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ON THE MODE OF ACTION OF ACTH ON THE ISOLATED PERFUSED ADRENAL GLAND

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SUMMARY

1. Isolated cat adrenal glands were perfused with Locke solution, and the corticosteroid outputs in response to adrenocorticotrophin (ACTH) were studied.

2. Steroid outputs varied with the ACTH concentration, as well as with the duration of exposure to a given ACTH concentration.

3. Omission of calcium from the perfusion medium markedly depressed ACTH-evoked steroid release. The steroid output was directly related to the extracellular calcium concentration up to 0·5 mM.

4. During a constant exposure to ACTH, steroid output was maintained for at least 2–3 hr, provided that calcium was present in the perfusion medium.

5. Strontium, but not barium or magnesium, replaced calcium in maintaining the secretory response to ACTH.

6. Magnesium depressed ACTH-evoked secretion in the presence of calcium, and this depression of secretion was antagonized by increasing the calcium concentration.

7. Prolonged perfusion with sodium-free or potassium-free solutions did not markedly inhibit steroid output in response to ACTH. Excess potassium (56 mM) did not produce a consistent or marked increase in spontaneous steroid output and did not affect the response to ACTH.

8. The steroid content of adrenal glands perfused with Locke solution and exposed to ACTH was about 10% of the amount which was secreted. By contrast, adrenal glands perfused with calcium-free media and exposed to ACTH contained much higher amounts of steroid, despite the negligible amount secreted.

9. These data suggest that calcium plays a critical role in the mechanism of corticosteroid secretion from the adrenal cortex.

INTRODUCTION

In most secretory systems where the requirement for calcium in the mechanism of secretion has been ascertained, the secretory product is thought to be stored in intracellular membrane-bound organelles. Electron microscopic and chemical evidence suggest that secretion from such organs occurs by exocytosis, a process which involves the coalescing of the secretory granule membrane and the cell membrane, and the extrusion of the granule contents (for references, see Douglas, 1968). It has been proposed that calcium somehow activates the process of exocytosis (Douglas, 1968; Simpson, 1968). By contrast, little is known about the mechanism of secretion in the adrenal cortex. Pituitary ACTH enhances both the synthesis and release of corticoids from intact adrenal glands (Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Schenker & Pincus, 1951; Holzbauer, 1957), possibly by the mediation of adenosine cyclic 3′5′-monophosphate (3′5′-AMP) (Haynes, 1958), but electronmicroscopic studies have failed to provide evidence for the presence of intracellular organelles which might act as a vehicle for the storage or extrusion of corticosteroids (Long & Jones, 1967; Luse, 1967; Bloodworth & Powers, 1968).

In light of the presumed absence of secretory granules within cortical cells, the findings by Birmingham and her associates (Birmingham, Kurlents, Lane, Muhlstock & Traikov, 1960; Triller & Birmingham, 1965), that calcium is important for optimal steroid production and output from sectioned adrenal glands in response to ACTH and 3′5′-AMP, is of great interest. The present experiments explore the role of calcium in ACTH-evoked corticosteroid release from isolated perfused adrenal glands, in order to gain further insight into the nature of the action of calcium in the adrenal cortex and to understand more about the intimate mechanism by which calcium acts in the physiological process of secretion.

A preliminary account of some of our findings has already been reported (Rubin, Jaanus & Miele, 1969).

METHODS

Perfusion of the adrenal gland. Under intraperitoneal pentobarbitone anaesthesia, adrenal glands of cats (2–3 kg) were prepared for perfusion *in situ* at room temperature according to the method of Douglas & Rubin (1961). The perfusion medium was normal Locke solution of the following composition (mM): NaCl 154, KCl 5·6, $CaCl_2$ 2·0, $MgCl_2$ 0·5, $NaHCO_3$ 12, dextrose 10, or modified Locke solution (see Results). When either excess potassium or magnesium was added to the perfusion solution, the NaCl was reduced by an equivalent amount to maintain isotonicity. The perfusion medium was equilibrated with 95% oxygen and 5% carbon dioxide, and had a pH close to 7·0. The rate of flow was maintained between 1·0 and 1·5 ml./min by regulation of the perfusion pressure, and modification of the normal Locke solution either by alteration of the ionic constituents or by the addition of ACTH did not significantly alter the flow rate.

Steroid determinations. Samples of perfusate collected at 10-min intervals via a cannula placed in the adrenolumbar vein were assayed for 11-hydroxycorticoids according to the fluorometric method of Mattingly (1962). Samples of perfusate (2 ml.) were extracted with 10 ml. methylene chloride. The water layer was removed by suction, and 5 ml. of the methylene chloride extract was shaken with 3 ml. fluorescent reagent (sulphuric acid–ethanol 7 : 3 v/v). The organic layer was discarded and the fluorescence intensity read after 20 min in a Turner fluorometer. Since the major corticoid secreted by the cat adrenal gland is hydrocortisone (Bush, 1953), the outputs were expressed as μg hydrocortisone released per minute. When hydrocortisone standards, ranging from 0·2 to 2·0 μg, were carried through the assay procedure, a linear relationship was found between fluorescent intensity and steroid concentration.

