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THE EFFECT OF ACTH ON CALCIUM DISTRIBUTION IN THE PERFUSED CAT ADRENAL GLAND

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SUMMARY

1. Experiments were carried out to study the effects of adrenocorticotrophin (ACTH) on calcium distribution in the isolated cat adrenal gland perfused with Locke solution.

2. The addition of ACTH to Locke solution caused a small, but significant, increase in the radiocalcium (^{45}Ca) space and content of the adrenal cortex.

3. The average ^{45}Ca washout curve obtained after exposure to ACTH was significantly displaced above the average washout curve for the control glands during the first 20 min of washout. During the time that the two curves were significantly different, maximum corticosteroid secretion was attained.

4. The rate of ^{45}Ca efflux was slowed after ACTH was added to the perfusion medium.

5. The total calcium content of the cortex was not altered by ACTH.

6. Perfusion with calcium-free Locke caused a rapid decrease in the calcium content of the cortex to about 20 % of the control value. Dinitrophenol was required to deplete completely the cortical calcium content during perfusion with the calcium-deprived medium.

7. The results are consistent with the suggestion that a translocation of calcium occurs during stimulation of the cortex by ACTH. The source of the calcium ions needed to support the secretory response to ACTH is probably not the extracellular fluid; but ACTH may shift calcium from a rapidly exchanging to a more slowly exchanging cellular pool.

INTRODUCTION

Considerable evidence has accumulated to support the hypothesis that calcium entry into the secretory cell may be the critical link between the effect of a given stimulating agent and the extrusion of secretory product

from a diverse number of secretory organs (Douglas, 1968; Rubin, 1970). One line of evidence supporting this view is that calcium must be present in the solution perfusing or bathing a given organ for the maintenance of evoked secretion. In addition, stimulation of certain endocrine glands, including the adrenal medulla (Douglas & Poisner, 1962; Rubin, Feinstein, Jaanus & Paimre, 1967) and neurohypophysis (Douglas & Poisner, 1964; Ishida, 1967), causes a marked enhancement of calcium exchange between tissue and extracellular fluid.

In contrast to other secretory cells that store their secretory product before release, the cells of the adrenal cortex contain no readily visible granules or vacuoles of stored secretory product; and the mechanism by which corticosteroid leaves the cell is, at present, an enigma (Fawcett, Long & Jones, 1969). However, recent studies from our laboratory have shown that when an adrenal gland is perfused with a calcium-free solution, adrenocorticotrophin (ACTH) still increases steroid production within the gland, while hormone secretion is almost completely inhibited (Jaanus, Rosenstein & Rubin, 1970).

In the present study a quantitative correlation between the effect of ACTH and calcium distribution in the cat adrenal gland was attempted in order to gain further understanding of the role of calcium in adrenocortical secretion which might be related to a more general mechanism of calcium action in the secretory process. The main conclusion drawn from the present studies is that a translocation of calcium occurs during stimulation of the cortex by ACTH. The calcium ions necessary to support the secretory response to ACTH are probably not supplied by an increased influx of extracellular calcium, but rather by some readily exchangeable cellular fraction.

METHODS

Adrenal perfusion. Under intraperitoneal pentobarbital anaesthesia, the left adrenal gland of the cat was prepared for perfusion *in situ* at room temperature according to the method of Douglas & Rubin (1961). In certain experiments a modification of this original method was employed so that the left and right adrenals were perfused simultaneously. When both adrenals were perfused, the right gland and kidney were left *in situ* and collection of effluent was carried out by means of a cannula inserted into the vena cava after this vein had been tied off below the liver.

All perfusions were carried out with normal or modified bicarbonate-buffered Locke solution, equilibrated with 95 % oxygen and 5 % carbon dioxide, with a pH of 7·0. All glands were initially perfused with normal Locke solution for approximately 1 hr before a given experiment was begun. Modification of the normal Locke solution, either by omitting calcium or by adding ACTH, did not alter flow rate through the gland.

Steroid determinations. The perfusate was assayed for 11-hydroxycorticosteroids by fluorescence in alcoholic sulphuric acid (for details see Jaanus *et al.* 1970). The output of steroid was expressed as hydrocortisone (alcohol) μg released/min.

^{45}Ca space experiments. In this first series of experiments both adrenal glands were perfused with Locke solution containing radiocalcium [^{45}Ca], 0·25 μc/ml. The left gland was removed after 2·5 min exposure to radiocalcium, and the right gland was removed after a 20 min exposure to radiocalcium. In certain experiments ACTH was present during the final 17·5 min of exposure of the right gland to radiocalcium. The adrenal glands were trimmed and blotted on ashless filter paper; the cortex was separated from the medulla as completely as possible and weighed. The ^{45}Ca remaining in the cortex was determined after dry-ashing the cortex at 600° C overnight. The ash was resuspended in 1 ml. 0·1 N-HCl, evaporated to dryness on planchets, and radioactivity determined in a low background gas flow counter (Nuclear Chicago). The ^{45}Ca space (ml./100 g) was calculated by dividing the total cpm/wet weight of cortex by the cpm/ml. in ^{45}Ca-Locke solution. This space is expressed in terms of ml. of perfusion medium that would supply the same radioactivity in 100 g of tissue. The ^{45}Ca content (μ-mole/100 g) was obtained by multiplying the value for the ^{45}Ca space by the concentration of calcium (2 μ-mole/ml.) in the Locke solution.

