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Effect of (1–24)Adrenocorticotrophin Stimulation on the Rate of Corticosterone Synthesis by the Rat Adrenal Cortex

G. R. CAM AND J. R. BASSETT[1]

School of Biological Sciences, Macquarie University
North Ryde, N.S.W. 2113, Australia

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CAM, G. R. AND J. R. BASSETT. *Effect of (1–24)adrenocorticotrophin stimulation on the rate of corticosterone synthesis by the rat adrenal cortex.* PHARMACOL BIOCHEM BEHAV **25**(4) 753–756, 1986.—The rate of corticosterone synthesis by the rat adrenal gland was measured *in vitro*, using a cell-free system, following the *in vivo* administration of (1–24)ACTH. The doses of ACTH used were 50, 100 and 250 μg ACTH/kg body weight. With all 3 doses of ACTH there was a significant increase in the rate of corticosterone synthesis within the first 5 min; this initial increase in rate did not vary with the dose of ACTH given. With both the 50 and 100 μg/kg doses the new rate of synthesis was maintained without further change, up to 30 min post-injection. In the case of the 250 μg/kg dose there was a second significant increase in the rate of corticosterone synthesis observed after 20 min. The results are discussed in the light of the hypothesis that the differential response of the adrenal gland represents the binding of ACTH to two receptors; a high affinity receptor of low abundance and a low affinity receptor of greater abundance. The results are consistent with the initial steroidogenic response resulting from binding to the high affinity receptors. Because of their low abundance these receptors may be completely occupied even at low doses of ACTH, thus explaining the dose-independent nature of the initial response to ACTH. Binding to the more abundant low affinity receptors may be associated with the secondary dose-dependent enhanced synthesis rate, a response which may be mediated via c-AMP.

Adrenocorticotrophin Steroidogenesis Corticosterone Adrenal cortex

EXPOSURE of rats to unpredictable stress [1] or an intraperitoneal injection of (1–24)adrenocorticotrophin (ACTH) [3] has been shown to result in a biphasic rise in plasma corticosterone. Following an IP injection of ACTH, the first phase of the steroid increase was essentially the same for all doses of ACTH administered, whereas the amplitude of the second phase was dose dependent [3].

Since two types of ACTH receptor have been proposed for the adrenal gland; a high affinity receptor of low abundance, and a low affinity receptor of greater abundance [7,9], Cam and Bassett [3] suggested that the differential stimulation of these receptors may be involved in the differential plasma corticosterone response to ACTH. They proposed that the first phase of the adrenal response to ACTH may be due to the binding of ACTH to the high affinity receptors, facilitating the immediate release of stored corticosterone but having little effect on synthesis. The second phase of the adrenal response may be associated with occupancy of the low-affinity receptors resulting in an enhancement of steroidogenesis, the number of receptors occupied determining the rate of synthesis.

The present study was undertaken to investigate the effect of ACTH on the rate of steroidogenesis by the adrenal cortex, and to see if the pattern of steroidogenesis was dependent on the dose of ACTH administered. The rate of corticosterone synthesis was measured *in vitro*, using a cell-free system, following *in vivo* administration of the peptide hormone.

METHOD

Animals

Male CSF rats, 90±5 days old were used in this study. The animals were housed in groups of 3 under conditions of constant temperature and humidity (21±0.5°C; 46% humidity) and subjected to a 12 hr reversed night-day schedule (light 2000 to 0800 hr) beginning at least 14 days prior to the commencement of experimentation. Food and water were provided ad lib. All animals were pretreated with betamethasone (20 μg/ml) in the drinking water for a period of 24 hr prior to experimentation in order to suppress endogenous ACTH secretion.

Procedures

The rates of synthesis of corticosterone were determined at 3 doses of ACTH (50, 100 and 250 μg/kg body weight). Animals were subjected to an intraperitoneal injection of ACTH either 5, 10, 20 or 30 min prior to sacrifice by cervical

[1]Requests for reprints should be addressed to Dr. J. R. Bassett.