Adrenal glands removed immediately at the termination of a given experiment were homogenized in absolute methanol and centrifuged at 30,000 rev/min for 10 min. The supernatant was placed in Thunberg tubes and evaporated *in vacuo* at 68° C for approximately 2·5 hr. The dry residue was resuspended in 2·0 ml. water, and the samples were analysed for corticosterone and hydrocortisone according to the method of Van der Vies (1961). This procedure is based upon the high partition coefficient of corticosterone between carbon tetrachloride and water. The corticosterone was extracted from the aqueous phase by shaking twice with 5 ml. purified carbon tetrachloride. Approximately 80–90 % corticosterone in the concentration range of 0·5–5·0 μg can be extracted by this procedure. The corticoid remaining in the water phase was then extracted with methylene chloride. The carbon tetrachloride and methylene chloride phases (5 ml. of each) were separately analysed fluorometrically, as described above. The gland homogenates caused no non-specific fluorescence, and complete recovery of hydrocortisone standard could be obtained when added to the homogenate. The lack of any non-specific fluorescence manifested by the gland homogenate made it unnecessary to employ chromatographic separation before assay (see Moncloa, Peron & Dorfman, 1959). Samples of perfusate were, on occasion, also analysed for corticosterone by extraction with carbon tetrachloride.

Compound A (21-hydroxypregn-4-ene-3,11,20 trione), which is a major steroid in the cat adrenal cortex (Holzbauer & Newport, 1969), was not detectable by the fluorometric assay procedure employed in the present study. Of all the steroids isolated and identified in the cat adrenal cortex by Holzbauer & Newport (1969), only corticosterone, hydrocortisone and cortisone manifested fluorescence in an approximate ratio of 1, 0·35, 0·15, respectively. Cortisone, like hydrocortisone, was not extracted by carbon tetrachloride, but was extracted by methylene chloride. Thus, the adrenal steroids which were extracted with methylene chloride and were identified by fluorometric assay, are referred to as the 17-hydroxycorticoids (hydrocortisone and cortisone).

Adrenocorticotrophic hormone. Stock solutions of ACTH were prepared in 0·5 % albumin solution at pH 3·0 and stored at $-4°$ C in polyethylene vials. Perfusion bottles which came in contact with the hormone were provided with a silicone coating to prevent adsorption of the polypeptide onto the glass. Natural ACTH isolated from porcine pituitary (Calbiochem; Sigma) or synthetic ACTH (β 1–24 Synacthen), generously supplied by Dr J. J. Chart, of Ciba Pharmaceuticals, was used. In our hands the synthetic hormone exhibited greater activity than the natural hormone, so that lower concentrations of the synthetic material were employed. Glands were generally exposed to the hormone for 10 min except on occasions when either shorter (2·5 min) or more prolonged exposures were used (see Results).

Reagents. Reagents used were purified as follows: methylene chloride and carbon tetrachloride was washed and distilled as described by Mattingly (1962). Ethanol

was purified according to the method of Peterson (1957). Methanol was distilled over concentrated NaOH, and then re-distilled (Holzbauer & Vogt, 1961). Concentrated sulphuric acid, A.C.S., was used as supplied by the manufacturer.

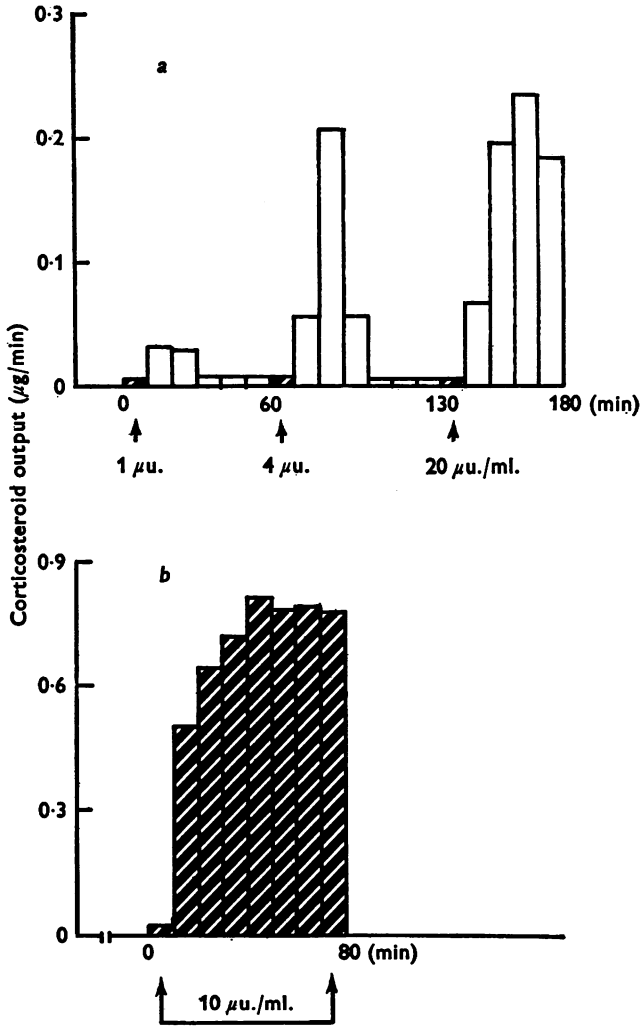

Fig. 1. The effect of ACTH (▨) on corticosteroid output.

(*a*) An adrenal gland was perfused with Locke solution, and varying concentrations of ACTH were added for 10 min every 60 or 70 min.

(*b*) An adrenal gland was perfused with Locke solution for 80 min in the presence of a constant concentration of ACTH.

