Inhibition of Binding of Corticotropin-(1-24)-tetracosapeptide (Synacthen) to Membrane Receptors of Adrenal Cortex by Cortisol

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The binding of Synacthen to partially purified bovine adrenocortical plasma membranes was shown to be inhibited by cortisol. The findings suggest that cortisol is involved in a peripheral feedback mechanism for the control of its release.

During attempts to establish a radio-receptor assay for corticotropin, it became apparent that ^{125}I -labelled Synacthen had different binding properties to unlabelled material. A variety of methods were used in the preparation of the samples of iodinated Synacthen studied, namely the chloramine-T method, the lactoperoxidase method, purification of labelled Synacthen by ion-exchange chromatography (Lefkowitz *et al.*, 1970a) and receptor purification (Smith & Hall, 1974). Alteration of binding properties by iodination would not be surprising since it is apparent that the corticotropin-binding site is dependent on a negative charge (Ways *et al.*, 1976) and iodination would significantly affect the charge on the corticotropin molecule. This would explain the emphasis placed by other authors on the use of monoiodinated corticotropin in radio-receptor assay systems. However, even with monoiodinated corticotropin we have not been able to achieve the sensitivity of radio-receptor assays reported by these other authors (Lefkowitz *et al.*, 1970a; Wolfson *et al.*, 1972; McIlhinney & Schulster, 1973).

We therefore decided to investigate unlabelled Synacthen by binding it to partially purified adrenal-cortex-membrane preparations, separating these, liberating the Synacthen with acid and determining the hormone liberated by means of radioimmunoassay. Here, binding to antibody is used and experience has shown that, unlike receptor binding, labelling of corticotropin with ^{125}I gives good reproducibility. Furthermore it is well recognized that, although labelling may affect ability to bind to anti-antibody, this is of no consequence in the radio-immunoassay, since the essential feature is the identity of standard and unknown. In the course of the present investigation, information has been obtained that indicated that cortisol appears to affect the binding of Synacthen to its receptors.

Materials and Methods

Dithiothreitol, cortisol, 11-deoxycortisol and β -mercaptoethanol were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. ^{125}I (product no. IMS-30) was from The Radiochemical Centre, Amersham, Bucks., U.K. Synacthen was from CIBA Laboratories, Horsham, Sussex, U.K. Horse serum and antiserum to human corticotropin (type RDO6) were from Wellcome Laboratories, Beckenham, Kent, U.K. Charcoal (Norit GSX) was from Hopkin and Williams, Chadwell Heath, Essex, U.K. Dextran T-70 was from Pharmacia (G.B.), London W.5, U.K.

Human serum albumin was a 10% (w/v) solution from the Blood Products Laboratory, Lister Institute of Preventive Medicine, Elstree, Herts., U.K.

The hand-held homogenizer was a 30ml-capacity Uniform Homogenizer (type H104/9) from Jencons (Scientific), Hemel Hempstead, Herts., U.K. The Microfuge and Microfuge tubes were from Precision Machining Engineers, Harrow, Middx., U.K. Other centrifuges were a Mistral 6L and a High Speed 18 (MSE Scientific Instruments, Crawley, Sussex, U.K.). The rotating mixer was an Eschmann Blood Cell Suspension Mixer (Eschmann Bros. and Walsh, Shoreham-by-Sea, Sussex, U.K.).

Radioactivity counting was performed on an ICN GammaGuard 150. Polystyrene tubes (12 mm $\times$ 75 mm) were from Walter Sarstedt, Leicester, U.K.

Preparation of membrane suspensions

Bovine adrenal glands were obtained immediately after slaughter and transferred to an ice-cold solution of 10 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-MgCl₂ and 0.3 M-sucrose. The cortices were dissected out within $\frac{1}{2}$ h of death and 20 g was homogenized in 60 ml of the same buffer in a hand-held homogenizer.

A partially purified plasma-membrane pellet was prepared from the homogenate by the method of McIlhinney & Schulster (1973). The pellet was resuspended in 20 ml of 10 mM-Tris/HCl buffer, pH 7.4, and stored at -20°C in 2 ml samples.