TABLE 1

EFFECT OF IN VIVO ADMINISTRATION OF (1–24)ACTH ON THE IN VITRO SYNTHESIS OF CORTICOSTERONE BY THE ADRENAL GLAND

Dose of ACTH (μg/kg)	Time (min)	Rate of corticosterone synthesis (mean ± S.D.) (ng steroid/mg tissue/min) 0	5	10	20	30
50	Rate	0.88 ± 0.07[a]	1.19 ± 0.17[a]	1.24 ± 0.17	1.24 ± 0.14	1.12 ± 0.16
100	Rate	0.88 ± 0.07[a]	1.15 ± 0.16[a]	1.35 ± 0.17	1.58 ± 0.28[c]	1.08 ± 0.21[d]
250	Rate	0.89 ± 0.06[a]	1.47 ± 0.26[a]	1.54 ± 0.16[b]	2.35 ± 0.26[b,c]	2.27 ± 0.29[d]

Time = time following IP injection of ACTH.
Zero (0) min represents a control group of animals without any prior treatment.
[a,b]Significant difference between times.
[c,d]Significant difference between doses (unpaired 't' test, $p<0.05$).

TABLE 2

LINEAR REGRESSION ANALYSIS (Y = mx + b) ON DATA FOR CORTICOSTERONE SYNTHESIS *IN VITRO* FOLLOWING IN VIVO STIMULATION WITH (1–24)ACTH

Dose of ACTH (μg/kg)	Time of ACTH stimulation (min)	y intercept (b) (μg corticosterone)	Regression coefficient (r)
50	0	0.06 ± 0.10	0.99
	5	0.04 ± 0.18	0.96
	10	−0.04 ± 0.14	0.96
	20	0.06 ± 0.09	0.97
	30	−0.18 ± 0.10	0.96
100	0	0.04 ± 0.10	0.98
	5	0.03 ± 0.17	0.94
	10	−0.02 ± 0.12	0.97
	20	0.19 ± 0.14	0.91
	30	−0.09 ± 0.14	0.93
250	0	−0.18 ± 0.10	0.99
	5	0.06 ± 0.21	0.93
	10	0.08 ± 0.09	0.96
	20	0.05 ± 0.18	0.91
	30	0.07 ± 0.09	0.93

(Mean + S.E.)
Gradients (m) given in Table 1. Values on y axis (μg corticosterone synthesised) range up to 10 μg for the 50 and 100 μg ACTH/kg doses, and up to 16 μg for the 250 μg ACTH/kg dose. There was no significant difference between any of the intercept values.

dislocation. These times were chosen as they covered both phases of the biphasic rise in plasma corticosterone in response to ACTH [3]. Five animals were used for each time period and their adrenal glands pooled. This procedure was repeated 6 times (n=6) for each time period. Animals were sacrificed between the hours of 0900 and 1100 in order to minimize circadian variation in corticosterone levels and synthesis rates. Both adrenal glands were immediately removed, transferred to Krebs-Henseleit solution at 4°C and cleaned of accessory tissue. Control animals, subjected only to betamethasone pretreatment, were removed from their home cage and sacrificed immediately. A control group (n=6) was used with each dose of ACTH. The results for the control groups are represented in Table 1 as the zero (0) min values.

Incubation Using a Cell-Free System

The method used was essentially that described by Koritz and Kumar [5]. Adrenal tissue from 5 animals was homogenized in neutralised 0.25 M sucrose (100 mg wet weight/ml). The homogenate was centrifuged at 900 g for 10 min and the pellet discarded. The supernatant was then centrifuged at 9000 g for 10 min and the mitochondrial pellet so obtained was washed twice by resuspending in 0.25 M sucrose and centrifuging at 7,300 g for 10 min each time. The

supernatant from the first mitochondrial pellet was centrifuged at 144,000 g for 45 min to sediment the microsomes. The mitochondrial and microsomal fractions were then resuspended in 0.25 M sucrose to a final volume equal to that of the original homogenate.