The striped and clear vertical columns represent steroid outputs obtained during 10-min collection periods in the presence and absence of ACTH, respectively.

RESULTS

The stimulant effect of ACTH in Locke solution

Glands were always perfused with normal Locke solution for 40–60 min before exposure to ACTH in order to obtain low basal rates of corticosteroid secretion. The initial high rates of secretion were probably a consequence of the residual effects of endogenous ACTH. During a 10-min exposure to the ACTH, there was little augmentation in the corticosteroid output; however, output was enhanced and reached a maximum 20–40 min after exposure to ACTH (Fig. 1*a*). Although there was variation in response to ACTH from preparation to preparation, it was found that a single gland

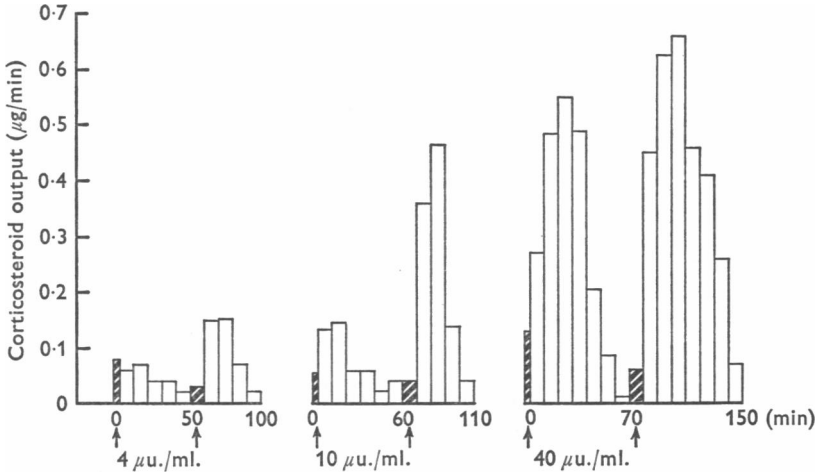

Fig. 2. The effect of exposure time on the response to ACTH (▨). Glands were perfused with Locke solution and exposed to varying concentrations of ACTH for both 2·5 and 10 min. A different preparation was used for each pair of responses to a given ACTH concentration.

responded in a graded manner to increasing amounts of ACTH given for a constant period of time (Fig. 1*a*). As the ACTH concentration was increased from 4 to 20 μu./ml., the peak output was not strikingly enhanced, but the increase in output was prolonged. When a single gland was exposed to a constant concentration of ACTH for a prolonged period of time, the output gradually reached a maximum within 50 min and then remained fairly constant over the next 30 min (Fig. 1*b*). The effect of varying the exposure time to ACTH was also studied. Glands which were exposed to a given concentration of ACTH for 10 min elicited higher and better sustained outputs than when they were exposed to the same concentration for 2·5 min (Fig. 2). Thus, a 2·5-min exposure to ACTH, 4 μu./ml., caused no

enhancement of corticosteroid output, in contrast to a 10-min exposure to the same concentration (Fig. 2). A 2·5- and 10-min exposure to ACTH, 10 μu./ml. produced steroid outputs of 3·93 and 10·38 μg, respectively,

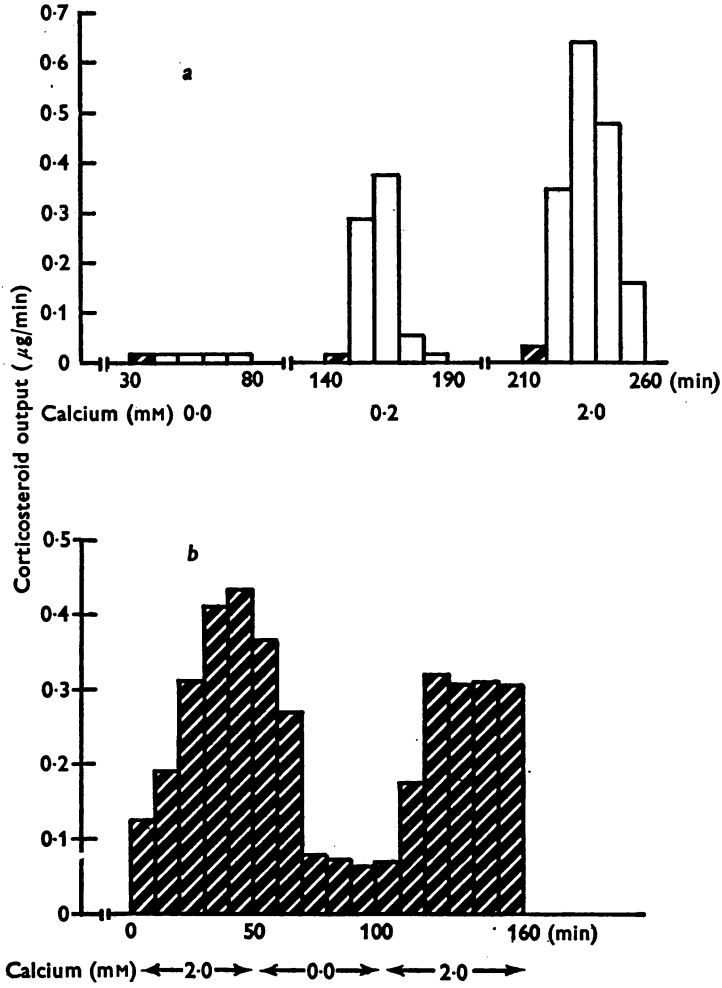

Fig. 3. The effect of extracellular calcium on the secretory response to ACTH, 10 μu./ml. (▨).