^{45}Ca washout experiments. In this series of experiments only the left gland was perfused, and the effluent was collected by means of a cannula in the adrenolumbar vein. The gland was perfused for 5 min with Locke solution containing ^{45}Ca (1 μc/ml.), in the presence or absence of ACTH (40 μu./ml.). The gland was then perfused with calcium-free Locke solution without ACTH for 60 min. The perfusate was collected in 5-min samples. Samples of effluent (1 ml.) were evaporated to dryness on planchets and the ^{45}Ca content determined in the gas flow counter. At the termination of the experiment the radioactivity of the cortex was also determined as described above. Washout (desaturation) curves were plotted from a summation of the ^{45}Ca present in each sample and the amount of ^{45}Ca remaining in the gland at the end of the washout period. Desaturation curves have been employed to indicate the amount of ^{45}Ca remaining in tissue after each washout interval as a percentage of the ^{45}Ca content at the beginning of the washout procedure (Hodgkin & Keynes, 1957; Shanes & Bianchi, 1959); these curves can be employed to detect and even quantitate the movement of radioactive ions in and out of different tissue compartments. The ^{45}Ca content at the end of the washout period was also determined by dividing the value for the residual ^{45}Ca (cpm/wet wt. cortex) by the ^{45}Ca (cpm/ml.) in Locke solution.

^{45}Ca efflux experiments. The experimental procedure employed in this study was essentially similar to that employed in the ^{45}Ca washout experiments. In five different experiments glands were perfused with Locke solution containing ^{45}Ca (1 μc/ml.) for 5 min, and then perfused for 60–90 min with non-radioactive Locke solution. After various intervals during the washout with the non-radioactive solution, ACTH (40 μu./ml.) was added to the perfusion solution. The 5-min samples of effluent were evaporated on planchets and the ^{45}Ca content of the perfusate determined. The radioactivity in the glands at the end of the experiment was also determined. The computed washout curves were expressed as ^{45}Ca efflux rate coefficient (percent/min), which is a measure of the amount of radioactivity leaving the gland as a percentage of the radioactivity in the gland at any given time.

Total calcium determinations. Adrenal glands were removed either after evisceration or at the termination of a given experiment. The glands were trimmed, the medulla removed as completely as possible, and the cortices weighed and ashed in nitric-perchloric acid (1:1 v/v) at 160° C for 18 hr. The ash was resuspended in 0·1 N-HCl and the fluid was analysed for calcium according to the fluorometric method of Hattingberg, Klaus, Lüllman & Zepf (1966), which involves the use of calcein dye. All samples were prepared in duplicate. Stock solutions and calcium standards (0·15–0·3 μ mole/ml.) were prepared using deionized water, and stored in poly-

ethylene bottles which previously had been rinsed with 10^{-4} M ethylenediaminetetraacetic acid (EDTA). All glassware used in these experiments was washed with concentrated acid to avoid extraneous calcium contamination. Values are expressed as μ-mole/100 g wet wt. of cortex.

Inulin space determinations. Both adrenal glands were perfused for 10–25 min with Locke solution containing [^{14}C]inulin (0·1μc/ml.) plus 'cold' inulin, 0·25 mg/ml. In some experiments, ACTH (4 μu./ml.) was added to the perfusion medium either before or during the period of perfusion with inulin. At the termination of the experiment the glands were removed, the medulla and cortex separated, blotted on ashless filter paper, and weighed. The medullae and cortices were homogenized separately in 0·2 N perchloric acid and centrifuged at 3000 rev/min for 15 min. A 1·0 ml. aliquot of the supernatant was placed in 1 ml. of scintillating solution and the radioactivity counted in a liquid scintillation counter (Packard). The scintillation mixture employed was Scintisol-Complete (Isolab), which consisted of a toluene base plus solubilizing agent. No quenching was observed when known amounts of radioactivity were counted with this mixture. The inulin space was determined from the counts in the tissue homogenate and in the perfusion medium.

Substances used. Synthetic β-1-24 ACTH (Ba 30920) was generously supplied by Dr J. J. Chart of Ciba Pharmaceuticals. ^{45}Ca was obtained from Tracerlab, and [^{14}C]inulin was obtained from Calbiochem and Mallinckrodt.

RESULTS

The effect of ACTH on radiocalcium distribution and exchange

^{45}Ca space measurements. The initial experiments were designed to study the distribution of radiocalcium [^{45}Ca] in the perfused adrenal gland and to examine whether ACTH causes a change in this distribution. A determination of the ^{45}Ca space gives an indication of the amount of radioactivity in tissue relative to that in the perfusion fluid.

