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A CORRELATIVE STUDY OF THE BINDING OF DEXAMETHASONE IN HYPOTHALAMIC BLOCKS *IN VITRO* WITH ITS ABILITY TO INHIBIT THE RELEASE OF BIOACTIVE CORTICOTROPHIN-RELEASING FACTOR

M. C. HOLMES*§, U. BECKFORD†¶, B. D. GREENSTEIN‡, B. GILLHAM†‖ and M. T. JONES*

Departments of *Obstetrics and Gynaecology, †Biochemistry and ‡Pharmacology, St Thomas' Hospital Medical School, London, SE1 7EH, U.K.

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Summary—Rat hypothalamic blocks incubated *in vitro* were used to study the characteristics of binding of [^{3}H]dexamethasone and other steroids to cytosolic binding sites. Cytosols prepared following incubation of the tissue with [^{3}H]dexamethasone for 2 h contained specifically bound steroid in amounts that depended upon the concentration of potassium (but not sodium) ions in the extracting buffer. There was an increase in bound [^{3}H]dexamethasone extracted as the potassium ion concentration increased up to 0.1 M, but not beyond. Dexamethasone, when added to hypothalami *in vitro* caused a biphasic inhibition of bioactive corticotrophin-releasing factor (CRF) release, and the extent of the second phase of inhibition was dose-related. 11-Epicortisol, when added in a 100-fold molar excess over dexamethasone was able to prevent the second phase of inhibition caused by the latter steroid, as in the binding studies it was able to cause a 50% reduction in the binding of [^{3}H]dexamethasone. In the functional studies it was shown that 11-epicortisol was able to "rescue" the tissue from dexamethasone-mediated delayed inhibition of CRF secretion if added to the blocks 30 min (but not later) after the agonistic steroid.

INTRODUCTION

The presence of binding sites for glucocorticoids in all tissues, including the brain, is relatively easy to demonstrate. Such binding sites have been shown to be present in cytosolic preparations obtained from many discrete areas of the brain including the hypothalamus and also the anterior pituitary gland [1–5]. The functional correlates that allow such binding sites to be designated receptors have proved much more difficult to adduce. However, evidence has been provided to show that the ability of glucocoriticoids specifically to inhibit the secretion of adenocorticotropin (ACTH) is related to their occupation of cytosolic binding sites in the pituitary gland [6, 7].

Glucocorticoids and their synthetic analogues have been shown to cause a direct inhibition of the secretion of corticotropin releasing factor (CRF) from hypothalamic blocks maintained *in vitro* [8–13]. In the present study we have used this technique of incubating hypothalami *in vitro* to compare the characteristics of the binding of the synthetic steroid dexamethasone to hypothalamic cytosolic sites with the ability of the steroid to inhibit the release of bio-active CRF from that tissue. In addition the displacement of dexamethasone from its binding sites by 11-epicortisol has been compared with the ability of the latter steroid to antagonise the functional activity of the former in respect of the inhibition of CRF secretion. Strategies for extracting bound steroid from tissues are discussed.

EXPERIMENTAL

Animals

Wistar-derived, male albino rats weighing 110–150 g were used. They were housed in an air-conditioned room with a controlled lighting cycle (lights on from 07.00 to 21.00 h). Rat food and tap water were available *ad libitum* and the tap water was made 0.9% (w/v) in NaCl when the animals were adrenalectomized. This operation was performed on animals previously anaesthetized by using sodium pentobarbitone (50 mg/kg i.p.; Sagatal, May & Baker Ltd, Dagenham, Essex) and was conducted *via* a dorsal midline approach. At least 7 days elapsed between the operation and use of the animals in the experiments.