(*a*) A gland was perfused for 30 min with Locke solution containing either 0·0, 0·2 or 2·0 mM calcium. ACTH was then added to the perfusion medium for 10 min, and perfusion with the given calcium concentration continued for an additional 40 min.

(*b*) A gland was perfused with Locke solution plus ACTH (10 μu./ml.) for 160 min. After 50 min, perfusion was switched to calcium-free Locke solution for 50 min, and then calcium was restored during the final 60 min of perfusion.

during the 40 min after exposure to the ACTH (Fig. 2). With higher ACTH concentrations, the disparity between the outputs obtained with the two periods of stimulation became less striking. Steroid outputs of 17·94 and 21·91 μg were obtained during the 40 min after exposure to ACTH, 40 μu./ml., for 2·5 and 10 min, respectively (Fig. 2).

The role of divalent cations in the action of ACTH

Extracellular calcium. The secretory response to ACTH was profoundly reduced by the omission of calcium from the perfusion solution (Fig. 3*a*). In fact, in certain experiments ACTH was unable to elicit any discernible corticosteroid output in the absence of calcium. In general, the ACTH-induced outputs were reduced by 85% after calcium-deprivation. ACTH-induced release was readily restored by the returning calcium to the perfusion medium. However, the reintroduction of calcium did not enhance steroid output in the absence of ACTH. The response of a single adrenal gland to a given concentration of ACTH could be correlated with the extracellular calcium concentration (Fig. 3*a*). The peak 40-min steroid output in response to a 10-min exposure to maximal stimulating ACTH concentrations was obtained with 0·5 mM calcium. Thus, calcium concentrations of 0·0, 0·2, 0·5 and 2·0 mM gave mean outputs of 4·53 ($\pm$2·11 S.E.), 12·22 ($\pm$2·93), 45·45 ($\pm$11·52) and 30·12 ($\pm$4·95) μg, respectively. During a constant exposure to ACTH when the corticosteroid output had reached maximal levels, the removal of the extracellular calcium caused a decrease in hormone release which reached a basal level in 30 min; the re-introduction of calcium produced a restoration of secretion which attained a maximal rate within 30 min (Fig. 3*b*). The addition of the calcium chelating agent EGTA (10^{-4} M), together with calcium deprivation, did not enhance the rate of decline of the response, but depressed the minimal rates of secretion obtained in the absence of calcium to below measurable levels.

The effect of magnesium. Magnesium, even in concentrations as high as 20 mM, was unable to restore the response to ACTH in the absence of calcium. In the absence of calcium plus 20 mM magnesium, the outputs obtained following a 10-min exposure to ACTH (40 μu./ml.) were below the sensitivity of the assay method. The addition of 20 mM magnesium to Locke solution containing calcium (2·0 mM) produced about a 35% inhibition of ACTH-evoked corticosteroid release (Fig. 4*a*). Magnesium (10 mM) was unable to cause a discernible depression of ACTH-induced release in the presence of 2·0 mM calcium; however, in the presence of 0·5 mM calcium, a 50% inhibition of evoked output was obtained with 10 mM magnesium, which was reversed by the addition of 2·0 mM calcium (Fig. 4*b*).

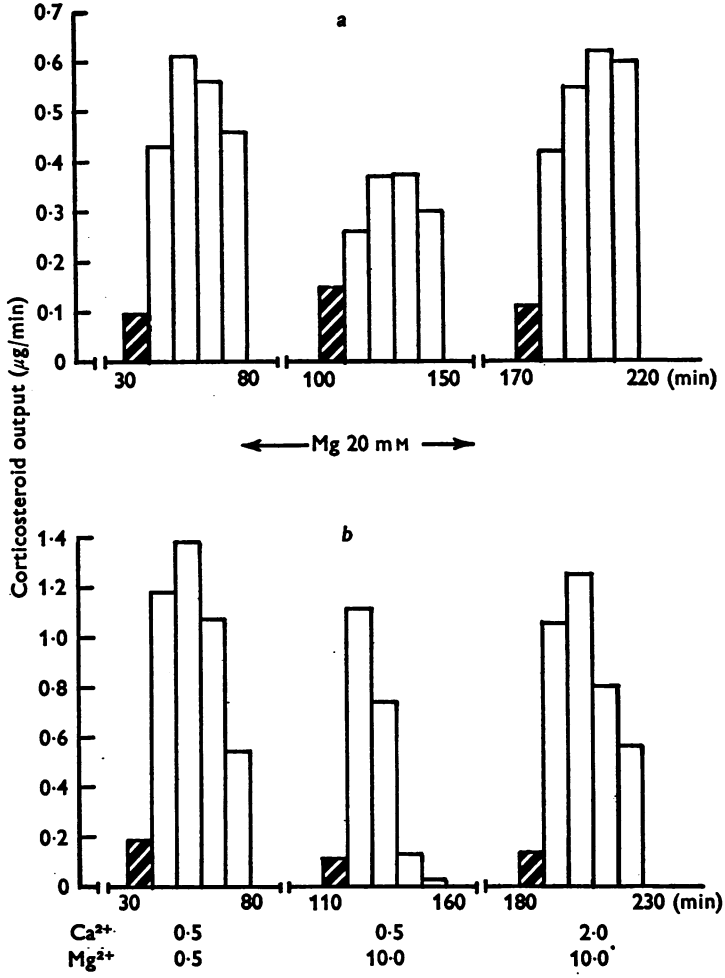

Fig. 4. The effect of magnesium on the secretory response to ACTH, 10 μu./ml. (▨).