For synthesis of corticosterone from naturally occurring precursors, the following incubation medium was set up: 0.4 ml of microsomal suspension, 0.3 ml of 0.3 M Tris buffer at pH 7.5, 0.15 ml of generated NADPH, 0.1 ml of 0.01 M NAD^+, 0.15 ml of 0.1 M Mg Cl_2, 0.15 ml of 0.2 M phosphate at pH 7.5, 0.15 ml of 0.25 M succinate at pH 7.5, and 0.7 ml of 0.25 M sucrose. Chemicals were obtained from Sigma, St. Louis, MO. NADPH was generated by prior incubation of a solution containing NAD^+ (25 μmoles/ml) and glucose-6-phosphate (70 μmoles/ml) with 0.025 ml glucose-6-phosphate dehydrogenase for 15 min at 21°C (NAD^+, glucose-6-phosphate and glucose-6-phosphate dehydrogenase supplied by Calbiochem-Bohring, Australia). The incubation medium above was maintained at 21° C in a shaking water bath. To start the synthesis reaction 0.4 ml of mitochondrial suspension was added to the incubation medium. Samples (100 μl) were removed at 0, 2, 4, 6 and 8 min after addition of mitochondria and the steroids immediately extracted with 1 ml dichloromethane (DCM). The 0 min value represents endogenous corticosterone levels existing in the gland before separation of the mitochondrial and microsomal fractions. This value is subtracted from the corticosterone levels obtained following 2, 4, 6, and 8 min incubation.

Chromatographic Separation of Corticosterone

Aliquots of the DCM extracts were evaporated to dryness in a water bath at 37°C. The extracted steroids were resuspended in 20% ethylacetate in iso-octane (20% mobile phase) and added to a micro-celite column. The celite (acid washed celite 545, Altech Associates, Sydney, Australia) was prepared by heating at 700°C for 24 hr, then stored in a dessicator. The celite was mixed with 0.33 ml of stationary phase (80:20 ethylene glycol:water) per gram of celite, then packed into micro columns (0.5 g/column). The column was flushed with 20% mobile phase, then the resuspended steroids were added. The column was again flushed with 3.5 ml of 20% mobile phase to remove the two immediate precursors to corticosterone (progesterone and deoxycorticosterone). Recovery studies using labelled progesterone and deoxycorticosterone showed that 84.5±4.8% and 96.8±1.9% respectively of these steroids were eluted in this fraction. A 30% mobile phase (3.5 ml) was then run through the column to elute the corticosterone. Recovery studies using labelled corticosterone showed that 88.3±2.4% of the corticosterone was eluted in this fraction. Three 1 ml aliquots of the corticosterone fraction were dried under nitrogen and assayed for corticosterone using a competitive protein binding (CPB) assay. Recovery estimates for corticosterone were carried out with each run, and the amount of corticosterone extracted was corrected for recovery efficiency. The mean % recovery for all estimates was 86.2±2.1% (mean±S.E.M.).

Corticosterone Assay

The method used was a modification of the competitive protein binding assay described by Murphy [8]. A corticosterone-binding globulin-isotope solution, 8% human control serum (Hyland Diagnostics) containing sufficient 1–2 [H^3] corticosterone (Amersham International) to saturate available binding sites, was added to the separated extract and vortexed. The tubes were then incubated at 37°C for 15 min, followed by 45 min at 4°C. Unbound steroid was removed using 0.2 ml of a dextran coated charcoal suspension (625 mg activated charcoal and 62 mg dextran in 100 ml phosphate buffer at pH 7.0 containing 30% glycerol). After centrifugation an aliquot of the supernatant (bound hormone) was transferred to scintillation fluid and counted in a Packard 3375 scintillation counter. Standard curves were prepared using known quantities of unlabelled corticosterone (Sigma Chemical Co.) and plotted as Free/Bound against the amount of corticosterone. The inter- and intra-assay variabilities were both approximately 4%.

