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STRUCTURE–ACTIVITY RELATIONSHIP AND THE MODE OF ACTION OF CORTICOSTEROID FEEDBACK ON THE SECRETION OF CORTICOTROPHIN-RELEASING FACTOR (CORTICOLIBERIN)

M. T. JONES and E. W. HILLHOUSE
Sherrington School of Physiology, St. Thomas's Hospital Medical School, London, SE1 7EH, England

SUMMARY

Corticosteroid feedback mechanisms were investigated at the hypothalamic level using the technique of the rat hypothalamus *in vitro* and at the pituitary level using basal hypothalamic lesioned rats. Both fast and delayed corticosteroid feedback effects were demonstrated at the hypothalamic and pituitary levels with doses of corticosteroids within or near the physiological range. These two phases of feedback were separated temporally by a 'silent period' during which no feedback was apparent.

Studies on the mechanism of action of corticosteroids at the hypothalamic level showed that the fast feedback mechanism acts *via* the inhibition of release whilst the delayed feedback mechanism acts *via* inhibition of both synthesis and release. The fast feedback action of corticosterone does not appear to act *via* the excitation of neuroinhibitory pathways since neither picrotoxin nor phentolamine prevented the feedback action of corticosteroids *in vitro*.

Corticosterone inhibition of corticotrophin-releasing factor (CRF) release was overcome by depolarization of the membrane with K^+ suggesting that the mechanism of action of the fast feedback of corticosteroids is via membrane stabilization. Increasing the concentration of Ca^{2+} in the medium from 0–6 mM caused an increase in the release of CRF which was mimicked by Str^{2+} (6 mM) and blocked by Mn^{2+} (12 mM). Corticosterone inhibited the CRF release due to Ca^{2+} suggesting the possibility that the fast feedback action of corticosteroids is due to an effect on Ca^{2+} flux. The fast feedback receptor in the hypothalamus is an unique example of a steroid acting via an immediate effect on the cell membrane.

Structure–activity studies showed that different steroids have different effects on both fast and delayed feedback at the hypothalamus. Models of the basic steroid structures required, for affinity and efficacy, are presented.

INTRODUCTION

The secretion of ACTH in the rat is under the control of two temporally and dynamically distinct phases of corticosteroid negative feedback inhibition [8, 18]. The first phase of inhibition occurs immediately after the adminstration of corticosteroids and exhibits unidirectional rate-sensitivity [18] whilst the second phase of inhibition does not occur until some hours after corticosteroid administration and exhibits proportionality to the administered dose [8]. The two phases of feedback have been designated fast or rate-sensitive feedback and delayed or level-sensitive feedback respectively and the period in between the two phases of feedback is termed 'the silent period' [42].

Studies on these two periods of feedback inhibition have suggested that they are mediated by different corticosteroid receptor mechanisms [19]. However, the actual site and mechanism of action of the corticosteroids is undetermined and remains the subject of some controversy [23, 24]. Most studies implicate the pituitary gland as the major locus of feedback action [23, 32] but a great deal of circumstantial evidence suggests that corticosteroids also feedback at the level of the hypothalamus. This hypothesis is supported by the studies of De Kloet, Wallach and McEwen [25] who have reported that $[H]^3$-corticosterone binds to cell nuclei and to soluble macromolecules in both the hypothalamus and the anterior pituitary.

In a previous study we have shown that adrenalectomy causes an increase in basal and stimulated CRF secretion [3, 17], and this hypersecretion can be suppressed by pretreatment with corticosteroids. We have now extended these studies to examine directly the effect of corticosteroids on the secretion of CRF from the rat hypothalamus *in vitro* by their addition to the incubation medium. By so doing we could test for both fast and delayed feedback at the hypothalamus.

The viability of the preparation has been established [3, 14] and electronmicrographs of the tissue following incubation have revealed a good preservation of the fine structure of the median eminence [20]. Studies with the hypothalamus of the rat *in vitro* have shown that it is able to secrete CRF [16, 20], vasopressin and oxytocin [4, 5] and radioimmunoassayable LH-RH and TRH [Jeffcoate *et al.*, unpublished observations]. The rat hypothalamus also secretes

substances which modulate the secretion of radioimmunoassayable GH *in vitro* [Hamilton *et al.*, unpublished observations].

In view of the striking temporal separation of the two periods of feedback it seems likely that they exert their effects *via* different mechanisms of action. We therefore tested this possibility using the hypothalamus *in vitro*. We also examined structure–activity relationships of various steroids on the two corticosteroid feedback mechanisms.

We investigated corticosteroid feedback at the anterior pituitary level by pretreating basal hypothalamic lesioned rats with corticosteroids and testing the effect of such treatment on CRF-induced release of ACTH.

To estimate the doses of corticosterone which are physiologically relevant, we cannulated the left adrenal vein (the right is less accessible) and estimated corticosterone in the adrenal effluent under stress conditions.

A preliminary report of these studies has been published previously [15].

METHODS

Male Wistar-derived rats weighing 100–150 g from a specific pathogen-free colony bred in the Medical School Animal House were used in these experiments. They were allowed free access to rat chow and water, or, in the case of adrenalectomised rats, 0.9% NaCl. The animals were housed in an air-conditioned room with a controlled light cycle (lights on 07.00–21.00 h). The operation of bilateral adrenalectomy was performed by the dorsal midline approach.

Corticosterone secreted by the left adrenal estimated by cannulation and collection of effluent blood

200–250 g rats (male) were anaesthetised with pentobarbitone (60 mg/kg.p.) and tracheotomised. The left adrenal vein was exposed for cannulation proximal to its entry into the left renal vein. The inferior phrenic veins were ligated. A stab wound was made in the left flank and the cannula, consisting of fine polythene tube tipped with a No. 18 gauge hypodermic needle with a 90° bend passed through this aperture. 250 units of heparin was given and the venous ligature was tied. The laparotomy incision was closed. Adrenal venous effluent was collected from 65 rats over a period from 10–80 min. The flow rate was measured in successive 10 min intervals. The blood was spun and the supernatant plasma was collected for corticosterone analysis.

Removal and incubation of hypothalami

The removal and incubation of rat hypothalami *in vitro* was carried out as described previously [14–22]. The blocks of hypothalamic tissue used in these studies included the whole hypothalamus extending from the preoptic area to the mammillary bodies. This is important since preliminary studies have shown that the site of action of acetylcholine is in the preoptic region (Bock *et al.*, unpublished observations).

Assay of CRF

The CRF activity in hypothalamic incubating fluid or in neutralised hypothalamic extracts was measured using 48 h basal hypothalamic-lesioned rats [16]. The CRF was administered via the femoral vein and the end-point of the assay was the *in vitro* corticosterone production of adrenals excised 15 min following the i.v. injection. The corticosterone was estimated fluorometrically.

Hypothalamic extracts for CRF activity

Extracts of the hypothalamus were prepared according to the method of Chan, Schal and Saffran [7]. The hypothalamus was removed and immediately placed in 100 μl of 0.1 N HCl in a tissue homogeniser. The extract was stored in the refrigerator and was neutralised with sodium bicarbonate before use.

Protocol in studies on the fast feedback mechanisms

(*a*) *At the hypothalamus.* In these experiments acetylcholine (3 pg/ml) and various doses of steroids were added simultaneously to the hypothalamic medium and the release of CRF during the subsequent 10 min incubation measured. Hypothalami used in these experiments were obtained from 1-day-adrenalectomised rats unless otherwise stated.

When a steroid was tested for antagonism, it was given 5 min prior to the simultaneous addition of antagonist, corticosterone and acetylcholine. The medium was then assayed for CRF activity.