Removal and incubation of tissues

Hypothalami were removed and incubated *in vitro* in a rat cerebrospinal fluid (CSF)-like medium as described previously [14]. For binding studies the tissues were pre-incubated for 30 min at 37°C before

§Present address: ERRB-NICHD, Building 10, Room 8C-407 NIH, National Cancer Institute, Bethesda, Md 20205, U.S.A.
¶Present address: The Wellcome Research Laboratories, Langley Court, Beckenham, BR3 3BS, U.K.
‖To whom correspondence should be addressed.

the addition of 1,2,6,7-[^{3}H]dexamethasone (*ca* 70 Ci/mmol, Amersham International plc, Bucks) for 2 h. A range of concentrations of the radioactive steroid (approx 1 to 40 nM) was used in order to obtain binding isotherms. Non-specific binding was assessed by incubating in the presence of a 100-fold molar excess of unlabelled dexamethasone (Sigma [London] Chemical Co. Ltd, Poole, Dorset).

For functional studies hypothalami were pre-incubated for 30 min prior to the addition of dexamethasone (either labelled or not) in the concentration range 0.25–5 nM for a further 30 min. The steroid-containing medium was then replaced by fresh CSF-like medium for a total period of 90 min, changing that medium every 30 min. The tissue was then challenged with acetylcholine (5 pg/ml in CSF-like medium) for 5 min. This stimulation medium was collected and assayed for corticotropin releasing biological activity by adding it to quartered anterior pituitary glands incubated *in vitro* [15]. The consequent release of ACTH during a 15 min period of incubation with test medium was determined by the cytochemical assay for that hormone [16]. In experiments in which it was not possible to remove the steroid from the hypothalamic medium before its addition to the pituitary quarters changes in pituitary ACTH content were used as an index of corticotropin releasing activity as explained previously [8].

Preparation of cytosols

After incubation with [^{3}H]dexamethasone, hypothalami (5 per ml) were homogenized in either buffer A (Na_2HPO_4, 8.2 mM; NaH_2PO_4, 1.8 mM; sucrose, 0.25 M; pH 7.4) or buffer B (K_2HPO_4, 80 mM; NaH_2PO_4, 10 mM; $MgCl_2$, 1 mM; sucrose, 58 mM; adjusted to pH 7.0 with HCl). Both buffers contained 2-mercaptoethanol (5 mM) and 0.25% (w/v) bovine serum albumin. Homogenization was carried out in a 1 ml Jencon's glass homogenizer (radial clearance: 0.25 mm) at 2–4°C.

The homogenates were centrifuged at 105,000 g_{av} for 1 h at 4°C and samples of the supernatant (100 μl) were run through small columns of Sephadex LH-20 (Pharmacia (U.K.) Ltd, Uxbridge) at 2–4°C to separate macromolecular bound radioactive steroid from free and measure the bound as described previously [17, 18].

Functional antagonism by 11-epicortisol

Two types of experiments were carried out to assess the ability of the antagonistic steroid, 11-epicortisol, to prevent the delayed ability of dexamethasone to inhibit the secretion of CRF. In the more straightforward of these experiments the conditions were exactly described under *Removal and incubation of tissues*; however, during the 30 min period of incubation in the presence of dexamethasone (10 nM) various concentrations of 11-epicortisol were present (1 nM–1 μM). In the second type of experiment the protocol for the addition and removal of dexamethasone (0.8 nM) remained the same, but 11-epicortisol (0.1 μM) was added to the tissue for 30 min at time intervals that varied from 5 to 90 min after the dexamethasone. In all cases the final diagnostic test of the functional activity of the tissue consisted of a 5 min period of stimulation with acetylcholine (5 pg/ml) in the absence from the medium of any added steroid.

Specificity of the binding of [^{3}H]dexamethasone

These experiments were also carried out as described under *Removal and incubation of the tissues* and the final concentration of dexamethasone added to the tissue *in vitro* was 10 nM whereas the concentrations of possible competing steroids ranged from 0.5 nM to 1 μM. The unlabelled steroids tested were dexamethasone, corticosterone (Sigma [London] Chemical Co. Ltd), testosterone (Organon Laboratories Ltd, Morden, Surrey) and 11-epicortisol (Schering Corporation, Bloomfield, NY, 07003). After incubation the hypothalami were homogenized in buffer B prior to centrifugation and determination of bound radioactive dexamethasone.

Determination of protein

Protein determinations were carried out on the cytosol preparations by the method of Lowry *et al.*[19] using bovine serum albumin as the standard.

