Self-inhibition of steroid secretion by amphibian adrenocortical cells is not mediated through glucocorticoid receptors

P. Netchitailo, A. Larcher, F. Leboulenger, M. Feuilloley, D. Philibert* and H. Vaudry

Groupe de Recherche en Endocrinologie Moléculaire, CNRS URA 650, Unité Affiliée à l'INSERM, Université de Rouen, 76134 Mont-Saint-Aignan Cedex, France
*Roussel-Uclaf Laboratories, Route de Noisy, 93230 Romainville, France

(Requests for offprints should be addressed to H. Vaudry)

REVISED MANUSCRIPT RECEIVED 3 January 1991

ABSTRACT

To investigate a possible direct action of glucocorticoids on adrenal steroidogenesis, the effect of corticosterone on the conversion of pregnenolone into various metabolites by frog adrenal tissue was examined. Frog interrenal slices were incubated with \(^3\text{H}\)pregnenolone (1 mCi/ml) and the various labelled metabolites analysed by reverse-phase high-performance liquid chromatography. With the methanol gradient used, five identified steroids were resolved: progesterone, 11-deoxycorticosterone, corticosterone, 18-hydroxycorticosterone and aldosterone. Corticosterone (10 µg/ml) induced a 45–80% decrease in all steroids synthesized from \(^3\text{H}\)pregnenolone. In contrast, the glucocorticoid agonist dexamethasone did not reduce the rate of conversion of pregnenolone into its metabolites. In addition, the inhibitory effect of corticosterone was not reversed by the specific glucocorticoid antagonist RU 43044. These results show that corticosterone exerts a direct inhibitory effect on adrenal steroid secretion. In addition, our data indicate that the ultra-short regulation induced by corticosterone is not mediated through glucocorticoid receptors. Journal of Molecular Endocrinology (1991) 6, 249–255

INTRODUCTION

There is now considerable evidence that end-product glucocorticoids can modulate adrenocortical steroid secretion. Most of the studies dealing with this issue indicate that glucocorticoids reduce the capacity of adrenal cells to secrete corticosteroids by inhibiting both corticotrophin-releasing factor release from hypothalamic neurones (Jones, Hillhouse & Burden, 1977; Moldow & Fischman, 1982) and adrenocorticotropic hormone (ACTH) release from anterior pituitary corticotrophs (Arimura, Bowers, Schally et al. 1969; Kraicer & Milligan, 1970; Giguère, Labrie, Côté et al. 1982). In addition, several studies indicate that glucocorticoids suppress steroidogenesis through direct negative-feedback regulation of adrenocortical cells (Birmingham & Kurlents, 1958). For instance, it has been shown that glucocorticoids inhibit steroid production from isolated mammalian and avian adrenocortical cells (Carsia, Scanes & Malamed, 1987). However, other reports indicate that glucocorticoids exert a facilitatory effect on steroid secretion (Kowal, Mattson & Cheng, 1985). In particular, chronic exposure to dexamethasone has been found to enhance both the formation of cyclic AMP (cAMP) and the secretion of corticosteroids induced by ACTH in ovine adrenocortical cells (Darbeida & Durand, 1988, 1990).

Since the rate of corticosteroid production in the absence of ACTH is very low in mammals, the effect of glucocorticoids on spontaneous corticosteroid secretion has never been investigated. In mammals, therefore, the ultra-short feedback exerted by glucocorticoids on ACTH-stimulated adrenocortical cells can be ascribed either to a reduction in the sensitivity of the cells to ACTH or to an inhibitory effect on the biosynthetic pathway of corticosteroids.

We have previously shown that frog adrenocortical (interrenal) tissue produces high amounts of corticosteroids, even in the absence of corticotrophic factors (Leboulenger, Delarue, Bélanger et al. 1982), and thus represents an ideal model to
investigate the mechanism of action of substances which inhibit spontaneous release of corticosteroids (Netchitailo, Delarue, Perroteau et al. 1985; Netchitailo, Lihrmann, Perroteau et al. 1986). In the present study we have used frog adrenal slices to examine the effect of exogenous glucocorticoids on the conversion of labelled pregnenolone.