(*b*) *At the anterior pituitary.* 48 h basal hypothalamic-lesioned rats were injected s.c. with 70 μg corticosterone and their response to a standard i.v. dose of CRF was tested 5, 10, 20, 30 and 50 min later. CRF for these experiments was obtained from hypothalami stimulated by 5-hydroxytryptamine to release CRF as described previously [12]. 0.5 ml of 5-hydroxytryptamine stimulated hypothalamic medium was injected into each animal.

Protocol on studies on the delayed feedback mechanisms

(*a*) *At the hypothalamus.* (i) In vivo. The steroid was suspended in arachis oil and administered s.c. 4 or 24 h prior to the removal of the hypothalamus. The hypothalami were subjected to 3 incubation periods of 10 min in the presence of acetylcholine (3 pg/ml) during a total incubation period of 2 h.

(ii) In vitro. The hypothalami were exposed to the steroids for a period of 30 min. The medium was then replaced by fresh, steroid-free medium for 90 min, with a change of fresh medium every 30 min. The hypothalami were then challenged with acetylcholine and the CRF released during the subsequent 10 min incubation period measured.

The procedure for antagonism *in vitro* was similar except that the putative antagonist was given 15 min prior to the 30 min incubation with corticosterone and the putative antagonist.

(*b*) *At the anterior pituitary.* The steroids were administered in arachis oil to 48 h basal hypothalamic-lesioned rats. ACTH release was stimulated with either a median eminence extract or with 0.5 ml of incubating medium obtained from hypothalami stimulated to release CRF by 3 pg acetylcholine [16]. This medium contains ADH and oxytocin as well as CRF but the amounts of neurohypophysial hormones present do not affect the pituitary response to CRF [17]. Animals were tested 4 and 24 h after steroid pretreatment.

Silent period studies

Hypothalami were exposed to a steroid for 30 min and the medium was then replaced by fresh, steroid-free medium. The hypothalami were challenged with a standard dose of acetylcholine (3 pg/ml) at various time intervals when the steroid was either present or absent and the release of CRF during the subsequent 10 min incubation period measured.

Calculation and expression of results

CRF activity was expressed in nmol corticosterone/100 mg adrenal/h. The differences in mean CRF activity were tested for significance by Student's *t*-test.

RESULTS

Corticosterone secreted by the left adrenal

Table 1 shows the plasma flow rate and plasma corticosterone concentration in adrenal venous blood over the 80 min period.

Hypothalamic studies

(1) *Fast feedback (FFB).* (*a*) *Agonists.* Table 2 shows the effect of various doses of corticosterone (compound B) added simultaneously with acetylcholine (3 pg/ml) on the secretion of CRF from the hypothalamus of the rat *in vitro.* Corticosterone in a dose of 0.1 pg/ml had no effect upon the release of CRF in response to acetylcholine. However, increasing the dose of corticosterone to 1 pg/ml reduced the CRF response to acetylcholine ($P < 0.01$) but had no effect upon the basal secretion.

Table 2. The effect of acetylcholine (ACh) and corticosterone (B) on the secretion of CRF from the hypothalamus *in vitro.* Each value represents the mean ± S.E.M. of CRF activity released into the incubation medium during a 5 min. incubation period. The numbers in parentheses refer to the number of observations

Treatment	CRF activity (nmol B/100 mg adrenal/h)	
Basal	6.25 ± 0.5*	(10)
3 pg ACh/ml	15.6 ± 1.3	(14)
3 pg ACh/ml + 0.1 pg B/ml	15.9 ± 1.2	(10)
3 pg ACh/ml + 1.0 pg B/ml	7.8 ± 1.0*	(18)

* $P < 0.005$ as compared to 3 pg ACh/ml.

A wide variety of steroids were tested for their activity upon fast feedback and the results are shown in Fig. 1. The predominant glucocorticoids secreted by the adrenal cortex (cortisol and corticosterone) and dexamethasone (a synthetic, fluorinated glucocorticoid) were the only steroids tested which were able to suppress the acetylcholine-induced release of CRF. Thus, the loss of either the 11- or 21-hydroxyl group renders the steroid inactive which suggests that these groups must be essential for the efficacy of the steroid molecule on the fast feedback receptor.

(*b*) *Antagonists.* Several steroids were found to antagonise the inhibitory action of corticosterone and the results are illustrated in Fig. 2. The 11-β OH group is not required for binding of the steroid to the receptor since 11-deoxycorticosterone (DOC) and 11-deoxycortisol (S) are both antagonists. An additional hydroxyl group in the 18-position does not interfere with the binding to the receptor since 18-hydroxy-11-deoxycorticosterone (18-OH DOC) also antagonises the inhibitory action of corticosterone. Neither the 17α-OH nor the 21-OH group is

Table 1. The measurement of corticosterone secretion into adrenal venous blood collected from one adrenal of anaesthetised rats

Time period from adrenal vein cannulation	No. of animals	Plasma flow rate (ml/min)	Adrenal venous plasma Corticosterone concentration (nmol/ml)	Corticosterone secretion rate (nmol/min)
0–10 min	10	0.050 ± 0.008	120.0 ± 4.4	6.00 ± 0.22
10–20 min	9	0.039 ± 0.003	87.2 ± 8.3	3.40 ± 0.13
20–30 min	9	0.044 ± 0.003	76.9 ± 6.7	3.38 ± 0.11
30–40 min	9	0.043 ± 0.007	79.7 ± 8.9	3.42 ± 0.16
40–50 min	9	0.048 ± 0.005	75.3 ± 8.6	3.61 ± 0.14
50–60 min	8	0.047 ± 0.006	79.5 ± 7.8	3.74 ± 0.14
60–70 min	7	0.051 ± 0.004	62.4 ± 8.6	3.18 ± 0.25
70–80 min	4	0.046 ± 0.008	73.6 ± 12.8	3.39 ± 0.19

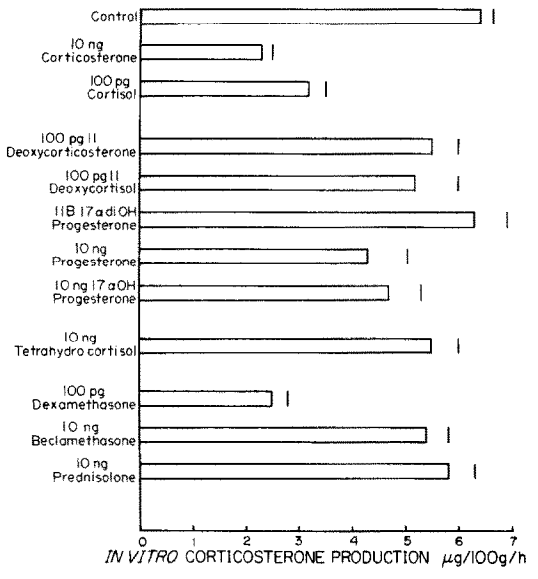

Fig. 1. The first column shows the effect of acetylcholine (3 pg/ml) on the release of CRF activity from the hypothalamus *in vitro*. The subsequent columns show the effect of various steroids added simultaneously with the acetylcholine. Each column represents the mean ± S.E.M. of a minimum of 10 observations.

essential since both progesterone and 17α-OH progesterone were antagonists on FFB. Tetrahydrocortisol was also tested for antagonism and found to be ineffective.

(2) *Silent period.* Figure 3 shows that the addition of corticosterone (100 ng/ml) to the incubation medium resulted in a significant inhibition of the acetylcholine-induced release of CRF during the first 10 min. This inhibitory action of corticosterone was not so great when tested 20 min after the addition of the steroid. When the corticosterone was removed and replaced with steroid-free medium then the acetylcholine-induced release of CRF returned to levels which were not significantly different from the control stimulation. During subsequent challenges of the tissue with acetylcholine there was a gradual decline of the acetylcholine response so that at 60 min after the removal of the corticosterone there was a significant inhibition ($P < 0.01$) of the response as compared to 10 min after removal of the steroid. Control experiments using similar incubations but with no exposure to steroids showed that there were only minor variations in the acetylcholine response throughout the experimental period.