Statistical analysis

Statistical significances of differences between groups were tested for by using either Student's *t*-test or by a single analysis of variance followed by Duncan's test for multiple comparisons [20].

RESULTS

Attainment of binding equilibrium

Preliminary studies showed that apparent equilibrium between bound and free [^{3}H]dexamethasone was attained within 2 h of incubation of hypothalamic blocks at 37°C with 10 nM radioactive steroid (Fig. 1), when homogenization was carried out in buffer B, and hence subsequent incubations were for that period.

[^{3}H]Dexamethasone binding isotherms

Hypothalamic blocks were incubated with various concentrations of [^{3}H]dexamethasone (1–40 nM) and the amount of steroid bound to macromolecules was determined following homogenization in buffer A or B. The results were expressed as the percentage of specifically bound steroid per mg protein (Fig. 2a). Non-specific binding in these experiments never exceeded approx 1% of the total bound. Significantly more bound radioactivity was recovered when the cytosol preparation was carried out using buffer B for homogenization and this was true for all concen-

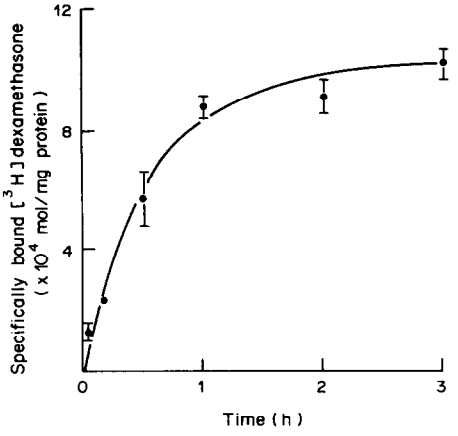

Fig. 1. Attainment of binding equilibrium between [^{3}H]dexamethasone (10^{-8} mol/l) and saturable binding sites in rat hypothalami incubated at 37°C. Bound steroid was extracted by homogenization in buffer B. Results are expressed as the mean value for specifically bound steroid ±SEM in 5 experiments.

trations of steroid used. The maximum amount of steroid bound (B_{max}) in buffer A was 60.9 ± 12.0 fmol/mg protein (mean ± SEM for 5 determinations) as compared to 180.0 ± 27.0 fmol/mg protein in buffer B ($P < 0.001$).

Influence of potassium ions on the extraction of [^{3}H]dexamethasone binding sites

The concentration of added potassium ions in buffer B was varied in the range 0–160 mM by reducing the concentration of that cation and making compensatory changes in the concentration of sodium ions so that the ionic strength of the buffer did not vary, and the ability of sodium ions to substitute for potassium in the extraction of steroid-labelled binding sites was determined. Figure 2b shows that the amount of bound dexamethasone found in the cytosol was a function of the concentration of potassium ions in the homogenisation medium. Analysis of variance of the data indicated that significantly ($P < 0.01$) more bound [^{3}H]dexamethasone was extracted at a concentration of potassium ions of 0.1 M when compared with the buffer not containing that cation ($F(3,18) = 3.914$; $P < 0.01$). A further increment in the potassium ion concentration to 0.16 M caused no further significant increase in the amount of dexamethasone bound. Addition of potassium ions to a cytosol preparation obtained by using buffer A for the extraction had no effect on the binding reaction (Holmes, unpublished observations).

All subsequent experiments were performed using cytosol prepared following homogenization in buffer B.

Effect of various unlabelled steroids on [^{3}H]dexamethasone binding

Of the steroids tested, unlabelled dexamethasone was best able to complete with its tritiated counterpart, (the latter used at 10 nM) for binding sites in the hypothalamus (Fig. 3). Corticosterone, although less potent than dexamethasone, was able to displace the tritiated steroid from all of its binding sites. 11-Epicortisol was less potent than corticosterone in its ability to compete with tritiated dexamethasone