Determination of Synacthen binding

Synacthen binding to adrenal membranes was studied at 4°C in 10 mM-Tris/HCl buffer, pH 7.4, containing 1 mM- MgCl_2 , 1 mM-dithiothreitol and 1% human serum albumin. Resuspended membranes (0.1 ml, 1 mg of protein) were added to 0.7 ml of buffer, followed by an appropriate concentration of the substance under test in 0.1 ml of the same buffer. After 15 min 10 ng of Synacthen in 0.1 ml of the same buffer was added.

After 1 h incubation, the tubes were centrifuged at 14000 g in a Microfuge to sediment the adrenal tissue. The supernatant was removed, 1 ml of 0.1 M-HCl was added, the tube vortexed for a few seconds and then mixed for 30 min at 4°C on a rotary mixer and finally centrifuged again. A 0.1 ml portion of the acid supernatant was diluted to 1 ml with radioimmunoassay buffer. The Synacthen content of the diluted fraction was determined by radioimmunoassay.

Synacthen removed by the acid was considered to be that bound by the adrenal membranes. No binding of Synacthen was observed with membranes from bovine kidney, liver or muscle. Significant recovery of Synacthen was achieved from bovine adrenal-cortex tissue, varying between about 250 and 3000 pg (from the original 10 ng added) in individual adrenal preparations. Binding was completely unaffected by the presence, in large excess, of parathyrin, pig calcitonin, lutropin or follitropin. This would indicate the peptide specificity of the receptor studied.

Radioimmunoassay of Synacthen

Assay of Synacthen was performed with antiserum (Wellcome type RDO6) to human corticotropin. Standard solutions of Synacthen were prepared in assay buffer containing 0.1 M-sodium phosphate, pH 7.4, containing 0.5% human serum albumin and 0.5% β -mercaptoethanol. Standard or unknown solutions (0.2 ml) were added to 0.3 ml of assay buffer in polystyrene tubes. Antiserum (0.1 ml) in assay buffer at a dilution of 1:60000 was added and incubation carried out for 3 days at 4°C . ^{125}I -labelled Synacthen (12.5 pg) in 0.1 ml of buffer was then added and incubation continued for a further 24 h. Iodination was performed by the chloramine-T method as described by Landon *et al.* (1967). Bound and free Synacthen were then separated by adding 0.3 ml of a suspension of coated charcoal. The suspension was prepared by mixing 17.5 ml of 0.1 M-sodium phosphate buffer, pH 7.4, with 7.5 ml of horse serum and adding 0.25 g of charcoal and 0.0625 g of Dextran T-70. The suspension was cooled to 4°C and stirred

continuously while being added to the tubes. The tubes were allowed to stand for 5 min and then centrifuged for 5 min at 500 g in a Mistral 6L centrifuge at 4°C . The supernatant was removed by aspiration and the charcoal, containing the free Synacthen, counted in the γ -counter for sufficient time to accumulate 10000 counts (2–4 min).

Duplicate control tubes to determine incubation damage (Berson & Yalow, 1968) were included in each batch of analyses, by adding 0.1 ml of assay buffer in place of antiserum. Damage was always less than 10% of total counts added. The percentage of antibody binding of ^{125}I -labelled Synacthen was calculated by reference to the damage-correction tubes.

Sensitivity and precision of the assay were determined by including ten observations of each point in a standard curve.

Investigation of Synacthen binding

The effects of cortisol and 11-deoxycortisol on the binding of Synacthen to the adrenal-cortex membranes were investigated by adding appropriate concentrations of these substances in 0.1 ml of buffer to the incubation media 15 min before the addition of Synacthen. We have also carried out observations in which the cortisol incubation followed that with Synacthen.

Results

Radioimmunoassay of Synacthen

A typical standard curve for the radioimmunoassay of Synacthen is shown in Fig. 1, which shows the means ($\pm 2\text{s.d.}$) for ten separate standard tubes of Synacthen at concentrations of 0, 12.5, 25, 50 and 100 pg/ml. Duplicates only at 200 and 400 pg/ml were determined.