(*a*) A gland was perfused with Locke solution for 220 min. During the 80–150th min of perfusion, magnesium 20 mM (40 × normal) was added. ACTH was added during the 30–40th, 100–110th and 170–180th min of perfusion.

(*b*) A gland was perfused initially with Locke solution containing 0·5 mM calcium for 80 min. Perfusion was then switched to Locke solution containing magnesium 10 mM (20 × normal), with 0·5 and 2·0 mM calcium present during the 80–160th and 160–230th min of perfusion, respectively. ACTH was added to the perfusion medium during the 30–40th, 110–120th and 180–190th min of perfusion.

The effect of strontium and barium. Strontium (2 mM) restored the response to ACTH (2 μu./ml.) in the absence of calcium. Thus, after a 10-min exposure to ACTH, the 40-min steroid output in strontium calcium-free Locke solution was 13·94 μg, as compared with 1·61 μg in the absence of strontium. By contrast, barium (2 mM) was unable to maintain the secretory response to ACTH (2 μu./ml.) in the absence of calcium. The 40-min outputs obtained with calcium-free and barium-substituted Locke solution were 3·50 and 1·91 μg, respectively, as compared to 12·26 μg with normal Locke solution.

The role of monovalent cations in the action of ACTH

Sodium deprivation. Perfusion with a sodium-deprived or sodium-free solution for 40–50 min did not depress the secretory response to ACTH. When an iso-osmotic equivalent of sucrose replaced the sodium chloride, so that the only sodium remaining in the solution was 12 mM-$NaHCO_3$ (< 10% of normal), the 50-min output obtained with a 10-min exposure to ACTH (10 μu./ml.) was 29·76 μg, as compared to 15·68 μg in normal Locke solution. When equimolar concentrations of choline and potassium bicarbonate were employed to replace both sodium chloride and sodium bicarbonate, the response to ACTH was strikingly similar to that observed with normal Locke solution (Fig. 5*a*).

Potassium deprivation. When glands were perfused for 40–50 min with potassium-free solutions, maximum stimulating concentrations of ACTH (10–30 μu./ml.) were still able to elicit high rates of corticosteroid release. Thus, in three experiments employing potassium-free solutions, a 10-min exposure to ACTH elicited an average 40-min output of $34·80 \pm 6·29$ μg, which compares favourably with the average output of $30·12 \pm 4·95$ μg obtained with ACTH in normal Locke solution. However, it was very difficult to obtain a control response after stimulation in a potassium-free medium, for, when potassium was restored to the perfusion medium, output was enhanced (Fig. 5*b*). In one gland where a control response to ACTH could be obtained, the ACTH-induced corticosteroid output in normal Locke solution was some 35% higher than that obtained in the potassium-free medium (Fig. 5*b*).

Excess potassium. Potassium, in the concentration range of 28–84 mM, was unable to elicit a consistent or striking increase in the rate of corticosteroid release. The increase in secretion, when present, was observed during the 10–20th min after the addition of excess potassium to the perfusion medium. In one experiment, 28 mM potassium increased output from 0·08 to a maximum of 0·14 μg/min during the 10–20th min. The maximum response obtained with three experiments with 56 mM potassium was a rise from a basal level of 0·05 to 0·14 μg/min. In one of two