In all of these studies the left and right glands were exposed to ^{45}Ca for 2·5 and 20 min, respectively. The effect of ACTH on the ^{45}Ca space of the cortex was determined during a constant exposure of the right gland to a maximal stimulating concentration of ACTH (40 μu./ml.), at a time when the gland was responding to ACTH with near maximal corticosteroid outputs (Fig. 1). Glands which were exposed to ACTH had a slightly higher average ^{45}Ca space than glands perfused for an identical period with ^{45}Ca in the absence of ACTH (Table 1). The average increase in the space produced by ACTH was only 5·4 ml./100 g, but this difference was statistically significant ($P < 0·05$). By contrast, the ^{45}Ca spaces of the left glands in both groups, which were similarly treated, were not significantly different ($P = 0·5$).

Determination of the extracellular space. Studies were also carried out to determine the magnitude of the extracellular space of the perfused adrenal gland in the presence and absence of ACTH. These data would allow a more accurate estimation of the distribution of calcium in the cortex, and would determine whether any observed changes in the distribution of

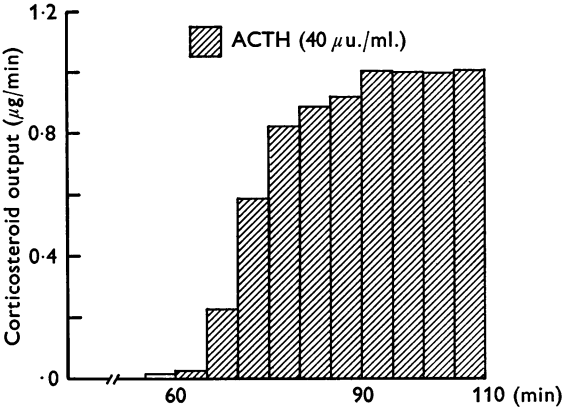

Fig. 1. The time course of corticosteroid release during continuous perfusion with ACTH. The left adrenal gland was perfused with normal Locke solution for 60 min. ACTH was then added to the perfusion medium and successive 5 min samples were collected.

The vertical bars represent the rate of corticosteroid release in the absence (clear bar) and presence (striped bar) of ACTH during a given 5 min interval.

TABLE 1. The effect of ACTH on ^{45}Ca distribution in the adrenal cortex

	^{45}Ca space (ml./100 g)	
	Left gland	Right gland
	Control	
	44·7	48·1
	42·0	47·6
	38·1	49·9
	30·1	53·8
	40·5	47·2
Mean ± S.E.	39·1 ± 2·5	49·3 ± 1·2
	Experimental	
	48·3	56·0
	36·6	58·1
	41·5	50·5
	40·2	54·3
Mean ± S.E.	41·7 ± 2·4	54·7 ± 1·6

The glands were perfused simultaneously. In control animals the left gland was removed after 2·5 min exposure to ^{45}Ca and the right gland was removed after an additional 17·5 min exposure to ^{45}Ca. In the experimental animals the left gland was removed after 2·5 min exposure to ^{45}Ca, and ACTH (40 μu./ml.) was added to the perfusion solution during the final 17·5 min exposure of the right gland to radiocalcium.

calcium produced by ACTH might be ascribed to changes in the size of the extracellular space. Inulin space appears to be a reliable measure of extracellular fluid volume, since this substance moves into tissues by physical diffusion and is not taken up by cells (see Bohr, 1964).

The left gland was usually removed after a 10 min exposure to inulin, and the right gland perfused for an additional 5–15 min. Equilibration of inulin with the adrenal gland appeared to be completed within 10 min, since longer periods of perfusion with inulin gave similar values for the inulin space. Some variation in the inulin space was observed from animal to animal, but little or no variation was observed from right to left gland within a given animal. The mean inulin space of the Locke-perfused cortex was $31{\cdot}2 \pm 2{\cdot}7$ ml./100 g (six glands). The mean inulin space after the addition of ACTH (40 μu./ml.) was $29{\cdot}0 \pm 0{\cdot}8$ ml./100 g (four glands). ACTH produced no discernible alteration in the inulin space when added either for 10 min before the addition of the radioactive inulin or during the final 10 min of a 20 min perfusion period with inulin.

If one assumes that the inulin space is a reasonable measure of the extracellular volume, then by comparing the difference between the inulin space (31·2 ml./100 g) and a given ^{45}Ca space, an estimate can be made of the amount of ^{45}Ca that penetrates from the extracellular fluid into tissue. Such an estimate (determined from the ^{45}Ca spaces of 39·1 and 49·3 ml./100 g) revealed a quantity of 15·8 and 36·2 μ-mole/100 g which was, at least in part, taken up by cortical tissue after 2·5 and 20 min exposures to radiocalcium, respectively. An estimate of 47·0 μ-mole ^{45}Ca/100 g was calculated to be present in various intracellular compartments in the presence of ACTH. This 23% increase in the ^{45}Ca content of the cortex observed in the presence of ACTH cannot be ascribed to changes in the extracellular volume, since the inulin space was not altered by ACTH. Although total calcium content of glands was determined more completely in later experiments, a limited number of calcium determinations was done in this group of experiments, and it was found that approximately 45 and 60% of the ^{45}Ca exchanged with cortical calcium during the 2·5 and 20-min exposures, respectively.