(3) *Delayed feedback (DFB).* (*a*) *Agonists.* (i) In vivo. A wide variety of steroids were tested for a delayed feedback (DFB) action on the release of CRF from the hypothalamus *in vitro* following pre-treatment *in vivo*. The results are illustrated in Fig. 4. The hatched columns show responses which are significantly different from vehicle treated groups and it can be seen that corticosterone, cortisol and several of their precursors show DFB activity. The 11β-OH group is not essential for activity as both 11-deoxycorticosterone and 11-deoxycortisol exhibit DFB activity. The 21-OH group is also not required as both 11β-17α-dihydroxyprogesterone and progesterone itself were active. However, the presence of a 17α-OH group weakens the activity as 17α-OH progesterone showed no DFB. Other steroid hormones, such as testosterone, estradiol and dehydro-epiandrosterone were inactive. If the 11-OH group is in the α position

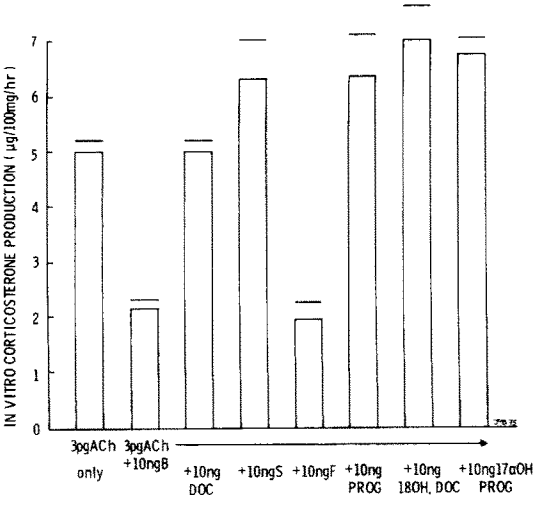

Fig. 2. The effect of various steroids tested for antagonism of the inhibitory action of corticosterone (B) on acetylcholine (ACh)-induced CRF release. Each column represents the mean ± S.E.M. of a minimum of 10 observations. Abbreviations: DOC = 11-deoxycorticosterone; S = 11-deoxycortisol; F = cortisol; Prog. = progesterone; 18-OH DOC = 18-hydroxy-11-deoxycorticosterone; 17α-OH prog = 17α hydroxyprogesterone.

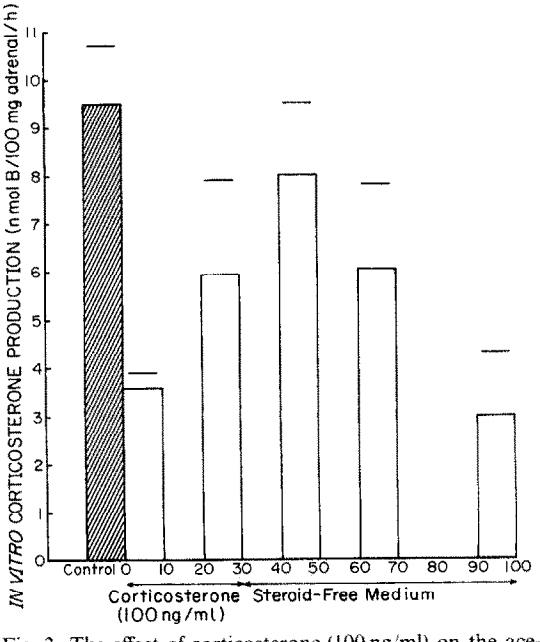

Fig. 3. The effect of corticosterone (100 ng/ml) on the acetylcholine (100 ng/ml) induced release of CRF. Each histogram gives the mean ± S.E.M. of observations in 9–12 animals.

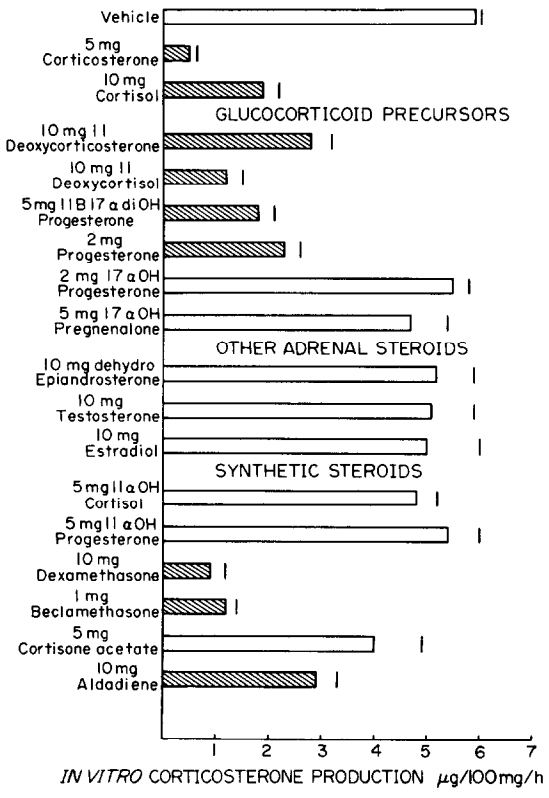

Fig. 4. The effect of pre-treatment with various steroids *in vivo* on the acetylcholine (3 pg/ml) induced release of CRF. Each column represents the mean ± S.E.M. of a minimum of 10 observations.

(11α-OH cortisol) all activity is abolished as also occurs if this group is replaced by a keto from (cortisone). Synthetic anti-inflammatory steroids with extensive structural modifications also show DFB activity, i.e. dexamethasone, beclamethasone diproprionate and aldadiene. DFB was also tested at 4 h and similar results were obtained.

It is possible, however, that since the animals were pretreated with corticosteroids the changes in CRF secretion were due to a primary effect in the limbic system which then modulated CRF secretion.

Alternatively the results may have been due to metabolism to more active compounds *in vivo*. To test for these possibilities we also added steroids directly to the rat hypothalamus *in vitro*.

(ii) In vitro. Figure 5 shows the results obtained when several steroids were tested for a delayed feedback action *in vitro*. Corticosterone (B) in doses of 10 and 100 ng/ml significantly ($P < 0.025$) reduced the CRF output in response to acetylcholine as compared to the control hypothalami which only received the vehicle (medium alone) 2 h prior to testing. 11-Deoxycorticosterone (DOC) also exhibited a delayed feedback action *in vitro* ($P < 0.01$) whereas progesterone (100 ng/ml) did not reduce the CRF output in response to acetylcholine to values significantly lower than the control.

(*b*) *Antagonists.* (i) In vitro. Putative antagonistic steroids were added prior to and simultaneously with corticosterone. The results are illustrated in Fig. 6 and show that both 17α-OH progesterone and 11α-OH cortisol antagonised the inhibition of CRF *in vitro* produced by an equimolar dose of corticosterone. Progesterone, however, did not affect the inhibitory action of corticosterone suggesting that the 17α-OH group is essential for binding. The 3 keto and 4-5 ene groups are also necessary as 11α-OH pregnenalone was also inactive. Similarly 18-OH deoxycorticosterone, which causes an exaggerated stress response *in vivo* [40] did not antagonise the delayed feedback action of corticosterone *in vitro*.