Analysis of Results

Synthesis of corticosterone in the cell-free system commenced with the addition of the mitochondrial fraction to the microsomal fraction. The amount of corticosterone synthesised/mg adrenal tissue was measured following 0, 2, 4, 6 and 8 min incubation time. The amounts of corticosterone synthesised were plotted against the time of incubation (0–8 min), and linear regression lines fitted by the method of least squares. Each line was tested statistically and found to be linear and the gradient was calculated. The gradient represents the rate of corticosterone synthesis in response to ACTH stimulation in ng corticosterone/mg of adrenal tissue/min. The mean rates obtained for each post-injection time and for each dose of ACTH were compared statistically using unpaired t-tests. ($p<0.05$ was taken as a significant difference.)

RESULTS

The results are shown in Table 1. With all 3 doses of (1–24)ACTH (50, 100 and 250 μg/kg), there was a significant increase in the rate of corticosterone synthesis within the first 5 min after the IP injection (unpaired t-test, $p<0.05$ in all cases). With both the 50 and 100 μg/kg dose of ACTH, this new rate of synthesis was maintained up to 30 min; there was no further significant increase in rate over this time. In the case of the 250 μg/kg dose, however, there was a second significant increase in the rate of corticosterone synthesis observed at 20 min (unpaired t-test, $p<0.05$) which was maintained up to 30 min. There was no significant difference in the rate of synthesis between the 3 doses of ACTH at either the 5 or 10 min times. At 20 and 30 min however, the rate of synthesis induced by 250 μg ACTH/kg was significantly greater than the corresponding rates following the injection of either 50 or 100 μg ACTH/kg ($p<0.05$ in both cases). Table 2 shows the intercept values and mean regression coefficients obtained from the linear regression analysis.

DISCUSSION

The IP administration of ACTH resulted in a very rapid increase in steroidogenesis by the adrenal gland. The initial increase in synthesis rate was similar for all doses of ACTH examined. However with the larger dose of ACTH (250 μg/kg) there was a second more substantial increase in rate after 20 min post-injection. Such a secondary increase in rate

was not observed with either of the lower doses, although there was a tendency for the rate to rise at 20 min with the 100 μg/kg dose. Comparing the present observations with the findings of Cam and Bassett [3], regarding plasma corticosterone elevation in response to an IP injection of ACTH, it should be noted that 50 μg of ACTH/kg did not produce any secondary rise in plasma corticosterone concentrations. Both the 100 and 250 μg/kg dose, however, did produce secondary rises in plasma corticosterone concentration, the effect being greatest with the latter dose.

The present results would tend to support the hypothesis of Cam and Bassett [3]. The first phase of the adrenal response to ACTH may be due to the binding of ACTH to a limited number of high affinity receptors. Because of their low abundance, these receptors may be completely occupied even at low doses of ACTH, thus resulting in the relatively dose-independent nature of the initial response to ACTH. However, while binding to these receptors may facilitate the immediate release of stored corticosterone as suggested by Cam and Bassett [3], the present study clearly demonstrates that there is also an enhanced synthesis rate at this stage. The proposal that the second phase of the adrenal response may be associated with occupancy of the low-affinity high abundance receptors, resulting in a dose-dependent enhancement of steroidogenesis, it is also supported by the findings of the present study. With the 250 μg/kg dose of ACTH there was a secondary enhancement in synthesis rate, a response not seen with the lower doses. As would be expected this enhanced steroidogenesis preceded the secondary increase in plasma corticosterone, which was not apparent until after 25 min post-injection [3].

At this stage one can only speculate as to the nature of the two different types of receptors involved. Two different ACTH receptors have been associated with the two intracellular chemical messengers, c-AMP and cGMP [2]. Sharma, Ahmed, Sutliff and Brush [10] considered that ACTH induced steroidogenesis was facilitated via both c-AMP and cGMP; the former at high doses of ACTH, the latter at lower doses. The mechanism of steroidogenic action of ACTH mediated via c-AMP formation may occur in conditions where large and prolonged stimulation with ACTH activates the whole adrenal machinery in response to an emergency [4]. Such activation of steroid synthesis would require the synthesis of protein(s) of short half-life from a stable messenger RNA [9] and would explain the delay in initiation of the secondary enhancement in synthesis rate.

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