^{45}Ca desaturation curves. When the glands are exposed to ^{45}Ca and then washed out with a calcium-free medium, the relative rates of removal of ^{45}Ca from perfused adrenal glands can be represented as desaturation curves. Averaged curves illustrating the time course of ^{45}Ca washout, expressed as the percent ^{45}Ca initially present in the glands, are shown in Fig. 2. The washout of ^{45}Ca can be approximated as the sum of at least two separate components, a rapid one with a half-time of less than 1 min, and a slower one with a half-time of 15 min. The fast component represents, at least in part, washout from the extracellular space of the gland; however,

this component also comprises ^{45}Ca washout from some non-glandular tissue present in the perfusion system, including a segment of aorta and arteries supplying the gland, plus variable amounts of adhering fat and connective tissue. The slower component is believed to represent mainly ^{45}Ca washout from various intracellular tissue fractions. The half-times of the two components of the washout curves obtained from glands exposed to ACTH were very similar to those of the control—less than 1 min and 13 min for the fast and slow components, respectively.

During an early phase of washout the average desaturation curve obtained after exposure to ACTH was significantly displaced above the average desaturation curve obtained in the absence of ACTH. The probability, calculated by Student *t* test, that the differences between the mean values for the two groups of glands are due to chance, is less than 5% for the 0–15 min time period, 5% for the 15–20 min time period, and greater than 10% for the 20–60 min time period. No differences in flow rates were observed in these two groups of glands during the time periods under consideration. After a 60-min washout with the calcium-free medium, only about 2% of the radioactivity remained in the control and ACTH-treated glands. Thus, under both experimental conditions a major portion of the radioactivity was washed out during the course of the experiment.

If we assume that the slower linear phase (on a semi-log plot) of ^{45}Ca washout represents calcium coming from the intracellular space, then one can obtain an approximation of the original ^{45}Ca content of the gland by extrapolation of the lines back to the points at which they intercept the ordinate. The extrapolated values for percent of total ^{45}Ca in the intracellular space was about 40% for control and 50% for ACTH-treated glands. The extrapolated values of percent ^{45}Ca localized intracellularly for both control and ACTH-treated glands are probably somewhat lower than the true value, due to the fact that some washout of ^{45}Ca occurs from non-glandular tissue components as well as from the gland itself.

The differences in the percent of ^{45}Ca remaining in the control and ACTH-treated glands during washout can be compared with rates of corticosteroid secreted during this time period (Fig. 2). In the presence of ACTH, secretion of steroid increased up to 15 min after exposure to ^{45}Ca. Thereafter, secretion slowly declined due to the effects of calcium deprivation, and by 30 min it was again near control levels. During the periods up to the time that maximum steroid secretion was attained, the respective points of the two desaturation curves were significantly different. As the secretory rate declined, the curves tended to approach one another.

After a 60-min washout with non-radioactive solution, it is reasonable to assume that most of the residual radioactivity was intracellular. Analysis

of the ^{45}Ca content of control and ACTH-stimulated glands after washout showed that ACTH-stimulated glands did not retain more ^{45}Ca than control glands (Table 2).

^{45}Ca efflux. The effect of ACTH on ^{45}Ca efflux was examined in several experiments. Glands were loaded with ^{45}Ca for 5–10 min and then perfused with calcium-free Locke solution, as previously described, so that efflux (as measured by the rate coefficient) would represent loss of radio-

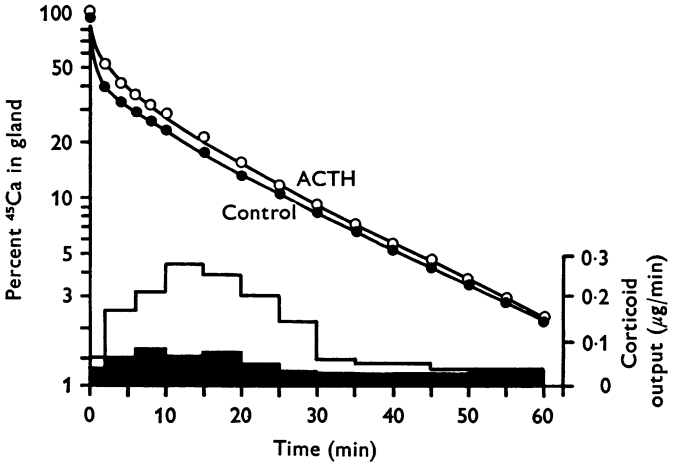

Fig. 2. ^{45}Ca washout from the adrenal gland in the presence and absence of ACTH. The left gland was perfused with ^{45}Ca Locke solution for 5 min with or without ACTH (40 μu./ml.); then, beginning at time 0, perfusion was switched to calcium-free Locke solution for 60 min. The radioactivity in the perfusate and that remaining in the gland at the end of 60 min were determined as described in the Methods. The curves depict the time course of ^{45}Ca washout, expressed as a percentage of ^{45}Ca initially present in the glands. Each point represents mean values obtained from seven control glands and six ACTH-treated glands.

The clear and black bars represent the mean rates of corticosteroid secretion in the presence and absence of ACTH, respectively. These outputs were determined from the perfusates obtained during washout of ^{45}Ca.