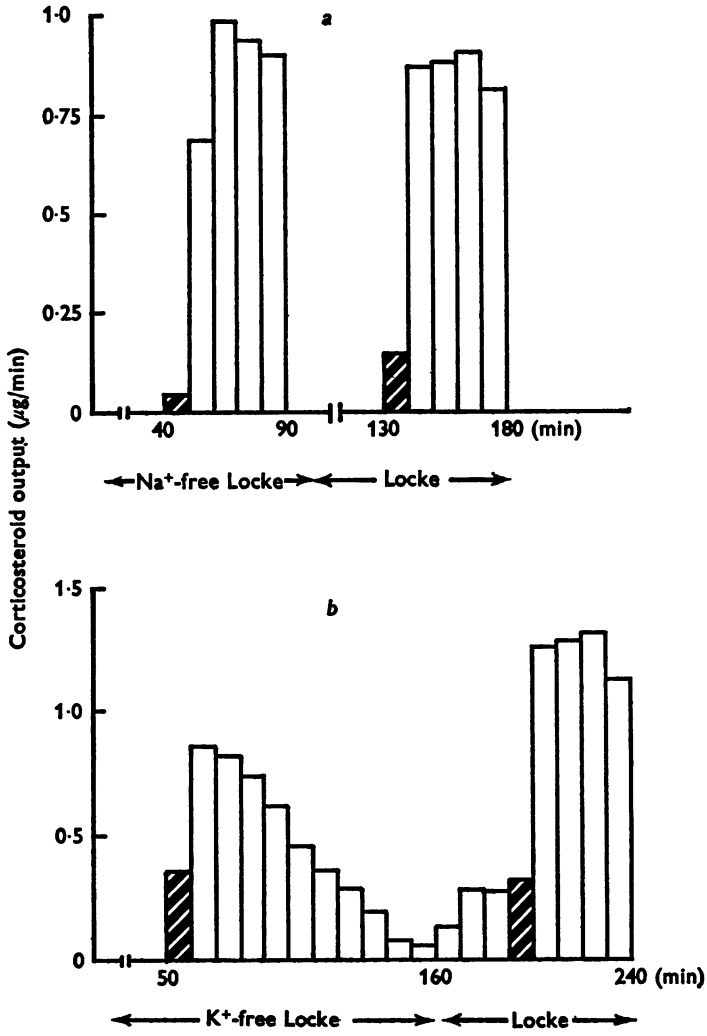

Fig. 5. The effect of sodium and potassium deprivation on the secretory response to ACTH, 2 μu./ml. (▨).

(*a*) A gland was perfused with sodium-free Locke solution for 90 min, and synthetic ACTH (2 μu./ml.) was added during the 40–50th min of perfusion. Perfusion was then switched to normal Locke solution, and ACTH was added during the 130–140th min of perfusion. In the sodium-free solution the NaCl and $NaHCO_3$ were replaced by an equivalent amount of choline and $KHCO_3$ respectively.

(*b*) A gland was perfused with potassium-free Locke solution for 160 min, and synthetic ACTH (2 μu./ml.) was added during the 50–60th min of perfusion. Perfusion was then switched to normal Locke solution and ACTH added during the 190–200th min of perfusion.

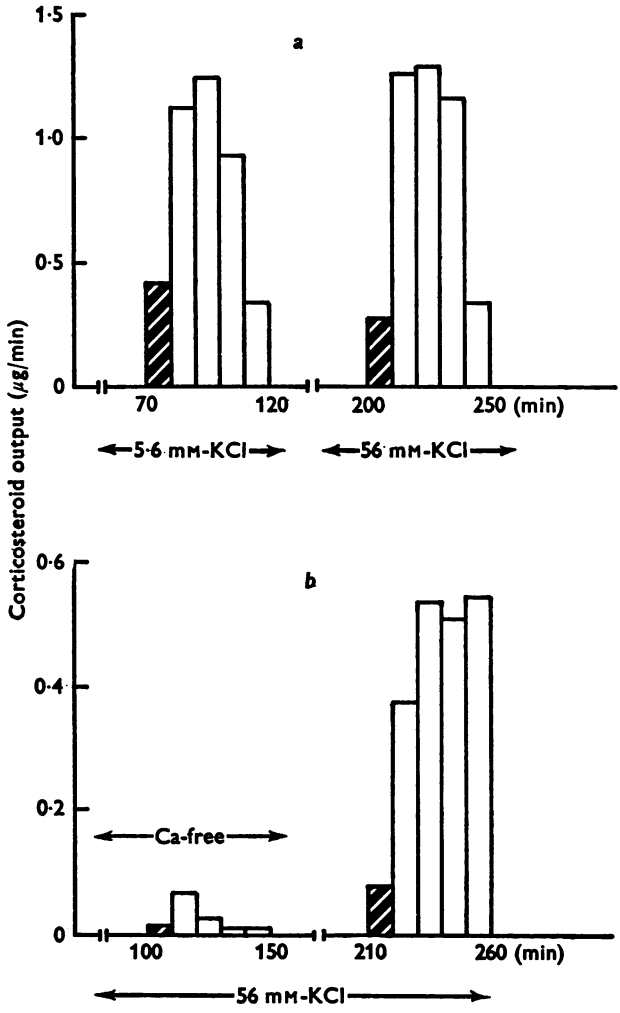

Fig. 6. The effect of excess potassium on the secretory response to ACTH (▨).

(*a*) A gland was perfused with Locke solution for 140 min. Perfusion was then switched to Locke solution containing 56 mM potassium (10 × normal) for an additional 110 min. Synthetic ACTH (2 μu./ml.) was added during the 70–80th and 200–210th min of perfusion.

(*b*) A gland was perfused with 56 mM potassium for 260 min. During the 70–150th min of perfusion, calcium was omitted from the perfusion medium. ACTH (10 μu./ml.) was added during the 100–110th and 210–220th min of perfusion.

experiments employing 84 mM potassium, steroid output rose from 0·19 to 0·56 μg/min during the 10–20 min. In the other experiment where 84 mM potassium was used, no augmentation in output was observed.

Excess potassium did not affect the response to ACTH. The output obtained in response to ACTH in the presence of 56 mM potassium was very similar to that obtained with the normal potassium concentration (5·6 mM) (Fig. 6*a*). In the presence of excess potassium, ACTH-induced steroid release was almost completely abolished when calcium was omitted, and ACTH-induced secretion could be readily restored by the reintroduction of calcium (2 mM) to the perfusion medium (Fig. 6*b*).