MATERIALS AND METHODS

Animals

Adult male frogs (Rana ridibunda), weighing 40–60 g, were purchased from a commercial source (Couétard, St-Hilaire de Riez, France). They were maintained in glass tanks at 8 ± 1 °C, under running water, on a 12-h light/dark cycle for at least 8 days before the experiments.

In-vitro incubations

Frogs were killed by decapitation and the interrenal tissue was carefully dissected. The tissue fragments were then preincubated in 700 μl fresh Ringer (15 mM Hepes buffer, 112 mM NaCl and 20 mM CaCl2) in the absence or presence of corticosterone, dexamethasone (Sigma Chemical Co. Ltd, St Louis, MO, U.S.A.; 10 μg/ml each) or corticosterone (10 μg/ml) plus RU 40344 (an anti-glucocorticoid; Roussel-Uclaf Laboratories, Romainville, France; 100 μg/ml) in Teflon vials for 2 h. Each vial contained fragments from six interrenal glands (8.2 ± 1 mg fresh tissue). At the end of the preincubation, the tissues were rinsed and then incubated with 0.1 μM tritiated pregnenolone (Δ5-[4,7-3H] pregnenolone; 19 Ci/mmol; Amersham International plc, Amersham, Bucks, U.K.) for 4 h in the presence or absence of the test substances cited above. The incubation was conducted at 24 °C in 700 μl Ringer supplemented with 30 μl propylene glycol to increase the solubility of the radioactive tracer.

Extraction procedure

At the end of the incubation, the medium and interrenal fragments were separated by filtration (Whatman 3M) and the fragments rinsed with 1 ml Ringer. Tissue fragments were transferred in 5 ml distilled water, sonicated and then submitted to three successive freeze–thawing steps. Steroids contained in the tissue and in the incubation medium were extracted three times with dichloromethane (v/v) and the extracts washed with 0.1 M NaOH (v/v), 0.1 M HCl (v/v) and distilled water. Each sample was evaporated under an air stream, and the dry residue kept frozen until chromatographic analysis. Recovery of steroids through the extraction procedure was >70%.

High-pressure liquid chromatography (HPLC)

The dry samples were redissolved in 100 μl of a solution of methanol and 0.1% trifluoroacetic acid in water (1:1, v/v). Tritiated steroids contained in each sample were separated by reverse-phase HPLC on a Gilson liquid chromatograph (model 302) equipped with a Zorbax ODS/C18 column (0.46 × 25 cm) as previously described (Feuilloley, Netchitailo, Delarue et al. 1987). The gradient used (50–100% methanol over 55 min with a 10 min isocratic step at 75% methanol) is presented in Fig. 1. The flow rate was 1 ml/min, and 0.5 ml fractions were collected. A 400 μl sample of each HPLC fraction was mixed with 6 ml liquid scintillator (Hydroluma, Lamuc, The Netherlands) and counted in a liquid scintillation counter (LKB 1211 Minibeta). Synthetic standards (100 ng) were analysed using the same gradient, and their retention time was characterized by u.v. absorption at 240 nm using an ISCO u.v. detector (model 1840).

Calculations

To standardize the experiments, the relative levels of each steroid, calculated as the fraction of the total amount of labelled steroids (sum of the radioactive steroids contained in the tissue and incubation medium, except pregnenolone for the incubation medium), were used for analysis of the data. Student’s t-test was employed to determine statistical significance between control and treatment experiments.