TABLE 2. The effect of ACTH on the ^{45}Ca and total calcium content of adrenal glands after washout with calcium-free Locke solution

Expt.	Total cpm/ 100 g × 10^{-4}	^{45}Ca content (μ-mole/ 100 g)	Total Ca (μ-mole/ 100 g)	Specific activity
Locke	88·8 ± 19·8	6·4 ± 1·5	48·8 ± 13·6	0·14 ± 0·04
Locke + ACTH	73·3 ± 8·6	5·0 ± 0·8	69·9 ± 7·4	0·08 ± 0·03

All values were obtained from experiments described in Fig. 2. None of the differences between the mean values of the control and ACTH-treated glands were significant, as determined by the Student t test ($P > 0·1$).

calcium from the gland. The rate coefficient for ^{45}Ca efflux reached a fairly stable level about 30 min after the onset of washout. When ACTH was added to the perfusion medium there was a gradual, but marked, decline in the rate at which radiocalcium left the gland (Fig. 3). The ^{45}Ca efflux rate decreased to one third of its previous level after the addition of ACTH. The corticosteroid secretion elicited by ACTH in such an experiment was markedly attenuated, but not abolished, by the omission of calcium. In a typical experiment, ACTH (40 μu./ml.) released 4·1 μg corticosteroid

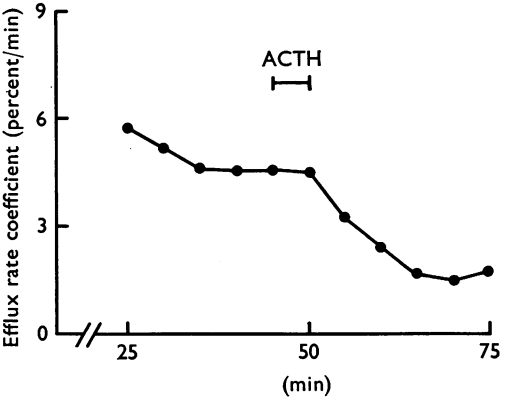

Fig. 3. The effect of ACTH on ^{45}Ca efflux. The left adrenal was exposed to ^{45}Ca and then perfusion was switched to calcium-free Locke solution at time 0. Between the 45th and 50th min ACTH (40 μu./ml.) was added to the perfusion medium.

in the absence of calcium. By contrast, in another gland the same concentration of ACTH released 17·7 μg of steroid in the presence of calcium.

ACTH also produced a decrease in the rate of ^{45}Ca efflux when washout was carried out with normal Locke solution. However, the observed decrease (approximately 40 %) was less marked than that observed when calcium was omitted from the perfusion medium.

Studies on total calcium content of cortex

Relationship between calcium content and calcium concentration of perfusion medium. In the initial experiments, the left gland was perfused with Locke solution before calcium analysis was carried out. The right gland was removed during preparation of the left gland for perfusion, and analysed for calcium content in a manner identical to perfused glands. The mean value for total calcium content of glands perfused with normal Locke solution was 172 ± 14 μ-mole/100 g (twenty glands). Varying the period of perfusion with Locke solution from 30 to 90 min did not alter the cal-

cium content of the cortex. The mean calcium content of adrenal glands taken directly from animals and analysed without prior exposure to Locke solution was 139 ± 13 μ-mole/100 g (eleven glands), which was not significantly lower ($P > 0{\cdot}05$) than the calcium values of Locke-perfused glands.

Glands perfused for 30 min with Locke solution of various calcium concentrations were also analysed for calcium content. Glands exposed to calcium concentrations of 0·0, 0·5 and 2·0 mM gave mean cortical calcium contents of 22·9 ($\pm$ 1·9), 94·7 ($\pm$ 18·5), and 112 ($\pm$ 14) μ-mole/100 g, respectively, when corrected for extracellular calcium. The contribution of the extracellular calcium was determined by multiplying the given calcium concentration of the Locke solution by the inulin space (31·2 ml./100 g).

Glands were also perfused for varying time periods with calcium-free Locke solution in order to obtain additional information as to the time course of calcium loss from the gland. During the first 3 min of perfusion with the calcium-deprived solution, the calcium content of the cortex fell to 77·4 μ-mole/100 g, which is 45% of the control value (Fig. 4*a*). After 30 min of calcium-free perfusion, the calcium content of the cortex was about 14% of that found in control glands, viz. 22·9 μ-mole/100 g. More prolonged periods of perfusion with calcium-free medium did not produce a further diminution in the calcium content of the cortex. The mean calcium content of glands after 60 and 90 min of calcium-free perfusion was 48·8 and 39·2 μ-mole/100 g, respectively (Fig. 4*a*).

The addition of 2,4-dinitrophenol to the calcium-free perfusion medium caused an almost complete disappearance of tissue calcium. Thus, two glands were perfused for 45 min with calcium-free Locke solution, and during the first 30 min of perfusion, dinitrophenol (1 mM) was present in the calcium-deprived perfusion solution. The calcium content of these two glands was 2·9 and 5·3 μmole/100 g, respectively.