Sensitivity is calculated by taking the concentration that represents binding 2 s.d. less than the mean of the zero tubes. This point is about 5 pg/ml. The only slight overlap of the spread of the observations occurs between standards of 12.5 and 25 pg/ml. Overlap would not occur for the same spread of observations at 10 and 30 pg/ml, indicating that a confident distinction can be drawn between low concentrations as close as 30 and 10 pg/ml from duplicate observations. These points represent recoveries of 300 and 100 pg from the 10 ng of Synacthen added originally to the membranes since the acid extract was diluted tenfold before assay (3 and 1% respectively). Much higher recoveries were usually achieved, and, if recovery exceeded 2000 pg (equivalent to the 200 pg/ml point on the standard curve), the extracts were re-assayed at greater dilution.

Binding of Synacthen to adrenal-cortex cell membrane

Increased concentrations of cortisol progressively inhibited Synacthen binding, as shown in Fig. 2,

Fig. 1. Standard curve for Synacthen radioimmunoassay. Points at 0, 12.5, 25, 50 and 100 pg/ml show means $\pm$ 2 S.D. for ten observations.

Fig. 2. Inhibition of binding of corticotropin to adrenal-cortex membranes by cortisol (●).

The control binding was 1350 pg of corticotrophin; points are the means $\pm$ S.E.M. for eight determinations. The control value for 11-deoxycortisol (Δ) was 2675 pg of corticotrophin; points are the means $\pm$ S.E.M. for four estimations.

which gives the results of one experiment, each point being determined by eight separate observations. It is interesting, however, that 11-deoxycortisol had no

Fig. 3. Failure of cortisol in increasing concentrations to dissociate Synacthen bound to membranes. Points are means $\pm$ S.E.M. for eight determinations.

effect. The inhibitory effect of cortisol was abolished by adding EDTA to the medium. This experiment has been repeated on five separate adrenal-cortex-tissue preparations. There was variation in maximum binding ability, but in each case similar inhibition was demonstrated. In the experiment in which cortisol was added after Synacthen had been bound to the membrane, no releasing effect was observed, as shown in Fig. 3.

Discussion

These experiments have demonstrated that cortisol, but not its immediate metabolic precursor, 11-deoxycortisol, inhibited binding of Synacthen to adrenal-cortex membranes. This binding was specific, since it was found only with the preparations from adrenal cortex and not from other tissues. Moreover, in spite of the fact that partially purified membrane preparations were used, the binding specificity indicates that binding involved the specific corticotropin receptors of the membranes. The binding inhibition process appeared to be dependent on critical ionic concentrations, since it was abolished by EDTA. Lefkowitz *et al.* (1970b) have demonstrated inhibition of binding of labelled corticotropin to adrenal receptors by Ca^{2+} ions. The partially purified plasma membranes also contain some mitochondria, and these may possibly play a part in the plasma-membrane binding process. It is known that mitochondria contain large amounts of calcium, and that permeability of their membranes to Ca^{2+} is increased by cyclic AMP (Borle, 1974). Cyclic AMP inhibits binding of Synacthen, but Ca^{2+} ions both inhibit binding and release Synacthen bound to membranes (A. L. Latner, D. B. Cook & K. U. Solanki, unpublished work). However, we have found that cortisol does not release corticotropin already bound to the membrane, and it seems, therefore, unlikely that its inhibitory effect on binding is dependent upon Ca^{2+} release from mitochondria.

From our findings, it would appear that cortisol

could regulate its own synthesis by inhibiting binding of corticotropin to adrenal-cortex cells, since Synacthen contains the receptor binding site (Hofmann *et al.*, 1970).

Although these deductions are based on observations using partially purified adrenal-cortex-cell plasma membranes, we feel justified in assuming that the postulated mechanism would also apply to intact cells. There is no obvious reason to believe that the situation with native corticotropin would be different from that with Synacthen.

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