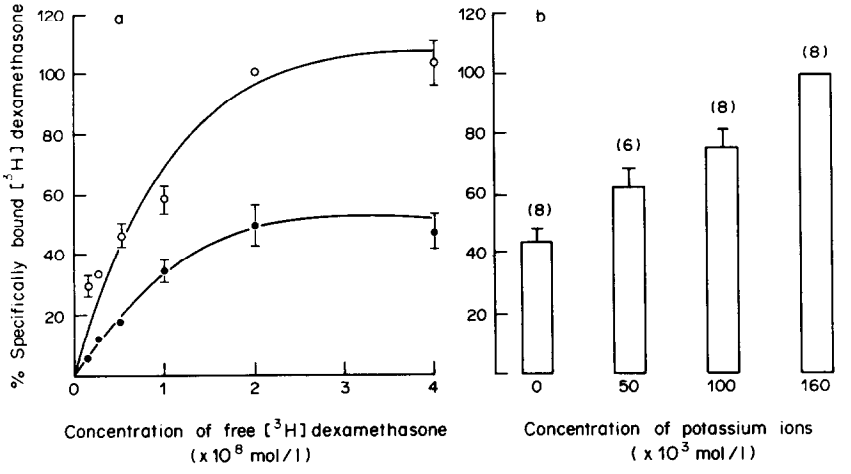

Fig. 2. a. Isotherms for the binding of [^{3}H]dexamethasone added to rat hypothalami, *in vitro* at 37°C, in the concentration range 1–40 × 10^{-9} mol/l. Steroid bound to cytosolic components was extracted by homogenization in either buffer A (●) or in buffer B (○, with a high concentration of potassium ions). Results are expressed as the percentage of steroid specifically bound per mg protein relative to the amount of bound steroid extracted using buffer B when the concentration of [^{3}H]dexamethasone was 10^{-8} mol/l. Values represent the mean ±SEM of 5 experiments. b. Effect of varying concentrations of potassium ions (0–0.16 mol/l) in the homogenization medium on the extraction of specifically bound [^{3}H]dexamethasone from rat hypothalami. Results are expressed as a percentage of bound radioactivity recovered relative to the amount bound using 0.16 mol/l potassium ions. Bars represent the mean ±SEM of the number of experiments shown in parentheses.

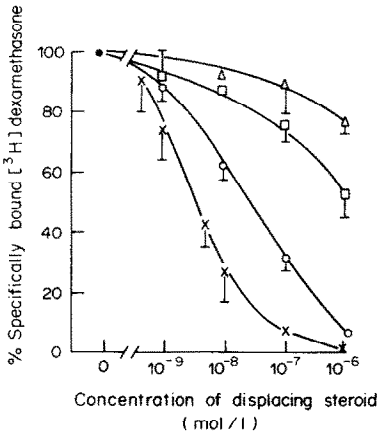

Fig. 3. The ability of various steroids to prevent the binding of [^{3}H]dexamethasone (10^{-8} mol/l) to cytosolic binding sites subsequently extracted from rat hypothalami incubated *in vitro*. The steroids tested were: dexamethasone (×), corticosterone (○), 11-epicortisol (□) and testosterone (△). Results are expressed as mean ±SEM of 5 experiments.

and the competition only reached significance when the former steroid was present at μM i.e. in 100-fold molar excess over the labelled steroid, at this point the competition amounted to 50%. Testosterone was much less able to compete for binding.

Biphasic inhibition of corticotropin factor secretion from the hypothalamus in vitro *by dexamethasone*

Following a 30 min period of preincubation of the tissue *in vitro* hypothalami were exposed to dexamethasone (1 nM) for 30 min and at times 0, 30, 45, 60, 80, 100 and 120 min after exposure to the steroid the blocks were challenged for 5 min with acetylcholine (5 μg/ml) and the release of bioactive CRF was determined. In addition, control tissues (not exposed to steroid) were challenged with the neurotransmitter for the first time at 150 min after their removal. The results shown in Fig. 4, show that the dexamethasone caused a biphasic inhibition of CRF secretion, the second phase of inhibition being most profound at 120 min after exposure to the steroid.