The effect of ACTH. Glands were exposed to ACTH for varying time intervals (10–50 min) after the initial equilibration period of perfusion with normal Locke solution. As the concentration of ACTH was increased and the exposure time prolonged, there was no discernible increase in the calcium concentration of the cortex (Fig. 4*b*). In another experiment a gland was continuously perfused for 140 min with ACTH in increasing concentrations from 10 to 200 μu./ml., during which time 100 μg of corticosteroid was secreted. At the termination of perfusion the calcium content of the cortex was 181 μ-mole/100 g, a value which approximates very closely the mean value of the control perfused glands. The mean value of all glands exposed to ACTH was 165 ± 12 μ-mole/100 g.

Since there is some variation in the calcium content of the cortex from

cat to cat, additional experiments were carried out to compare the calcium content of left and right adrenal glands in the same animal, in the presence and absence of ACTH. The calcium content of the right gland was very similar to the corresponding left gland, whether or not the right gland had been exposed to ACTH (Table 3).

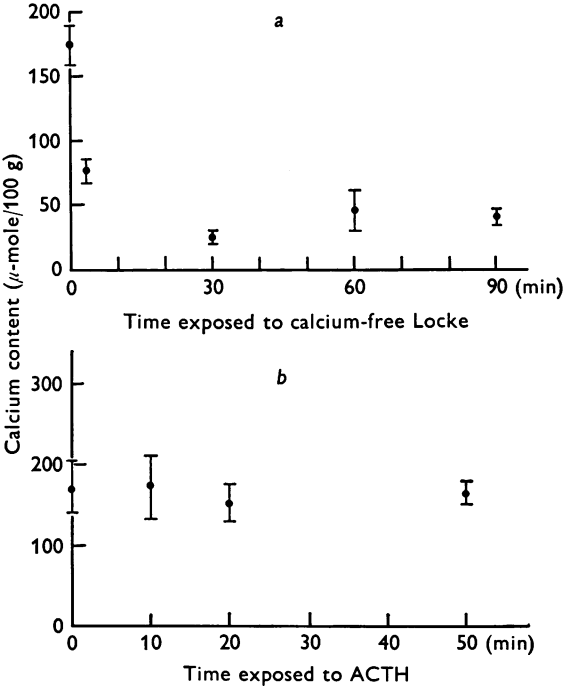

Fig. 4. The effect of (*a*) calcium-deprivation and (*b*) ACTH on the calcium content of perfused adrenal gland. Glands were perfused initially with normal Locke solution and then perfused for varying time intervals with either (*a*) calcium-free Locke or (*b*) Locke plus ACTH.

In *a*, calcium content of glands was determined after 3, 30, 60 and 90 min of perfusion with calcium-free Locke.

In *b*, calcium content of glands was determined after a 10-min exposure to ACTH, 10 μu./ml. and 20- and 50-min exposures to ACTH, 40 μu./ml.

All mean values (± S.E.) were derived from at least three different glands. The control values, shown at zero time, were derived from twenty different glands.

Cortical calcium content was also determined after prolonged washout with calcium-free Locke solution so that the residual calcium would in all probability be mainly intracellular, and any potential small differences in intracellular calcium would not be masked by the relatively large extracellular calcium pool. Glands which had been exposed to ACTH for 5 min before the 60 min washout contained a slightly higher calcium content than

control glands (Table 2); however, this difference was not statistically significant ($P > 0{\cdot}1$). Since these glands had been exposed to ^{45}Ca before washout, relative specific activity could also be determined. The relative specific activity gives an indication of the fraction of total tissue calcium which has undergone exchange with calcium in the medium. Although the relative specific activity of the perfused glands was very low after washout, there was no significant difference in the mean specific activity between control and ACTH-stimulated glands (Table 2).

TABLE 3. The effect of ACTH on the total calcium content of the adrenal cortex calcium content (μ-mole/100 g)

	Control		
	Left gland (a)	Right gland (b)	$\frac{(b)}{(a)} \times 100$
	115	130	113
	104	110	106
	191	150	79
	162	196	121
Mean ± S.E.	143 ± 20	147 ± 18	105 ± 9
		ACTH-treated	
	161	177	110
	100	104	104
	121	148	122
	193	190	98
Mean ± S.E.	144 ± 21	155 ± 19	109 ± 5

The glands were perfused with normal Locke solution simultaneously. After the left gland was removed, perfusion of the right gland was continued in the absence or presence of ACTH (40 μu./ml.) for an additional 20 min.

Calcium distribution in the adrenal medulla

Although our primary interest concerned the adrenal cortex, a limited amount of information was obtained on the distribution of calcium in the medulla for comparison with the data on the cortex. The mean calcium content of medulla was 197 μ-mole/100 g, a value obtained from two separate determinations, with each determination made from four pooled medullae. On the basis of the inulin space (33·2 ml./100 g) and ^{45}Ca spaces after 2·5 min (45·9 ml./100 g) and 20 min (51·5 ml./100 g) of perfusion with radiocalcium, an estimate of 25·4 and 36·6 μ-mole/100 g was made for ^{45}Ca uptake into medullary cells during the two time periods under consideration. These values for medullary ^{45}Ca are similar to those obtained for the cortex. When a left and right gland were perfused with ^{45}Ca in the presence of acetylcholine (2×10^{-5} g/ml.), the ^{45}Ca in the left and right

medullae were estimated to be 90·8 and 78·6 μ-mole/100 g after 2·5 and 20 min, respectively. The ^{45}Ca values of the medulla, in the presence of acetylcholine, were almost twice as high as those observed in the cortex after exposure to ACTH.