(ii) In vivo. As both 11α-OH cortisol and 17α-OH progesterone were able to antagonise DFB action *in vitro* we decided to examine the effect of these steroids on corticosteroid feedback inhibition of the hypothalamopituitary–adrenal system in the intact animal. The results are illustrated in Table 3 and show that neither of these steroids showed any significant DFB action *in vivo*. Neither did either of these steroids on their own, in the doses and time intervals used, show any significant interference with the DFB action of various glucocorticoids. However, when the two steroids were administered together as a single injection 30 min prior to the administration of a glucocorticoid there was a dose-dependent inhibition of the DFB action of the glucocorticoid.

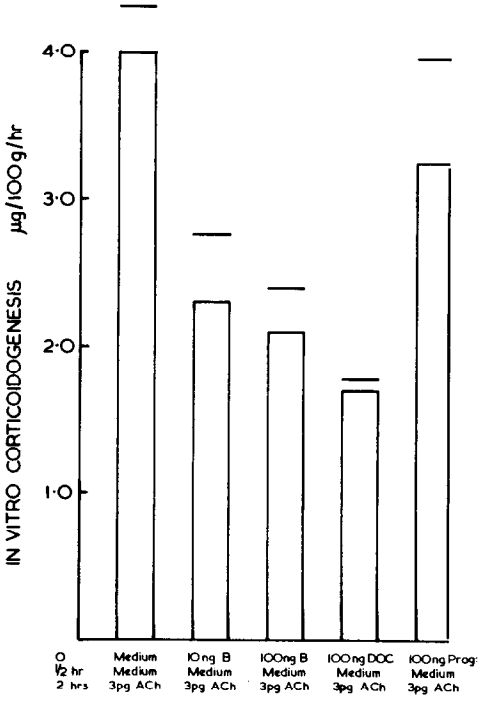

Fig. 5. The effect of pre-treatment with various steroids (100 ng/ml) on the acetylcholine (3 pg/ml) induced release of CRF. Each column gives the mean ± S.E.M. of a minimum of 10 observations.

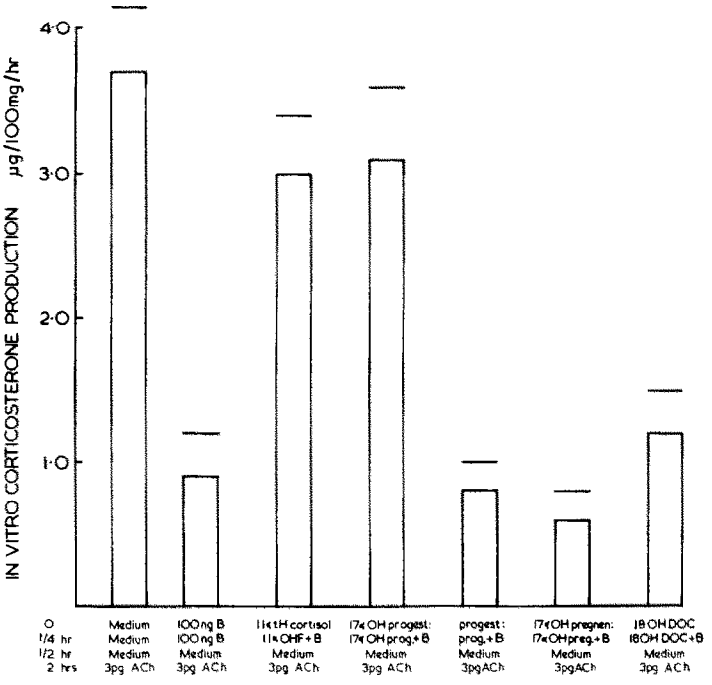

Fig. 6. The effect of various steroids tested for antagonism of the delayed feedback action of corticosterone *in vitro*. Each column shows the mean ± S.E.M. of a minimum of 10 observations. All steroids were administered in a dose of 100 ng/ml. Abbreviations: B = corticosterone; 11α-OHF = 11α-OH cortisol; 17α-OH Prog. = 17α-OH Progesterone; Prog. = Progesterone; 17α-OH preg. = 17α-OH pregnenalone; 18-OH DOC = 18-OH-11-deoxycorticosterone.

Studies on the mechanism of action of the two corticosteroid feedback mechanisms

(*a*) *Content studies.* In these experiments the release of CRF into the incubation medium was measured under various conditions and then at the end of the incubations the hypothalami were removed from the medium and the CRF content extracted with 0.1 N HCl for assay. The results of the experiments are summarised in Fig. 7. In the DFB experiments acetylcholine (3 pg/ml) caused a highly significant release of CRF activity which could be inhibited ($P < 0.001$) by exposure to a DFB signal (corticosterone 100 ng/ml) 2 h previously. However, the corticosterone did not significantly alter the CRF content suggesting that the corticosterone has inhibited both the synthesis and release of CRP.

In the experiments on the FFB mechanism, shown on the right of Fig. 7 the corticosterone significantly ($P < 0.001$) inhibited the release of CRF and at the same time caused an increase in the tissue content of the CRF activity ($P < 0.05$). This suggests that the fast feedback action of corticosterone is mediated *via* the inhibition of CRF release but has little or no effect upon the synthesis of CRP. Similar results were also obtained using cortisol (1 ng/ml) as the FFB signal.

(*b*) *Inhibitory pathways.* In previous studies we have shown that both noradrenaline [16, 20] and GABA [20, 21] cause the inhibition of CRF release *in vitro*. We therefore, tested for the possibility that the FFB action of corticosterone *in vitro* is acting via the release of either endogenous noradrenaline or endogenous GABA. The results are illustrated on the right-hand side of Fig. 8. This shows that corticosterone (10 ng/ml) reduced the acetylcholine-induced release of CRF to basal levels (FFB) and this inhibitory action of corticosterone was unaffected by either phentolamine (100 ng/ml) or picrotoxin (100 ng/ml). The antagonists were used in doses which have been shown to be effective in antagonising the inhibitory

Table 3. The effect of 11α-OH cortisol and 17a-OH progesterone (17α-OH PG) on the delayed feedback activity of corticosterone *in vivo*. Each value gives mean ± S.E.M. of a minimum of 10 observations

Pretreatment of intact rats	In vitro corticosterone production (nmol B/100 mg adrenal/h)
Vehicle	18.8 ± 1.0*
11α-OH Cortisol (2 mg)	17.6 ± 1.4*
17α-OH PG (2 mg)	18.2 ± 1.6*
Vehicle + B (400 µg)	3.9 ± 0.5
11α-OH Cortisol (2 mg) + B (400 µg)	3.4 ± 0.4
17α-OH PG (2 mg) + B (400 µg)	3.9 ± 0.6
11α-OH Cortisol (2 mg) + 17α-OH PG (2 mg) + B (400 µg)	11.8 ± 1.0*

* $P < 0.005$ as compared to vehicle + B (400 µg).

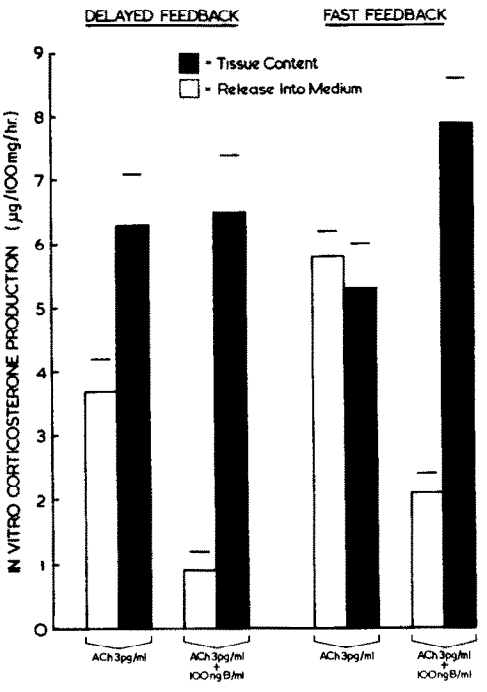

Fig. 7. The effect of either acetylcholine (ACh) or acetylcholine + corticosterone (B) on the release (□) and tissue content (■) of CRF. Each value gives the mean ± S.E.M. of a minimum of 10 observations.

action of noradrenaline and GABA *in vitro* [16, 20, 21]. These results suggest that the FFB action of corticosterone *in vitro* is not mediated *via* the release of endogenous GABA or noradrenaline.

