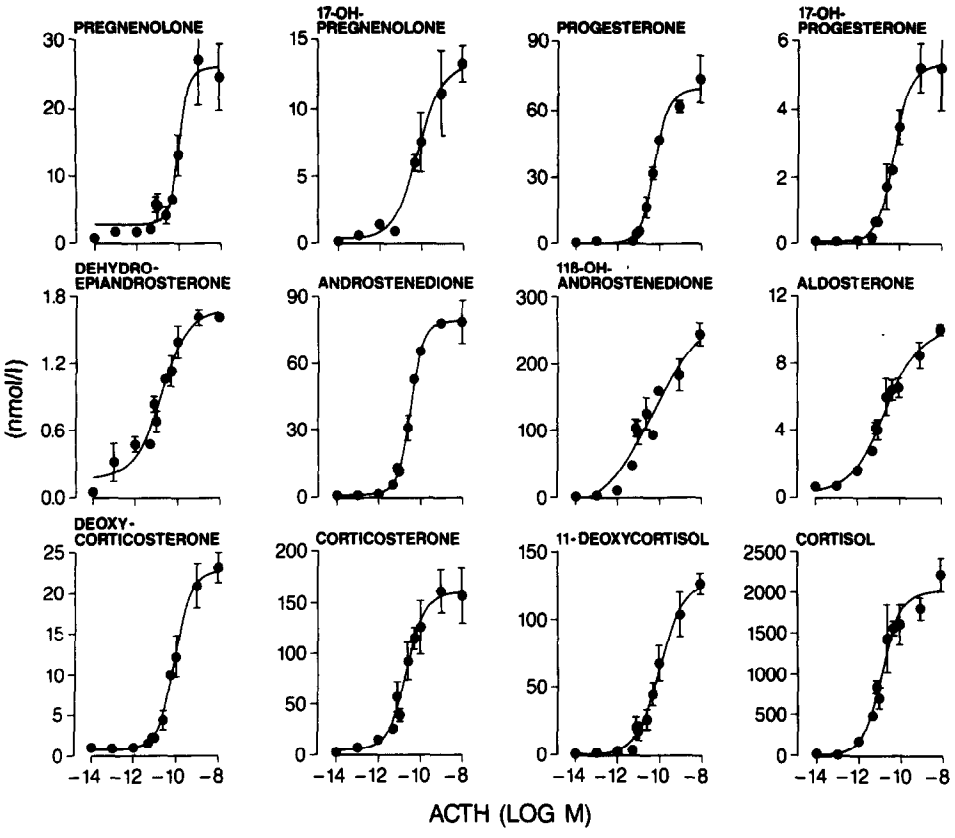

Fig. 2. Effect of ACTH on the secretion of 12 steroids by guinea-pig glomerulosa-fasciculata cells. Glomerulosa-fasciculata cells were maintained for 24 h in presence of ACTH at various concentrations (0.01 pmol/l–10 nmol/l). Each data point represents the mean ± SEM of results from triplicate incubations.

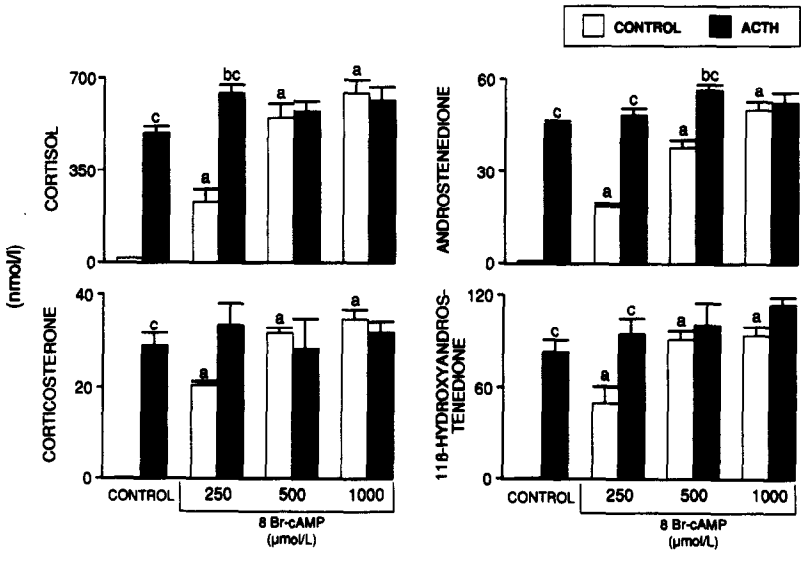

Fig. 3. Secretion of steroids by guinea-pig glomerulosa-fasciculata cells maintained in the presence of 250, 500 or 1000 μmol/l of 8-bromoadenosine 3′, 5′-cyclic monophosphate (8 Br-cAMP) alone (open columns) or in combination with 10 nmol/l ACTH (hatched columns) for a period of 24 h. Each data point represents the mean ± SEM of results from triplicate incubations. Statistical significance: (a) control vs 8 Br-cAMP ($P < 0.01$); (b) ACTH vs ACTH + 8 Br-cAMP ($P < 0.01$); and (c) 8 Br-cAMP vs ACTH + 8 Br-cAMP ($P < 0.01$).

dione was reached with 500 μmol/l 8 Br-cAMP, while the addition of 1 mmol/l 8 Br-cAMP was necessary to achieve maximal stimulation of androstenedione secretion. Concomitant incubation of 8 Br-cAMP (1000 μmol/l) and ACTH (10 nmol/l) did not further increase secretion of corticosterone, cortisol, androstenedione and 11β-hydroxyandrostenedione over those observed when cells were incubated with ACTH alone (Fig. 3). Similar results were obtained for pregnenolone, 17-hydroxypregnenolone, progesterone, 17-hydroxyprogesterone, corticosterone, 11-deoxycorticosterone and 11-deoxycortisol (data not shown).