The effect of dose on the dexamethasone-mediated, delayed inhibition of corticotropin releasing factor secretion

Hypothalamic blocks were exposed to dexamethasone (0.15–5 nM) for 30 min, 30 min after their removal from animals and preincubation *in vitro* and 2 h later they were challenged with acetylcholine (5 pg/ml) for 5 min prior to the determination of CRF release. Figure 5 shows that dexamethasone causes a delayed inhibition of CRF release that is dose-related. Similar results were obtained (results not shown) when [^{3}H]dexamethasone in the same range of concentrations was tested functionally in this way.

The ability of 11-epicortisol to antagonise the dexamethasone-mediated, delayed inhibition of corticotrophin releasing-factor secretion

In a preliminary experiment 11-epicortisol (3 μM) was shown not to act as an agonist on the delayed feedback mechanism as indicated by either CRF accumulation in the tissue or release into the medium (Table 1), when the ability was tested 90 min after a 30 min period of exposure of the hypothalamic blocks

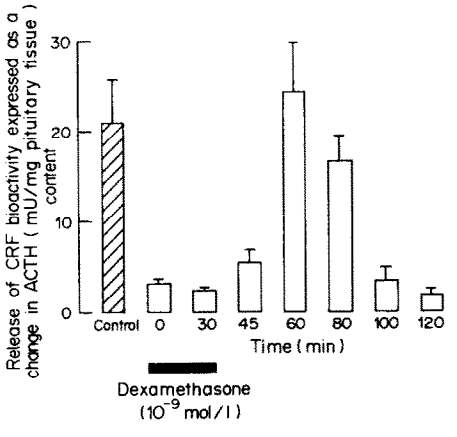

Fig. 4. Effect of dexamethasone (10^{-9} mol/l) on the acetylcholine (5 pg/ml)-induced secretion of bioactive corticotrophin-releasing factor (CRF) by the rat hypothalami *in vitro*. Release of CRF is expressed as a change in ACTH content in the pituitary. The control stimulation (hatched bar), which was of 5 min duration was performed at 120 min. Test stimulations, also of 5 min duration (open bars), were carried out during exposure to the steroid (solid bar) and after its removal. Values are means ±SEM of 5 determinations.

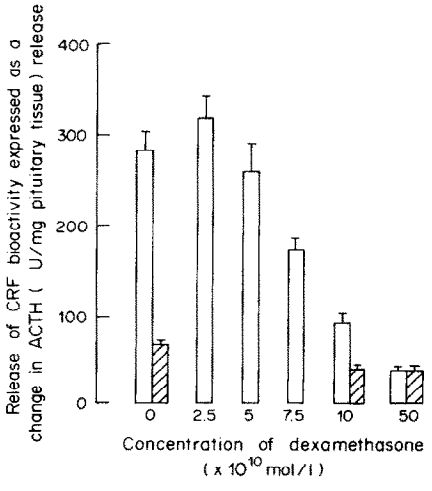

Fig. 5. Effect of exposure to dexamethasone for 30 min, 120 min previously, on the production of bioactive corticotrophin-releasing factor (expressed as ACTH release) induced in rat hypothalami *in vitro* by a 5 min period of stimulation with acetylcholine (5 pg/ml). Basal levels were determined for steroid-naive tissues and tissues previously exposed to 1 or 5 nmol/l dexamethasone (hatched bars). Values are means ±SEM of 5 determinations.

Table 1. Failure of 11-epicortisol to act as an agonist of dexamethasone in the delayed inhibition of changes in bioactive corticotrophin-releasing factor caused by the latter steroid

	CRF release	CRF content
	μU/per mg pituitary tissue	
Control	316.2 ± 77.0	110.4 ± 38.2
11-Epicortisol (3×10^{-6} mol/l)	301.5 ± 38.8	148.2 ± 36.4

Tissues were incubated with or without 11-epicortisol for 30 min, and 90 min after the removal of the steroid the tissues were challenged for 5 min with acetylcholine (5 pg/ml). Results are expressed as the release of ACTH from adenohypophysial fragments *in vitro*. Values are means ±SEM of 5 determinations.

to the steroid. On the other hand, when hypothalami were exposed simultaneously to dexamethasone (1 nM) and various doses of 11-epicortisol (1 nM/1 μM) it was found (Fig. 6) that the highest dose of the latter steroid prevented completely the inhibitory effect of dexamethasone i.e. when the antagonistic steroid was present in a 100-fold molar excess.