(*c*) *Potassium studies.* Hypothalami which were incubated in a medium which had a raised K^+ level (12–48 mM) showed higher basal secretion of CRF than hypothalami incubated in medium containing a normal K^+ concentration (6 nM). A summary of our studies on the effects of K^+ on CRF secretion are shown on the left-hand side of Fig. 8. Corticosterone in a dose of 10 ng/ml inhibited the acetylcholine-induced release of CRF but had no effect upon the basal release of CRF from hypothalami incubated in normal medium (6 mM K^+) or from hypothalami incubated in medium with a raised K^+ level (48 mM K^+). The depolarisation of the CRF cells with K^+ overcomes the inhibitory action of corticosterone on CRF release and this suggests that corticosterone may be acting *via* membrane stabilization. One possibility is that corticosterone may be acting by affecting K^+ flux and hence cell depolarisation. This was tested by incubating hypothalami in medium which had a low level of K^+ (2 mM). Hypothalami incubated in such a medium did not show any impaired ability to release CRF in response to acetylcholine (Fig. 8).

(*d*) *Calcium studies.* Since Ca^{2+} appear to play a role in stimulus–secretion coupling [9] we examined the effect of alterations in the Ca^{2+} concentration of

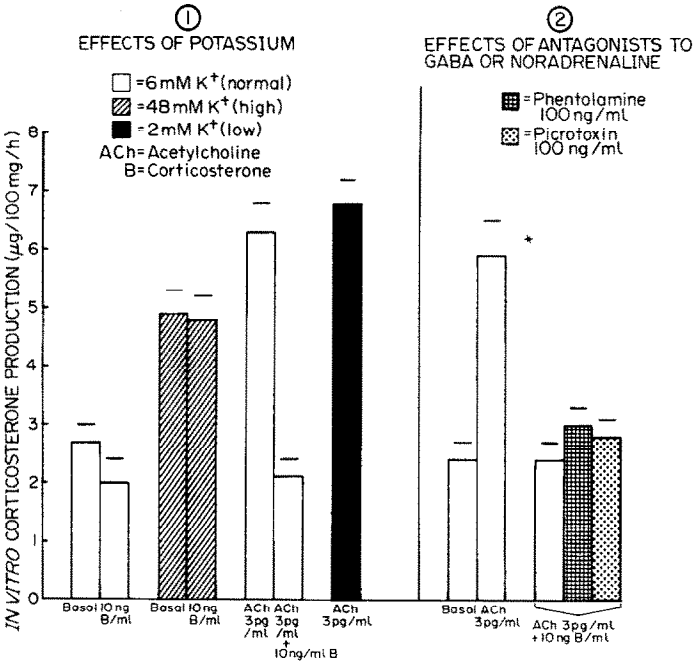

Fig. 8. The effect of acetylcholine (ACh) and corticosterone (B) on the release of CRF from hypothalami incubated in medium containing various concentrations of K^+. The effect of phentolamine (▨) and picrotoxin (▩) on the corticosterone (B) induced inhibition of the release of CRF in response to acetylcholine: Each histogram gives the mean ± S.E.M. of a minimum of 10 observations. * = $P < 0.01$ as compared to all other values.

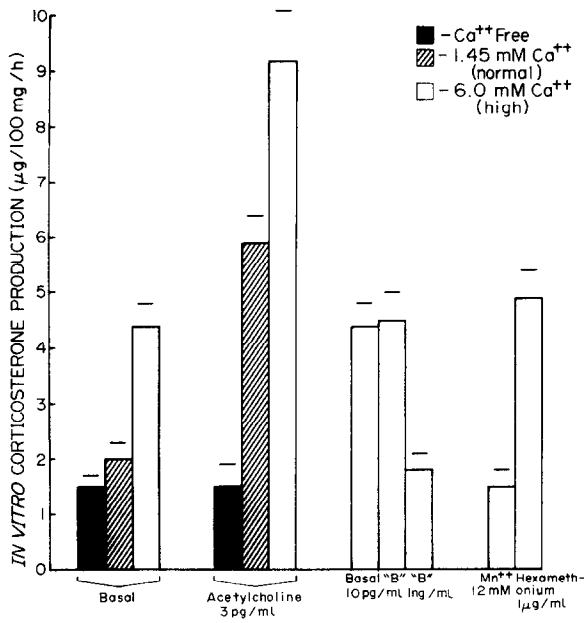

Fig. 9. The effects of acetylcholine (ACh), corticosterone (B), manganese (Mn^{2+}) and hexamethonium on CRF secretion from hypothalami incubated in medium containing various concentrations of Ca^{2+}. Each histogram gives the mean ± S.E.M. of a minimum of 10 observations.

the incubating medium on the secretion of CRF from the hypothalamus. The results are illustrated in Fig. 9 and show that increasing the Ca^{2+} concentration of the medium from 0–6 mM caused increased release of CRF activity into the medium. The amount of CRF released in the Ca^{2+} free medium was not significantly different from the base-line of the assay. The presence of Ca^{2+} in the medium is essential for the release of CRF since acetylcholine did not cause any significant release of CRF from hypothalami incubated in Ca^{2+} free medium. Further support for this hypothesis is provided by experiments in which the Ca^{2+} concentration of the medium was increased from 1.45 mM (normal) to 6.0 mM and this resulted in an increase in the release of CRF in response to acetylcholine.

It is possible that the enhanced release of CRF in high Ca^{2+} medium might be due to an increased release of endogenous acetylcholine. This would seem unlikely, however, since hexamethonium in a dose (1 μg/ml) which has been shown to be effective in blocking the acetylcholine-induced release of CRF *in vitro* [16] had no effect upon the CRF release in high Ca^{2+} medium.

Corticosterone (10 pg/ml) had no effect upon the basal release of CRF in medium containing a high concentration of Ca^{2+}. However, when the concentration of corticosterone in the medium was increased to 1 ng/ml there was a highly significant ($P < 0.001$) reduction in the release of CRF. This suggests that the FFB action of corticosterone might be mediated via an effect upon Ca^{2+} flux. This would appear to

Table 4. The effect of acetylcholine (ACh) and corticosterone (B) on the strontium (Str^{2+}) induced release of CRF. Each value gives the mean ± S.E.M. of a minimum of 10 observations

Treatment	CRF activity (nmol B/100 mg adrenal/h)
Basal	5.7 ± 0.5
Str^{2+} 6 mM	10.7 ± 1.0*
Str^{2+} 6 mM + B 1 ng/ml	8.6 ± 1.3 (N.S. from Str^{2+} alone)
ACh 3 pg/ml	19.0 ± 1.4
ACh 3 pg/ml + Str^{2+} 6 mM	24.7 ± 2.0**

* $P < 0.005$ as compared to basal.
** $P < 0.05$ as compared to ACh alone.

be confirmed by experiments which showed that the inhibitory action of corticosterone on CRF secretion in high Ca^{2+} medium can be mimicked by the presence in the medium of manganese, which is known to block Ca^{2+} channels, in a concentration of 12 mM (Fig. 9). Further support is provided by experiments which showed that the effects of Ca^{2+} on CRF secretion could be mimicked by the replacement of Ca^{2+} by strontium (Table 4). However, corticosterone in the same dose as used in the Ca^{2+} experiments did not block the CRF secretion in response to a high concentration of strontium (6 nM).