Since 11β-hydroxyandrostenedione appears to be the major C-19 steroid secreted by the glomerulosa-fasciculata cells, we carried out an experiment to determine its precursor. To this effect, glomerulosa-fasciculata cells were incubated for 48 h with labeled corticosterone, cortisol, 11-deoxycorticosterone and androstenedione. As shown in Table 3, analysis by HPLC of the medium indicated that only androstenedione can be converted into 11β-hydroxyandrostenedione. Moreover, incubation for 48-h with unlabeled androstenedione (250 and 2500 nmol/l), led to an accumulation of 11β-hydroxyandrostenedione as detected by radioimmunoassay, further confirming the rapid transformation of the substrate (data not shown).

DISCUSSION

A large number of experiments using freshly-dispersed adrenal cells from guinea-pig have shown that both C-21 steroids and C-19 steroids are secreted in cell medium in basal conditions as well as in response to ACTH [14–16]. By their high levels of production, cortisol, corticosterone, androstenedione and 11β-hydroxyandrostenedione contribute to a large extent to the adrenal steroid secretion in this animal species. Incubation of labeled precursors, namely pregnenolone, further confirmed the formation of both C-21 and C-19 steroids by dispersed guinea-pig fasciculata cells [24]. The possibility to maintain these cells in culture, as previously demonstrated by Black *et al.*[17], reduces the chance of cell alterations due to enzymatic treatment involved in cell isolation while further allowing the cells to be exposed to different compounds under controlled conditions. Interestingly, the qualitative analysis of steroidogenic secretion of glomerulosa-fasciculata cells in primary culture carried out by Black *et al.*[17] demonstrated that the steroidogenic pathway is quite comparable to that which operates in freshly-dispersed cells. In the present study, we have characterized the steroidogenic secretion of the guinea-pig glomerulosa-fasciculata cells in primary culture, confirming that both C-19 steroids and C-21 steroids are produced. Dehydroepiandrosterone, androstenedione and 11β-hydroxyandrostenedione were generated, while dehydroepiandrosterone and androstenedione were rapidly converted into 11β-hydroxyandrostenedione which becomes the marker of C-19 steroid production. Such data were in agreement with our previous observations showing that 11β-hydroxyandrostenedione is the predominant C-19 steroid in the guinea-pig adrenal gland as well as the major plasma C-19 steroid of adrenal origin [25]. While there is, in human adrenal, formation and secretion of 11β-hydroxyandrostenedione as observed in the guinea-pig, this steroid appears to be secreted in low amount in comparison to several other C-19 steroids, namely, dehydroepiandrosterone and its sulfate, indicating a marked difference in steroidogenic pathway between these two species.

Cortisol responsiveness to ACTH, with a K_m of approximately 50 pmol/l in our *in vitro* model, agrees with previously published data obtained with bovine adrenal cells in culture [26]. We further demonstrated herein that the ACTH responsiveness is not significantly different in regard with a series of C-21 steroids and, even more important, with the C-19 steroids indicating that ACTH is an important modulator of C-19 steroid production by guinea-pig adrenals and plays an equivalent role in the secretion of both C-21 and C-19 steroids. Our data also indicate that in relative terms, steroidogenic production of C-21 and C-19 steroids remained unchanged during the 7-day period in culture. Despite variations in the total steroidogenic production observed between experiments, the ratio between C-19 steroids and C-21 steroid secretion in basal conditions as well as after addition of ACTH remains quite constant. 8 Br-cAMP caused a dose-dependent stimulation of both C-19 and C-21 steroids; the addition of 8 Br-cAMP reproduced the effect of ACTH on steroid secretion. Our study does not however exclude the possibility that chronic effects of long-term ACTH stimulation may alter this mechanism. While it is difficult to explain a specific control of C-19 steroid production on the basis of the present data, it is suggested that if such a control exists, it might be located at a step distal with respect to ACTH generation of cAMP and to the side-chain cleavage of cholesterol. We previously observed that treatment with an antiandrogen in patients castrated for cancer of the prostate caused a marked reduction of adrenal 17,20-desmolase activity [9]. This concept can also be supported by the regulation of testicular 17,20-desmolase which has been found to be an important enzyme for C-19 steroid production [27].

Although it is generally believed that 11β-hydroxyandrostenedione originates from the hydroxylation of androstenedione, it was previously reported that cortisol can be transformed into 11β-hydroxyandrostenedione by the adrenal gland [28, 29]. Given the large amounts of cortisol and corticosterone produced by the adrenal, we can presume that even a low rate of 17,20-desmolase activity would have a tremendous effect on 11β-hydroxyandrostenedione production which is one order of magnitude lower

than that of cortisol. In such a case, evidently 11β-hydroxyandrostenedione could not serve as a marker of androstenedione formation by the adrenal. The rapid conversion of androstenedione into 11β-hydroxylated derivatives noted by Deshande *et al.*[29] is also observed in the present experiment while, contrary to these authors, we did not find any data supporting the view that cortisol undergoes transformation into C-19 steroids. In addition, incubation of 11-deoxycorticosterone and corticosterone further confirmed the experiment with cortisol. The production of 11β-hydroxyandrostenedione in our *in vitro* model thus reflects the adrenal C-19 steroid formation and since 11β-hydroxyandrostenedione is, in human, an exclusive adrenal C-19 steroid marker [30], it might be extremely important to understand well its mechanism of secretion.

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