When the addition of 11-epicortisol (0.1 μM) was delayed for various time periods after the previous addition of dexamethasone (0.8 nM), but the antagonist was still left in contact with the tissue for 30 min, and the functional activity was assessed at 2 h after the addition of the agonist, the results shown in Fig. 7 were obtained. At all times up to the end of the exposure to dexamethasone i.e. 30 min, the application of 11-epicortisol resulted in the prevention of inhibition by the agonist. A further experiment (results not shown) showed that reversal of the inhibition did not occur if the 11-epicortisol was added 45 min after the dexamethasone.

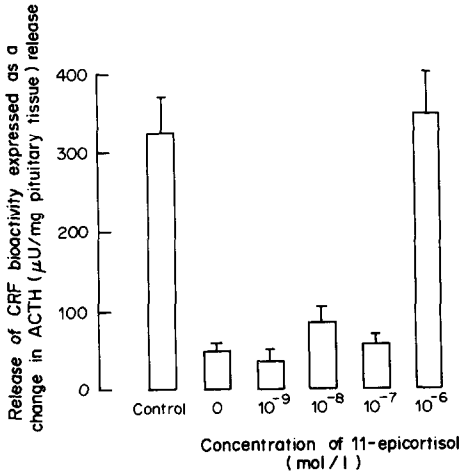

Fig. 6. Effect of exposure of rat hypothalami to dexamethasone (10^{-8} mol/l) in combination with 11-epicortisol for 30 min on the bioactive corticotrophin-releasing factor (expressed as ACTH release). The responses of control or steroid-exposed tissues to a 5 min period of stimulation with acetylcholine (5 pg/ml) applied 90 min after removal of the steroids are shown. Values are means ±SEM of 5 determinations.

DISCUSSION

The whole hypothalamus incubated *in vitro* in a CSF-like medium has proved to be very useful in studying the control of CRF secretion. The reasons for this are undoubtedly ascribable to the fact that the CRF containing neurones that project from the peraventricular nucleus to the median eminence remain intact, and that substances added to the incubating medium have rapid access to the nucleus *via* the third ventricle. The preparation has been used to determine the structure–activity relationships of various steroids in modulating CRF release [9, 11, 12, 13], and although these included dexamethasone detailed studies were only made of corticosterone. However, difficulties arise when using corticosterone in binding studies, and so a detailed investigation of the ability of dexamethasone to inhibit CRF release was undertaken in the present work to complement the binding data.

A serious criticism of the use of prepared cytosolic preparations for conducting glucocorticoid-binding assays is that the instability of the binding sites means that the binding has to be carried out at 4°C. Although glucocorticoid binding in experiments using intact tissue or cell preparations have been reported the present work appears to be the first in which the viability of the hypothalamus *in vitro* at 37°C has been taken advantage of for carrying out binding studies in that tissue.

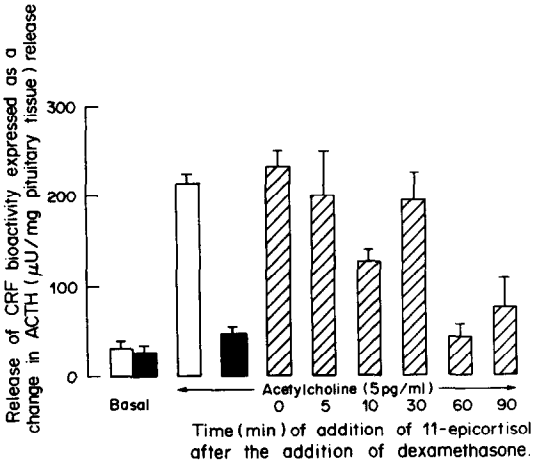

Fig. 7. The ability of 11-epicortisol (10^{-7} mol/l) to prevent the delayed inhibition by dexamethasone (8×10^{-10} mol/l) of the release of bioactive corticotrophin-releasing factor (expressed as ACTH release). The tissue was exposed to dexamethasone a time zero for a period of 30 min and at the time indictated *after* the addition of that steroid 11-epicortisol was added, also for 30 min. At 90 min after the removal of the dexamethasone the tissues were challenged by a 5 min period of exposure to acetylcholine (5 pg/ml). Open bars represent basal and stimulated release of CRF by control tissues, closed bars represent tissues exposed to dexamethasone alone and hatched bars represent tissues exposed to both steroids. Values represent means ±SEM of 5 determinations.