DISCUSSION

The stimulant action of ACTH on corticosteroid production has been extensively investigated by studies on adrenocortical tissue *in vivo* and *in vitro*. However, the intimate mechanism by which steroid is extruded from cortical cells is poorly understood. A previous study (Jaanus *et al.* 1970) has provided evidence that calcium is required for ACTH-induced corticosteroid release just as it is required for the evoked release of many other secretory substances (see Rubin, 1970). The present experiments demonstrate in a number of ways that stimulation of the adrenal cortex by ACTH is associated with an alteration in the distribution of radiocalcium. The change in calcium distribution produced by ACTH is temporally correlated with corticosteroid secretion; and, moreover, it precedes the secretory effect of ACTH, since marked differences in ^{45}Ca washout curves were observed before corticosteroid secretion reached peak levels.

Formulation of the hypothesis that extracellular calcium plays a critical role in stimulus-secretion coupling was, in part, based upon findings of an increased ^{45}Ca uptake and exchange during stimulation in such endocrine organs as the adrenal medulla (Douglas & Poisner, 1962; Rubin *et al.* 1967) and the neurohypophysis (Douglas & Poisner, 1964). The concept of stimulus-secretion coupling by an increased calcium influx implies either an increased uptake (or retention) of radiocalcium, or a higher total calcium content, or both. In the present investigation, extrapolation of the slow phase of ^{45}Ca washout curves indicated that the percent total ^{45}Ca in the intracellular space was some 20% higher in ACTH-treated glands. However, if additional calcium was taken up during exposure to ACTH it was lost during washout. The differences between the mean values of the control and ACTH-treated glands were significant only during the first 20 min of washout, and at the end of 60 min the percent and amount of ^{45}Ca remaining in the two groups of glands were very similar. By contrast, in the adrenal medulla where acetylcholine increases ^{45}Ca uptake, there is an increase in residual ^{45}Ca in glands exposed to acetylcholine (Douglas & Poisner, 1962). In addition, during ^{45}Ca washout from adrenal glands exposed to acetylcholine, with and without the inhibitor, tetracaine, the differences between the mean ^{45}Ca values for the two groups of glands were significantly different only for the 30–60 min time period, rather than for the 0–20 min time period (Rubin *et al.* 1967). The slight increase in the ^{45}Ca space of the cortex produced by ACTH might indicate either an in-

creased calcium uptake into cortical cells or a slowing of calcium efflux. The latter explanation is deemed more likely, since ACTH causes a marked decrease in ^{45}Ca efflux from the perfused gland. The finding that ACTH does not increase the total calcium content of the cortex further supports the idea that ACTH does not cause a net inward movement of calcium from the extracellular medium to the interior of the cortical cell. The lack of any discernible increase in the cortical calcium concentration during exposure to ACTH cannot be ascribed to a concomitant increase in calcium exchange since ACTH does not enhance, but, in fact, diminishes calcium efflux and exchange from the perfused gland. The possibility of calcium entry large enough to initiate secretion, but too small to be detected by the techniques employed, cannot be completely eliminated.

If there is no enhancement of extracellular calcium uptake in response to ACTH, then the effects of ACTH may be explained by the hypothesis that it causes a shift in calcium from a pool which is rapidly exchangeable to a pool which is less readily exchangeable. The idea that some cellular calcium pool is critical for ACTH action is in harmony with the finding that corticosteroid secretion was better correlated with the calcium content of cortices rather than with the calcium concentration of Locke solution. ACTH elicits similar rates of steroid secretion when glands are perfused with Locke solution containing 0·5 and 2·0 mM calcium (Jaanus *et al.* 1970); and the calcium content of cortices is very similar after perfusion with 0·5 and 2·0 mM calcium, when a correction is made for the extracellular calcium.

It is difficult to correlate the decrease in the response to ACTH with the fall in the calcium content of the gland during the washout of calcium, due to the very rapid fall in the calcium content as well as the latency of the secretory response to ACTH. Although the results of this investigation do not provide sufficient evidence for a precise localization of the critical calcium pool in the cortex, one can account for much of the calcium distribution within the cortical cell. The total calcium content of the perfused cortex (172 μ-mole/100 g) may be divided into three fractions: extracellular, cellular extractable, and non-extractable. The extracellular calcium, with an inulin space of 31 ml./100 g, was estimated to be about 60 μ-mole/100 g. Extracellular calcium is presumed to be of significance in the action of ACTH only in regard to its being in equilibrium with the more critical cellular calcium pool. During prolonged periods of perfusion with a calcium-free solution, approximately 40–50 μ-mole calcium/100 g cortex was still retained by the cortex. The locus of this non-extractable calcium is assumed to be the mitochondria. It is well known that mitochondria have a high affinity for divalent cations, due to the presence of an active pumping mechanism (Lehninger, Carafoli & Rossi, 1967), which