RESULTS

HPLC analysis of [3H]pregnenolone-prelabelled interrenal tissue indicated that frog adrenal slices have the capacity to synthesize five major identifiable steroids: progesterone, 11-deoxycorticosterone, corticosterone, 18-hydroxycorticosterone and aldosterone (Fig. 1). Comparison of the individual HPLC profiles clearly indicates that the absolute amounts of steroids contained in the tissue or released into the incubation medium were lower when the adrenal slices were incubated in the presence of corticosterone than in control conditions (Fig. 1a and b). In contrast, dexamethasone had no apparent effect on corticosteroid biosynthesis (Fig. 1c). Moreover, addition to the glucocorticoid antagonist RU 43044 did not reverse
corticosterone-induced inhibition of pregnenolone conversion (Fig. 1d).

A series of experiments similar to those presented in Fig. 1 were conducted to quantify the effect of corticosterone, dexamethasone and RU 43044 plus corticosterone on the biosynthesis and release of corticosteroids by frog adrenal slices. Figure 2a shows that addition of exogenous corticosterone (10 μg/ml) into the incubation medium caused a significant reduction in the amount of tritiated aldosterone (−67%), 18-OH-corticosterone (−48%), corticosterone (−45%) and deoxycorticosterone (−38%), while the level of [3H]progesterone was not significantly affected. In the extracellular compartment, the concentration of newly synthesized aldosterone (−62%), 18-OH-corticosterone (−68%), corticosterone (−52%), deoxycorticosterone (−39%) and progesterone (−69%) were markedly inhibited (Fig. 2b). Furthermore, addition of the antiglucocorticoid RU 43044 to the incubation medium did not modify the suppressive effect of corticosterone on steroidogenesis (Fig. 2). From Fig. 3 it is clear that dexamethasone did not significantly affect the rate of conversion of [3H]pregnenolone both in the tissue (Fig. 3a) and in the extracellular medium (Fig. 3b).

**DISCUSSION**

There is ample evidence that circulating steroids may exert a direct negative effect on the activity of
the cells from which they originate. In the case of glucocorticoids, it has been shown that corticoste-
on can regulate adrenal cell proliferation (Saito, Mukai, Muraki et al. 1979), inhibit adrenal protein
(Morrow, Burrow & Murrow, 1967) and DNA synthesis (Saez, Morera & Gallet, 1977) and exert local feedback inhibition of steroidogenesis (Carsia & Malamed, 1979, 1983; Kowal et al. 1985).

We have previously shown that, in amphibians, dexamethasone does not exert a direct suppressive effect on adrenal secretion in vitro (Netchitaio, Lihrmann & Vaudry, 1984) and that chronic dexamethasone treatment does not modify in vitro the responsiveness of interrenal tissue to corticotro-
phic stimuli (Leboulenger, Delarue, Jégou et al. 1980). In amphibians, it seems that dexamethasone
can inhibit corticosteroid production through an action on the central nervous system (Jégou, Tonon, Leroux et al. 1980). In mammals, the effect of dexamethasone on the hypothalamo-pituitary-adre-
nal axis is well documented (Bilizikjian & Vale, 1983; Suda, Tomori, Tozawa et al. 1984). These studies generally indicate that dexamethasone is less potent than corticosterone in inhibiting steroidogenesis.

To determine the possible effect of corticosteroids on adrenal steroid production, frog interrenal slices were incubated in the presence of a tritiated steroid precursor, and the newly synthesized steroids ana-
lysed by reverse-phase HPLC. The concentration of non-labelled steroids added to the incubation medium may be considered high when compared with circulating levels reported in the frog (Leboulenger et al. 1982). However, it is probable that concentrations of glucocorticoids in the vicinity of frog adrenocortical cells may be 10–100 times higher than plasma levels, as previously reported in
the rat (Morrow et al. 1967). Our results clearly show that incubation of frog interrenal fragments with exogenous corticosterone results in a marked decrease in the production of all steroids synthesized from [3H]pregnenolone. Furthermore, we have shown that dexamethasone does not affect the rate of adrenal steroidogenesis, in agreement with our previous studies (Netchitaio et al. 1984). These data confirm the results reported by Carsia & Malamed (1983), indicating that only natural glucocorticoids exert a marked inhibition on their own biosynthesis. The fact that dexamethasone did not mimic the suppressive effect of corticosterone on steroid bio-

Journal of Molecular Endocrinology (1991) 6, 249–255
synthesis strongly suggests that the inhibitory effect of corticosterone is not mediated through activation of glucocorticoid receptors. This hypothesis is supported by the observation that the selective glucocorticoid antagonist RU 43044 did not reverse the inhibitory effect of corticosterone on steroid formation.