The effect of ACTH and calcium deprivation on the corticosteroid content of the adrenal cortex

Glands which were perfused for 90 min with normal Locke solution contained low levels of corticosteroid, and no steroid could be detected in the perfusate during the final 40 min of perfusion (Table 1). Glands which were exposed to ACTH during the final 40 min of perfusion showed some increase in their steroid content; however, the amount remaining in these glands was about 10% of that which was detected in the perfusate during exposure to ACTH (Table 1). By contrast, ACTH caused a large increase in the steroid content of glands perfused with calcium-free Locke solution (Table 1). At the same time, there was a much smaller increase in the steroid output from glands exposed to ACTH in the absence of calcium (Table 1). The increase in the steroid content of calcium-deprived glands with ACTH was manifest whether the values were expressed in terms of either gland weight or total steroid content of gland (Table 1). The amount of steroid remaining in glands perfused with calcium-free solutions after the 40-min exposure to ACTH was equivalent to the amount which would have been released in 220 min; by contrast, the steroid content of glands perfused with normal Locke solution and exposed to ACTH was equivalent to the amount released in only 3·9 min. The latter value is similar to that found by Holzbauer & Newport (1969) in glands from stressed dogs and pigs.

In order to discern whether glands are able to retain large quantities of steroid in the presence of calcium, glands were perfused with normal Locke solution for 90 min and then exposed to ACTH (100 μu./ml.) for only 10 min before being immediately analysed. During the 10-min exposure to ACTH, there was only a very small amount of steroid released $-0{\cdot}28 \pm 0{\cdot}03$ μg (three experiments). The mean steroid content of these glands was $39{\cdot}07 \pm 3{\cdot}87$ n-mole/g. This value is equivalent to the steroid content of glands exposed to ACTH for 40 min, but is much lower than values obtained from glands exposed to ACTH during calcium deprivation.

TABLE 1. Corticosteroid content of adrenal glands and perfusate in the presence and absence of ACTH and calcium

Experiment	No. of expts.	Content of gland n-mole/g (corticoid)	μg	Percent 17-OH corticoids	Total output μg corticoid	Percent 17-OH corticoids
Locke	3	7·5 ± 2·2	0·3 ± 0·1	21·0 ± 4·2	—	—
Locke + ACTH	3	30·3 ± 8·2	1·2 ± 0·4	55·9 ± 3·7	11·9 ± 4·0	80·8 ± 1·5
Calcium-free	5	30·5 ± 3·0	1·4 ± 0·2	34·7 ± 4·6	—	—
Calcium-free + ACTH	5	108·0 ± 17·0	4·1 ± 1·3	37·8 ± 5·3	0·7 ± 0·3	81·8 ± 6·0

Glands were perfused with Locke solution for 90 min; in certain experiments, ACTH (100 μu./ml.) was added during the final 40 min of perfusion. In experiments with calcium-free solutions, calcium was omitted 30 min before the introduction of ACTH to the termination of the experiment. Total output from gland represents the amount of corticosteroid released during the final 40 min of perfusion, in the presence of ACTH with or without calcium. The outputs obtained in the absence of ACTH were below the limit of sensitivity of the assay method.

The corticosteroid content of adrenal glands is expressed in terms of both concentration per wet weight of gland and in total amount recovered.

All values are mean values (± S.E.).

The differential analysis of the corticosteroid content of the adrenal cortex and perfusate

Glands perfused with Locke solution contained a high percentage of corticosterone (carbon tetrachloride-extractable steroid) and a very low percentage of 17-hydroxycorticoids (methylene chloride-extractable steroid) (Table 1). Glands exposed to ACTH for 40 min showed approximately equal amounts of corticosterone and 17-hydroxycorticoids. The effect of ACTH in increasing the percentage of 17-hydroxycorticoids in the adrenal gland was not manifest in the absence of calcium (Table 1). Approximately 80% of the steroid in the perfusate of ACTH-stimulated glands was recovered in the methylene chloride extract and therefore identified as 17-hydroxycorticoids (Table 1).

DISCUSSION

The steroidogenic effect of ACTH. After an initial latency, ACTH was able to increase corticosteroid output from isolated perfused cat adrenal glands. The amount of steroid secreted depended upon the ACTH concentration, as well as the time of exposure to the ACTH. During the initial 10-min exposure to ACTH, when there was little enhancement of steroid output, there was an increase in the steroid content of the gland. After a 40-min exposure to ACTH, during which time some 12 μg steroid had been secreted, the gland still contained approximately the same amount of steroid as it did immediately after the 10-min exposure to ACTH. These findings suggest that when the steroid content of the adrenal gland accumulates to a certain critical level after exposure to ACTH, steroids are released into the circulation. Very little secretion can take place in the absence of synthesis, since excess potassium, which depolarizes cortical cells but does not increase steroid production (Matthews & Saffran, 1967), produced only a very small increment in output. These data also indicate that the adrenal cortex retains only a small fraction of the amount synthesized in response to ACTH, which is in agreement with the findings of Holzbauer & Newport (1969) *in vivo*.

Although the fluorometric assay method employed in the present study is somewhat limited in its ability to distinguish every adrenal steroid, analysis of the differential release of 17-hydroxycorticoids (hydrocortisone and cortisone) and corticosterone suggests certain general conclusions. The major corticosteroid secreted by the perfused cat adrenal gland after exposure to ACTH was a 17-hydroxycorticoid. Bush (1953), who employed chromatographic techniques to identify and quantitate the corticosteroids secreted from cat adrenal glands *in vivo*, found hydrocortisone as the major steroid, with corticosterone and cortisone present in the venous

effluent in only very small amounts. In the present study, corticosterone was found to be the predominant corticosteroid in unstimulated glands. During exposure to ACTH there was an increase in the percentage of 17-hydroxycorticoids within the adrenal cortex, which suggests that stimulation by ACTH enhances the synthesis of the releasable steroids, namely, hydrocortisone. The enhancement of 17-hydroxycorticoid formation in the presence of ACTH was not manifest in the absence of calcium, where little or no secretion was observed. The mechanism by which ACTH increases the synthesis of hydrocortisone at the expense of corticosterone production has been explained by an increase in the activity of 17α-hydroxylase (Fevold, 1969).