Anterior pituitary studies

(1) *Fast feedback.* Table 5 shows that 70 μg corticosterone administered subcutaneously to basal hypothalamus lesioned animals significantly reduced ($P < 0.01$) ACTH release induced by a standard dose of CRF when the response was tested 10–30 min after steroid administration. By 50 min the response had returned to normal.

(2) *Silent period.* Figure 10 shows that 20 min after the subcutaneous administration of corticosterone (500 μg) there was a significant inhibition of the release of ACTH in response to CRF. This inhibitory effect had disappeared by 40 min. However, 100 minutes after administration of the steroid there was

Table 5. The i.v. injection of CRF (½ medium eminence equivalent) to basal hypothalamic lesioned rats at various time intervals after the sc administration of 70 μg corticosterone (B). Each value gives the mean ± S.E.M. of a minimum of 10 observations

Time after corticosterone (min)	*In vitro* corticosterone production (nmol B/100 mg adrenal/h)
0	18.5 ± 0.8
5	14.6 ± 1.2
10	7.1 ± 1.0
20	7.0 ± 1.0
30	8.3 ± 1.2
50	19.2 ± 1.5

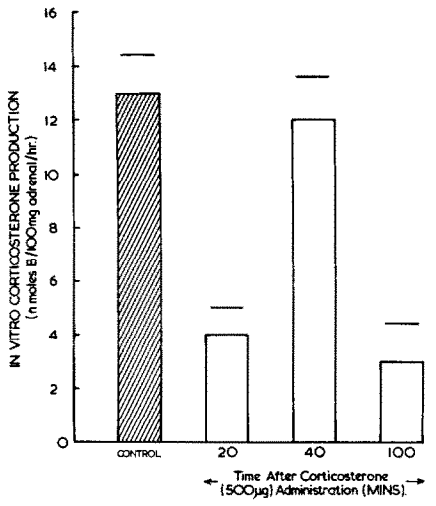

Fig. 10. The effect of the i.v. injection of CRF into basal hypothalamic lesioned rats at various time intervals after the subcutaneous administration of 500 µg corticosterone (B).

a further period of inhibition of the CRF response. This confirms that a similar biphasic inhibitory response to corticosteroids exists at the pituitary level as well as at the hypothalamic level.

(3) *Delayed feedback.* Table 6 shows that several steroids reduced CRF-induced release of ACTH at 4 and 24 h after steroid administration.

DISCUSSION

The adrenal vein cannulation results show that between 3–5 nmol (1–2 µg) corticosterone a min is secreted by the left adrenal gland, the amount decreasing to a steady level after the first 10 min. The two adrenals, together, secrete, under the maximum stress conditions, about 15.6 nmol (6 µg) during the first 10 min and 7.8 nmol/min (3 µg/min) thereafter. It can be assumed that these represent maximum stress values since the animal had been subject to cannulation stress as well as the slow haemorrhage associated with collection of the adrenal venous blood.

The data on adrenal vein cannulation estimate the maximum rate at which corticosterone can be secreted. Most adrenocortical stress responses last about 1–3 h, and the maximum amount secreted in this time should not exceed 1.3 µmol (500 µg). Therefore, feedback studies using doses above this range are investigating feedback outside the physiological limits. Fast feedback is shown only during the first 30 min after steroid administration [18] and the doses which would be within the physiological range would be 120 µg. Fast feedback must therefore be shown with doses at or below this value. Our current data have shown fast feedback effects at the anterior pituitary level with a dose well below this value.

Whilst the doses we have used for fast feedback studies are within the physiological range, this is not true of the doses we have used in our studies on delayed feedback. Delayed feedback occurs 1–2 h after steroid administration and we have used a dose of 2 mg corticosterone in our studies at the anterior pituitary. This dose is about 4 times the upper physiological limit and though it gave complete suppression of ACTH secretion it only indicates the presence of feedback at this site but says little of its physiological significance.

The other method of estimating the physiological significance of our experimental data is to relate the level of corticoids used in our *in vitro* system to the free, unbound moiety of the plasma. The resting level of plasma corticosterone in our animals is 1.042 nmol/l. (4 µg/100 ml) and the stress values are 10.42 nmol/l. (40 µg/100 ml). If it is assumed that the steroid is 95% bound then approximately 5.2 nmol/l. (2 ng/ml) corticosterone is free under basal conditions and about 52 nmol/l. (20 ng/ml) under stress conditions. Thus, in our hands fast feedback was initiated with doses well within the basal as well as the stress values and delayed feedback with values within the amount present under stress conditions. It must be concluded therefore that both mechanisms are of physiological importance and are active at both the hypothalamic and anterior pituitary levels.

The present data show that two temporally distinct periods of inhibition of CRF secretion can be shown *in vitro*. The first period of inhibition occurs immediately upon exposure to corticosteroids, lasts for 20 min, and requires the presence of corticosteroids in the medium. The second period of inhibition does not appear until much later at a time when the corticosteroids have been absent from the medium for a

Table 6. The steroids were administered subcutaneously in arachis oil to the basal hypothalamic-lesioned assay rat. ACTH release was stimulated with CRF obtained from incubated rat hypothalami in the presence of 3 pg acetylcholine/ml. Each value represents the mean ± S.E.M. and the number of animals used is given in parentheses.

Treatment	4 h pre-treatment of ME-lesioned rat (nmol B/100 mg adrenal/h)	24 h pre-treatment of ME-lesioned rat (nmol B/100 mg adrenal/h)
Vehicle	17.5 ± 1.4	17.2 ± 1.2
2 mg corticosterone	4.7 ± 0.8	2.2 ± 0.6
2 mg cortisol	3.3 ± 0.6	1.1 ± 0.6
2 mg 11-deoxycorticosterone	6.6 ± 1.7	7.2 ± 0.8

period of at least 60 min. These 2 phases of inhibition correlate with the fast and delayed feedback effect of corticosteroids and the period of no inhibition between the two phases is the same as the 'silent period' seen *in vivo* [8, 42].

The speed with which the corticosteroids exert their initial inhibitory action *in vitro* suggests that the mechanism of action of FFB is *via* the inhibition of release and not *via* an inhibition of synthesis. This is further confirmed by the observation that, following incubation with acetylcholine and a FFB agonist, the hypothalamic CRF content is significantly elevated compared to incubation with acetylcholine alone suggesting that the corticosteroid has inhibited release but not synthesis of CRF. In contrast, the DFB action of corticosteroids causes inhibition of both CRF release and synthesis. Recently these observations have been confirmed in studies measuring ACTH secretion and hypothalamic CRF content *in vivo* [34].

The FFB action of corticosteroids does not appear to be due to excitation of neuroinhibitory pathways since antagonists to noradrenaline and GABA, the neuroinhibitory transmitters to CRF [16, 20, 21], did not affect the fast feedback activity of corticosteroids *in vitro*.

The release of various hormones has been shown to be *via* a process of exocytosis which is Ca^{2+} dependent [9]. The present studies confirm previous observations that CRF release *in vitro* is Ca^{2+} dependent [3, 10]. Also, in electronmicroscopical studies, we have shown that both acetylcholine and 5-HT cause a significant decrease in dense core vesicles in nerve terminals in the external zone of the median eminence and this is associated with an increase in the number of microvesicles (W. Wittkowski, *et al.*, unpublished observations). These phenomena are consistent with current views on exocytosis. The morphological changes also show an increase in the number of neuro-vascular contacts in the median eminence.