The mechanism by which glucocorticoids suppress adrenocortical function is currently a matter of speculation. It has been proposed that glucocorticoids could inhibit the binding of ACTH (Latner, Cook & Solanski, 1977) or angiotensin (Campanile & Goodfriend, 1981) to their adrenal receptors. Several reports indicate that steroids may act on transduction mechanisms, for instance by suppressing ACTH-induced cAMP production (Carsia, MacDonald & Malamed, 1981; Kowal et al. 1985). In our model, however, self-inhibition of steroid synthesis cannot be ascribed to a negative action on the binding of corticotrophic factors or synthesis of their second messengers, since the present study was conducted in non-stimulated adrenocortical cells. Another possibility is that glucocorticoids may interact directly with enzymes involved in steroidogenesis through a mechanism of end-product inhibition (Pham Huu Trung, De Smitter, Biogyo & Girard, 1984). In this respect, Carsia, Scanes & Malamed (1984) have shown that self-inhibition of corticosterone production is partly mediated by an increase in 5α-reductase activity which is capable of metabolizing intracellular or extracellular corticosterone. However, our data do not substantiate a specific action of natural glucocorticoids on the activity of a steroidogenic enzyme such as 5α-reductase, since the HPLC data showed that the inhibition of steroids synthesized from pregnenolone was not

**Figure 3.** Effect of dexamethasone (Dexa; 10 μg/ml) on the conversion of [3H]pregnenolone into progesterone (P), deoxycorticosterone (DOC), corticosterone (B), 18-hydroxycorticosterone (OHB) and aldosterone (A) (a) contained in the cells or (b) released into the incubation medium. Values (means ± S.E.M. for three experiments) were calculated from high-performance liquid chromatograms similar to those presented in Fig. 1a and c and are expressed as a percentage of the total amount of radioactivity resolved by high-performance liquid chromatography (excluding [3H]pregnenolone in the incubation medium).
associated with increased output of any other steroid. It is noticeable that corticosterone had no
effect on the intracellular pool of newly synthesized progesterone, while it significantly reduced the
concentration of \(^{1}\text{H}\)progesterone in the incubation medium. Although the origin of this specific effect of
corticosterone on the extracellular pool of progester-
one is unknown, again, it is not attributable to stimulation of glucocorticoid receptors since it was
not affected by RU 43044.

In conclusion, our data indicate that, in the amphibian adrenal gland, exogenous corticosterone
directly inhibits steroidogenesis through a mechan-
ism which does not involve glucocorticoid receptors.

ACKNOWLEDGEMENTS

This study was supported by grants from the Centre
National de la Recherche Scientifique (URA 650)
the Direction des Recherches et Etudes Techniques
(grant No. 87/135) and the Conseil Régional de
Haute-Normandie. A.L. was a recipient of a fellow-
ship from the Direction des Recherches et Etudes Techniques.