Solutions in which the main osmotic support is non-ionic (e.g. sucrose, glycerol, mannitol) have frequently been used for the extraction of glucocorticoid binding sites from tissues. However, in the present work we have investigated the use of an isotonic buffer in which the osmotically active constituents are ions, constituted to resemble the intracellular *milieu* (buffer B). In such experiments use of buffer B leads to the recovery of 40% more binding sites in the cytosol when compared with the sucrose-based buffer A. K^+ are well-known to affect glucocorticoid receptors in several ways. Thus solutions containing KCl at 0.4 M have been used for extracting receptors from the nucleus [21], but such extraction shows no cation specificity and may be presumed to be the result of osmotic shocking of nuclei. Buffer B, even when containing as much as 0.16 M K^+ was still isotonic, and a substitution of a similar concentration of Na^+ for K^+ in buffer B did not increase the amount of bound [^{3}H]dexamethasone extracted above that found using sucrose/phosphate buffer. Increasing concentrations of K^+ in buffer B caused an increase in the amount of bound steroid extracted from the tissue until a plateau was reached at a concentration of K^+ equal to 0.1 M. These results suggest that buffer B prevents non-specific adsorption of [^{3}H]dexamethasone binding sites to membranes which would otherwise occur following homogenization. However, the alternative view that sugars in general (and sucrose in particular) help to preserve intracellular organelles and their contents during homogenization cannot be excluded.

Extraction with buffer B was then used further to characterize the dexamethasone binding site(s) in extracts of hypothalami previously incubated *in vitro* with added steroid. In competition studies both dexamethasone and corticosterones were potent in their abilities to prevent the binding of [^{3}H]dexamethasone with ED_{50} values 3×10^{-9} and 3×10^{-8} M respectively. At high concentrations, 11-epicortisol caused an approx. 50% reduction in the binding of [^{3}H]dexamethasone, whereas testosterone caused relatively little displacement even at the highest concentration used. The only concentration of 11-epicortisol able to compete significantly was one of 1 μM which caused a 50% reduction in the binding of 10 nM [^{3}H]dexamethasone i.e. when present in 100-fold excess.

From a functional point of view both dexamethasone and [^{3}H]dexamethasone were able to mimic corticosterone in its ability to cause biphase inhibition of CFR release from the hypothalamic. The second phase of inhibition (delayed feedback) was dose-related over the concentration range 0.5 to 5.0 nmol/l and this inhibition emerged despite the absence of added steroid from the medium for 90 min before challenge with acetylcholine. 11-Epicortisol was able to antagonise the delayed inhibitory effect of dexamethasone (10 nM), the dose–response curve (like that for dexamethasone itself) was very steep, such that no reversal of inhibition was registered at 100 nM but complete reversal was apparent at 1 μM. This finding accords well with the observation that 11-epicortisol only competes significantly with dexamethasone for binding sites when present in 100-fold molar excess. The fact that 11-epicortisol at 1 μM was able to complete for binding sites with dexamethasone at 10 nM only to the extent of 50%, yet this results in full reversal of the functional inhibition suggests that not more than 50% of the glucocorticoid binding sites extractable from the hypothalamus represent feedback sites.

The time course of the dexamethasone delayed inhibitory effect on CRF secretion seems to fall into two segments. In the first segment, lasting for between 30 and 45 min the tissue may be "rescued" from inhibition by the addition of 11-epicortisol, although this steroid has no effect on the fast inhibition currently seen in that time zone [22], thereafter despite the fact that the delayed inhibition has not yet become overt no prevention of the effect of dexamethasone is possible. This sort of time course is consistent with an activation of the receptor–steroid complex, its translocation to the nucleus and binding to an acceptor site on the DNA.

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