Thus, the fast feedback action of corticosteroids may be due to a membrane stabilization effect which somehow prevents the process of exocytosis taking place. The mobilization of calcium ions from intracellular pools or its influx from extracellular fluid is thought to occur by hormonal or electrical stimulation of the cell plasma membrane or by cAMP interactions. Cortisol has been shown to increase the binding of Ca^{2+} to the plasma membrane of rat liver cells [35], leading to a decrease in cell membrane permeability. It is possible that a similar mechanism mediates the fast feedback action of corticosteroids especially as we have shown that corticosteroids inhibit high Ca^{2+} induced release of CRF *in vitro*. The involvement of Ca^{2+} in CRF release *in vitro* would appear to be confirmed by the fact that Mn^{2+}, which blocks Ca^{2+} channels [2], prevents the CRF releasing action of Ca^{2+} and that strontium ions, which mimick many of the physiological actions of Ca^{2+} also release CRF.

Further differences in the two feedback mechanisms were shown by the structure–activity studies which showed that different steroids have different effects upon the two feedback mechanisms; this implies that the feedback effects are mediated *via* separate receptor mechanisms. The fast feedback receptor at the hypothalamus appears highly specific since of several steroids tested, only cortisol, corticosterone and dexamethasone were agonists. Thus, the structure essential for efficacy is highly specific, involving an 11β-OH group and an unblocked 21-OH group. These findings closely follow our previous findings *in vivo* [19]. However, the binding site does not appear to be very specific since several steroids showed antagonism. 18-hydroxy-deoxycorticosterone (18-OH DOC) is an antagonist which suggests that neither of the groups essential for efficacy are involved in the binding of the steroid to the receptor. The basic steroid structures required for affinity and efficacy on the fast feedback receptor at the hypothalamus are shown in Fig. 11.

It is interesting that it is the precursors of corticosterone and cortisol which have antagonistic fast feedback properties and not the metabolites. In man the circulating levels of these precursors are low [27] which suggests that they would not normally interfere with the fast feedback mechanism. However, in adrenogenital syndrome the level of antagonistic precursors is high (i.e. 17α-OH progesterone and 11-deoxycorticosterone) and under these conditions it is likely that these steroids would interfere with normal fast feedback control of ACTH secretion [22].

The delayed feedback mechanism requires either an 11β-hydroxyl group or a 21-hydroxyl group for efficacy since steroids containing either both of these groups (e.g. corticosterone or cortisol) or one of them (e.g. 11-deoxycorticosterone or 11β-OH progesterone) are active. However, for the binding to the receptor a 17α-hydroxyl group is required when the 11β- or 21-hydroxyl groups are absent as shown by the antagonistic activity of 17α-OH progesterone. Binding

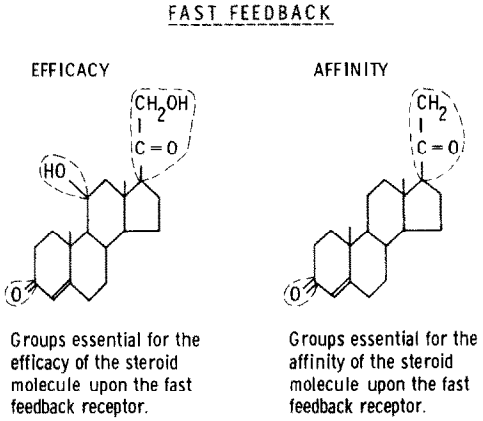

Fig. 11. The groups essential for the efficacy and affinity of the steroid molecule upon the fast feedback receptor of the hypothalamus.

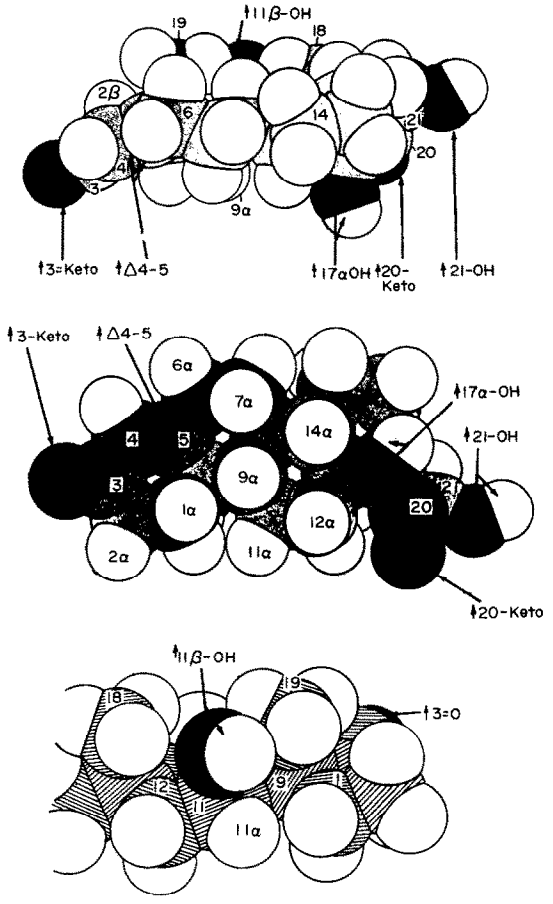

Fig. 12. The groups involved in the affinity and efficacy of steroids molecules for delayed feedback receptors in the hypothalamus. These diagrams are based on the Stuart Type Model of cortisol.

must also involve the 3 keto, 4-5 ene structure as steroids in which these are absent (e.g. 17α-OH pregnenolone) are inactive. A model of the basic steroid molecules required for affinity and efficacy are shown in Fig. 12.

The application of these studies to glucocorticoid therapy may prove extremely interesting. Our initial studies have shown that a combination of two antagonistic steroids (11α-OH cortisol and 17α-OH progesterone) can prevent the feedback suppression of ACTH release by glucocorticoids administered parenterally to rats [26]. Labelled corticosteroids are found concentrated in the pituitary and various brain regions, including the hypothalamus, with the greatest concentration being found in the hippocampus [25, 28, 39]. These corticosteroids are associated with binding proteins both in the nucleus and the cytosol [25, 29, 30]. Moreover, it has been shown that the natural corticoids, corticosterone and cortisol, compete for corticosterone sites in the brain as does progesterone which has no glucocorticoid activity [3].

There is considerable evidence in the literature supporting our contention that corticosteroids feedback at both the hypothalamus and the pituitary. The administration of corticoids both *in vivo* [1, 33, 41] and *in vitro* [1, 11, 32] has been shown to block CRF-induced ACTH release. However, the results presented in this study support the concept that the prime effects of the two corticosteroid mechanisms are on the secretion of CRF and some of the effects of ACTH secretion might be secondary to changes in CRF secretion.

Implantations of corticosteroids in the hypothalamus have been shown to inhibit the ACTH release [36, 37] and the systemic or microiontophore-

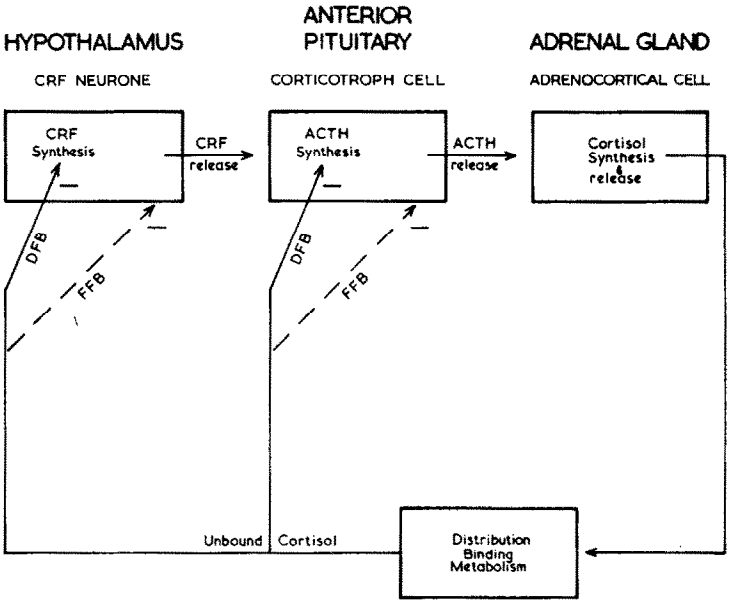

Fig. 13. A flow diagram of corticosteroid negative feedback control of CRF ACTH secretion. DFB = delayed feedback mechanism which inhibits the synthesis of CRF and ACTH. FFB = fast feedback mechanism which inhibits the release of CRF and ACTH.

tic application of dexamethasone phosphate has been shown to reduce the firing rate of steroid sensitive neurones in the hypothalamus [38]. Changes in morphological parameters such as nuclear volume [31] and in the number of granules in the median eminence [6] also suggest feedback at the hypothalamus.