would make it extremely difficult to deplete this fraction of calcium by washout. Furthermore, almost complete depletion of this calcium pool could be obtained with 2,4-dinitrophenol, an uncoupling agent which releases calcium from mitochondria (Carafoli, 1967). The non-extractable calcium pool does not appear to be involved directly with steroid secretion from the intact gland, since after 30 min of perfusion with a calcium-free medium, ACTH-induced corticosteroid release is profoundly attenuated although corticosteroid content of the gland is very high (Jaanus *et al.* 1970). On the other hand, studies *in vitro* have shown that calcium is important for certain steps in corticosteroid biosynthesis which take place in mitochondria (Peron & McCarthy, 1968). The presence of calcium in the mitochondria of the intact adrenal gland even after prolonged calcium deprivation would, therefore, allow corticosteroid synthesis to continue, although the depletion of a non-mitochondrial calcium pool would depress hormone release.

Since the results suggest that neither extracellular nor non-extractable calcium is critical for the release of steroid hormone, the calcium associated with the secretory process must be localized within some component of the extractable cellular fraction, which comprises less than half of the total calcium of the cortex and extracellular fluid. Whether all or only part of this pool is required for secretion cannot be determined at present. However, on the basis of the evidence cited, the critical calcium fraction is probably bound at or near the surface of the cell or localized in some readily exchangeable intracellular site.

The hypothesis that stimulation of the adrenal cortex by ACTH is not accompanied by an inward movement of extracellular calcium is supported by electrophysiologic studies. ACTH-induced steroidogenesis in rabbit adrenal glands is not associated with changes in membrane potential (Matthews & Saffran, 1967), which makes it unlikely that the action of ACTH involves marked changes in cell permeability to ions. Under normal conditions, ACTH increases corticosteroid production and the hormone is immediately liberated (Vogt, 1943; Holzbauer & Newport, 1969), although in the absence of calcium the effects of ACTH on steroid synthesis and release can be dissociated (Jaanus *et al.* 1970). The mechanism by which corticosteroid reaches the extracellular space is still a matter of conjecture. Electronmicroscopic studies have so far provided no evidence for the existence of granular structures within cortical cells which may store steroid hormone prior to secretion (Fawcett *et al.* 1969).

By contrast, the secretory process of the adrenal medulla has been more clearly elucidated. Although the cat medulla has a much lower and less stable membrane potential than the cortex (Matthews, 1967), it seems clear from the comparative values for ^{45}Ca and total calcium content of

medulla and cortex obtained in the present investigation that the resting permeability to calcium is not significantly higher in the medulla. However, stimulation of the adrenal chromaffin cells by acetylcholine results in cell membrane depolarization and a marked increase in permeability to calcium (Douglas & Rubin, 1961; Douglas & Poisner, 1962; Douglas Kanno & Sampson, 1967). The resulting movement of extracellular calcium into the chromaffin cell is presumed to trigger catecholamine secretion. The adrenal medulla contains a large amount of preformed catecholamine which is stored in granules together with ATP and soluble protein; and the release of the contents of these granules is presumed to occur by fusion of the granule membrane with the cell membrane, i.e. exocytosis (Douglas, Poisner & Rubin, 1965; Kirshner, Sage & Smith, 1967; Schneider, Smith & Winkler, 1967). In light of the proposed differences in the mechanism of secretion from cortex and medulla, it is of interest that calcium is required for the release of both catecholamine and corticosteroid, although the locus of the critical calcium pool may be different in these two systems. Thus, the action of calcium at the molecular level may be of a general character which is independent of the nature of the secretory process.

In the adrenal cortex there is morphological evidence that the smooth endoplasmic reticulum forms an anastomosing system of tubular elements which may play a role in the extrusion of the steroid to the cell exterior (Long & Jones, 1967). Tubular connexions running to the surface of the cell also have been proposed to exist in cholinergic nerves (Whittaker, 1968), mast cells (Padawer, 1969), and β cells of the pancreas (Lacy, Howell, Young & Fink, 1968); and the secretory activity of these systems depends upon the presence of calcium (Harvey & MacIntosh, 1940; Katz & Miledi, 1967; Mongar & Schild, 1958; Hales & Milner, 1968). It is conceivable that the action of calcium may be to alter somehow the structure of these proposed intracellular tubules to permit the egress of secretory product.

Finally, it should be emphasized that the conclusions drawn from the present findings are not intended to mitigate the importance of calcium in the process of stimulus-secretion coupling, which has been shown to exist in a large number of secretory systems (see Rubin, 1970). These results merely indicate that the calcium which couples the action of ACTH with the release of corticosteroid may come from some cellular source rather than by an influx of extracellular calcium. Parallels have previously been drawn between the role of calcium in stimulus-secretion coupling and in excitation–contraction coupling in muscle (Douglas & Rubin, 1961; Douglas & Poisner, 1962; Rubin, 1970). It may be reasonable to contemplate that the source of the calcium required for secretion may vary with

the type of secretory organ, just as the roles of cellular and extracellular calcium vary in effecting contraction of skeletal, cardiac and smooth muscle.

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