REFERENCES

Arimura, A., Bowers, C. U., Schally, A. V., Saito, M. & Miller,
M. C. (1969). Effect of corticotropin-releasing factor, dexam-
ethasone and actinomycin B on the release of ACTH from
rat pituitaries in vitro and in vivo. Endocrinology 85, 300–311.
inhibit corticotropin-releasing factor-induced production of
adrenocortical monophosphate in cultured anterior pituitary
ACTH by isolated rat adrenals and inhibition of corticoid
formation by adrenocortical hormones. Endocrinology 62,
47–60.
potential modulators of angiotensin receptors in bovine
dence for a regulatory role of end-product glucocorticoids in
corticosteroidogenesis. Program of the U.S. Endocrine Society,
Carsia, R. V. & Malamed, S. (1979). Acute self suppression of
corticosteroidogenesis in isolated adrenocortical cells.
Endocrinology 100, 1268–1275.
Carsia, R. V. & Malamed, S. (1983). Glucocorticoid control of
steroidogenesis in isolated rat adrenocortical cells. Biochimica
et Biophysica Acta 763, 83–89.
suppression of corticosteroidogenesis: evidence for a role of
adrenal 5α-reductase. Endocrinology 115, 2464–2472.
nal regulation of avian and mammalian corticosteroidogenesis
in vitro. Comparative Biochemistry and Physiology 88A,
131–140.

Journal of Molecular Endocrinology (1991) 6, 249–255

of glucocorticoid production by cultured ovine adrenocortical
cells. Biochimica et Biophysica Acta 272, 200–208.
Darbeida, H. & Durand, P. (1990). Mechanism of glucocorti-
coid enhancement of the responsiveness of ovine adrenocorti-
cal cells to adrenocorticotropic. Biochemical and Biophysical
Research Communications 166, 1183–1191.
Feuillolé, M., Netchitailo, P., Delarue, C., Lihrmann, I. &
requires the integrity of the microfilament network in
adrenocortical cells. Biochemical and Biophysical Research
Communications 148, 1354–1362.
Giguère, V., Lahrie, F., Côté, J., Coy, D. H., Sueiras-Diaz, J.
& Schally, A. V. (1982). Stimulation of cAMP accumulation
and corticotropin release by synthetic ovine corticotropin-
releasing factor in rat anterior pituitary cells: site of glucocor-
ticoid action. Proceedings of the National Academy of
Sciences of the U.S.A. 79, 3466–3469.
Jégou, S., Tonon, M. C., Leroux, P., Leboulechanger, F.,
Effect of dexamethasone treatment on ACTH and β-LPH
concentrations in frog pituitary and hypothalamus. Advances
in Physiological Sciences 13, 129–133.
Dynamics and mechanisms of corticosteroid feedback in the
hypothalamus and anterior pituitary gland. Journal of
Endocrinology 73, 405–417.
associated with ACTH action. Endocrine Reviews 10,
463–471.
release from adrenohypophysis by corticosterone.
Endocrinology 87, 371–376.
Inhibition of binding of corticotropin-(1–24)-tetracosapeptide
(Synacthen) to membrane receptors of adrenal cortex by
Leboulechanger, F., Delarue, C., Belanger, A., Perrotteau, I.,
Netchitailo, P., Leroux, P., Jégou, S., Tonon, M. C. &
corticosterone and aldosterone in frog. I. Validation of the
methods and evidence for daily rhythms in a natural
environment. General and Comparative Endocrinology 46,
521–526.
Leboulechanger, F., Delarue, C., Jégou, S., Netchitailo, P.,
study of frog (Rana ridibunda Pallas) interrenal function by
use of a perfusion system. III. Influence of previous
hypophysectomy or dexamethasone treatment of donors upon
corticosterone and aldosterone production. General and
Comparative Endocrinology 42, 389–400.
in rat hypothalamic CRF: circadian, stress and steroid
Inhibition of adrenal protein synthesis by steroids in vitro.
Endocrinology 80, 883–886.
Netchitailo, P., Delarue, C., Perrotteau, I., Leboulechanger, F.,
potency of five mineralocorticoid antagonists on aldosterone
biosynthesis in vitro. Biochemical Pharmacology 34,
189–194.
Netchitailo, P., Lihrmann, I., Perrotteau, I., Delarue, C.,
Leboulechanger, F. & Vaudry, H. (1986). Involvement of
cycloheximide-sensitive mediators in the steroidogenic action
of adrenocorticotropic and angiotensin II. Journal of Steroid
Biochemistry 25, 59–64.
effect of dexamethasone on corticosteroid production in the