Our conclusions concerning the site and mechanism of action of corticosterone are shown in Fig. 13. Fast and delayed feedback mechanisms are shown acting at both the hypothalamic and anterior pituitary levels. Both feedback mechanisms are important in the regulation of CRF–ACTH secretion, the fast feedback provides an immediate regulatory influence by inhibiting CRF and ACTH release whilst the delayed feedback controls CRF and ACTH synthesis. The fast feedback receptor in the hypothalamus is an unique example of a steroid acting *via* an immediate effect on the cell membrane involving Ca^{2+} flux which is too rapid in its onset to involve the nucleotides and must therefore involve a receptor which lies close to or in the membrane.

Our findings provide an estimate of the magnification involved from neuotransmitter excitation of CRF release to the secretion of corticosterone. CRF is secreted in response to 3 pg acetylcholine/ml, and corticosterone is secreted by the adrenal at a rate of about 6 μg/min; the magnification involved must therefore be about 2 million fold.

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DISCUSSION

Jungblut. Did you try to counteract the calcium effect with Isoptin?

Jones. No we have not.

Crabbé. I found these data of yours most interesting especially because it forces us to reflect on the key role ascribed to cytosol-protein receptors in all aspects of steroid hormone action. Indeed here you present us with a case that is almost impossible to reconcile with the set pattern of response of target tissues to steroid hormones. I would like to know whether the manipulations of calcium concentration in your incubation medium enable you to overcome the inhibiting effects of steroids that you show capable of interfering with the fast release of CRF. I have another question: Did you try ouabain as another means of depolarizing hypothalamic cells, in influencing the process you've described?

Jones. We intend investigating the relationship between calcium flux and the antagonists of fast feedback. I should point out that Schatz and Martinetti (*Science* **176** (1975) 175–177) have shown that calcium gets bound to plasma membrane of liver cells. This might be a general model for the mechanism of action of corticosteroids.

Martini. Have you tried to potentiate your "fast feedback" by giving cyclic AMP or phosphodiesterase inhibitors?

Jones. No, we have not investigated it.

Stumpf. What is your evidence that this is CRF and not Egdahl's "hindbrain factor"?

Jones. The following: The material selectively releases ACTH from the hemi-sected anterior pituitaries *in vitro* as well as from basal hypothalamic lesioned rats. This material has no direct action at the adrenal level. Does that answer your question?

Stumpf. I'm not so sure. You know the problem with the unspecific releasing agents in the brain. You can take out the hypothalamus and you increase the ACTH releasing mechanism by some unknown factor and if you take out the whole brain you increase it even more and, this hindbrain factor, which is not identified as far as I know, but possibly a tissue polypeptide may well be involved here. Did you have a control using instead of hypothalamus maybe mid brain or some other regions?

Jones. We have in fact done controls with cerebral cortex and with thalamus and we find no activity there. The other reason for believing this is CRF is that it is modified by such things as steroid treatment in exactly the same way as happens *in vivo*. Its secretion is also modified by ACTH short-loop feedback. Neurotransmitters which will release it from hypothalamus also do so *in vivo*.

Jungblut. I have a vague recollection that corticosteroids were used in a biochemical preparation of lysosomes. They stabilized membranes. Would you see any connection to what you're doing?

Jones. Yes, indeed. As far as I am aware cortisol is in fact the only physiological material that stabilises the membranes of lysosomes. This may indeed be a general property of cortisol.

Birmingham. I am very interested in your data, for we also found that 18-hydroxy DOC gave a positive feedback effect, and the other finding to our great surprise was that the most pronounced negative feedback effect we got in the rat was with aldosterone, much better than with corticosterone (*J. steroid Biochem.* **5** (1974) 789). I was wondering whether you had tried aldosterone. Since aldosterone is probably a phylogenetically old steroid this should be of interest.

Jones. No we have not used aldosterone, we intended using it but we never did.

Beyer. As shown in Fig. 1, 50 micrograms of 5β pregnanolone injected into the carotid elicits striking depression of neuronal activity and EEG synchronization with in a matter of seconds. The interest of this effect is that

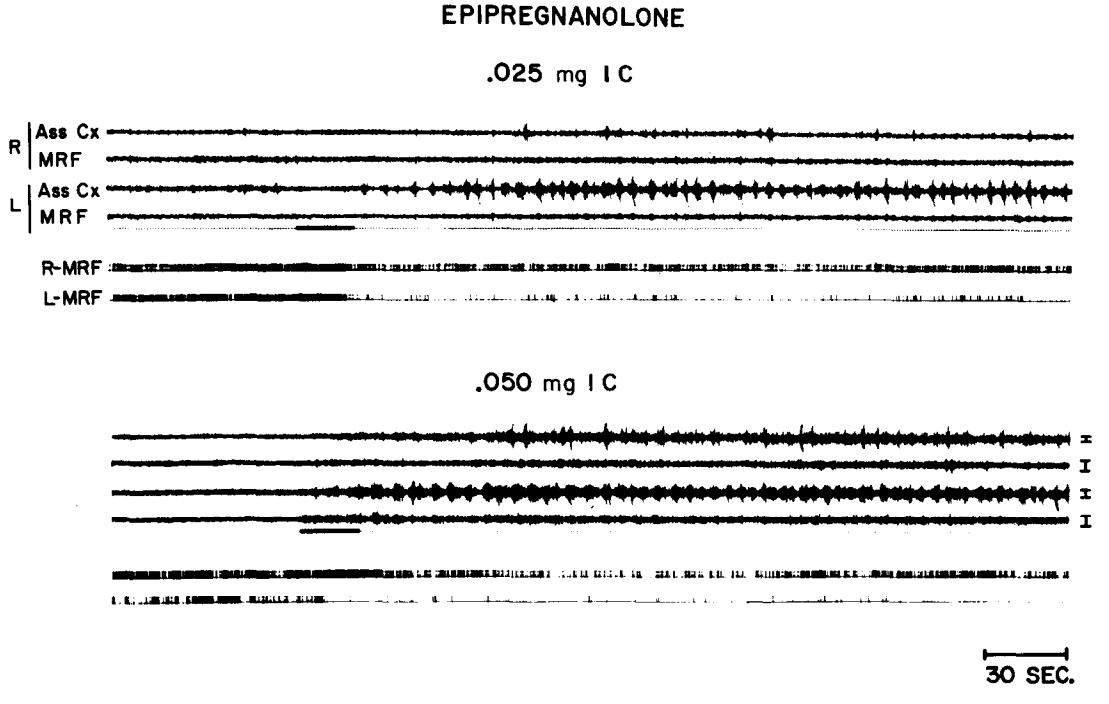

Fig. 1.

its time course excludes the possibility of participation of a classical receptor system.

Jones. Yes, the fact that we find a very clear and very specific structure activity relationship at the hypothalamus suggests surely that we are dealing with a receptor mechanism. That is a system we've been able to end up with a structure for activity, affinity and efficacy.