The interaction of ACTH and calcium. The present findings indicate that calcium is required for ACTH to increase corticosteroid secretion. The data also suggest that calcium and ACTH must both be present for steroid release. During constant stimulation with ACTH, secretion could be depressed and then increased by the omission and subsequent reintroduction of calcium. Furthermore, after a period of perfusion with a calcium-free medium plus ACTH, the re-introduction of calcium did not enhance steroid secretion in the absence of ACTH. The locus at which calcium is required for steroid secretion cannot, at present, be ascertained; however, since steroid output can be correlated with the extracellular calcium concentration, the critical calcium pool must be either extracellular or readily exchangeable with extracellular calcium.

Previous studies have already shown that calcium is needed for optimal steroid production and release *in vitro* (Birmingham *et al.* 1960; Triller & Birmingham, 1965), and it has been suggested from studies on broken cell preparations, which do not respond to ACTH, that the action of calcium is to promote the intramitochondrial synthesis of corticosteroids (see Peron & McCarthy, 1968). However, the present studies on intact glands show that in the absence of extracellular calcium, ACTH is still able to increase steroidogenesis, although steroid output is markedly depressed. Thus, in the adrenal cortex, as in other secretory organs, secretion appears to be inhibited by the absence of calcium. It should be stated that in the presence of calcium the sum of the steroid content of the adrenal gland plus the amount secreted during a 40-min exposure to ACTH, exceeded by about 60 % the amount of steroid obtained from adrenal glands stimulated with ACTH in the absence of calcium. However, this inhibition of the steroidogenic effect of ACTH observed during calcium deprivation might be ascribed to the accumulation of steroid within the cortical cell, i.e. end-product inhibition (Birmingham & Kurlents, 1958; Ferguson, Morita & Mendelsohn, 1967), although one cannot rule out that calcium deprivation might also directly suppress steroidogenesis in the intact adrenal, just

as it does in quartered adrenals (Birmingham *et al.* 1960), and in broken-cell preparations (Peron & McCarthy, 1968).

In the adrenal medulla there is strong evidence to support the idea that the depolarizing action of acetylcholine causes an increase in the permeability of the chromaffin cell membrane to inorganic ions, and as a consequence, calcium enters the cell to trigger catecholamine secretion (Douglas, 1968). Electrophysiological studies indicate that ACTH does not generally depolarize cortical cells in normal media (Matthews & Saffran, 1967), but can depolarize and even elicit action potentials in potassium-free media (Matthews & Saffran, 1968). However, the steroid output in response to ACTH was not markedly affected either by depolarizing potassium concentrations (see Matthews & Saffran, 1967), provided that calcium was present, or by potassium deprivation, which is in harmony with the suggestion that the stimulant effect of ACTH is independent of electrophysiological changes which accompany its action (Matthews & Saffran, 1967). Further investigations are required to discern whether the action of ACTH requires transmembrane ion fluxes.

The role of calcium in corticosteroid release. The present findings that calcium is required for ACTH-induced corticosteroid secretion is of some significance, since the secretory mechanism of the adrenal cortex may be somewhat different from that of many other secretory organs where the importance of calcium in the secretory process has been ascertained. In those tissues where a large portion of the secretory product is stored within granules (or vesicles), electronmicroscopic and chemical evidence provide strong support for the hypothesis that secretion occurs by the direct extrusion of the granule contents to the cell exterior (Douglas, 1968). The inability to detect similar membrane-bound organelles within adrenal cortical cells in electronmicroscopic studies has prompted an alternative hypothesis to explain corticosteroid secretion. Thus, ACTH is thought to enhance the conversion of cholesterol to corticosteroid mainly within the mitochondria of the cortex, and the steroid might then diffuse through the cytoplasm and across the cell membrane (Sabatini & De Robertis, 1961; Luse, 1967; Bloodworth & Powers, 1968).

Despite the presumed differences in the mode of secretion from the adrenal cortex, the present studies indicate that alterations in the ionic milieu affect secretion from the adrenal cortex in a similar manner to secretion from the adrenal medulla (Douglas & Rubin, 1961, 1963, 1964), endocrine pancreas (Curry, Bennett & Grodsky, 1968; Hales & Milner, 1968), neurohypophysis (Douglas & Poisner, 1964), and adrenergic (Kirpekar & Misu, 1967) and cholinergic nerve terminals (Hutter & Kostial, 1954; Katz & Miledi, 1967). Thus, ACTH-evoked corticosteroid secretion varied directly with the extracellular calcium concentration, and

was not markedly affected by monovalent cation deprivation. Furthermore, strontium could replace calcium, but magnesium inhibited secretion. However, unlike some other secretory systems, including the adrenal medulla (Douglas & Rubin, 1964), an equimolar concentration of barium was not able to maintain cortical secretion in the absence of calcium. These results do not necessarily indicate that calcium has a similar mechanism of action in promoting secretion from the adrenal cortex and from other secretory organs, such as the adrenal medulla, where there is strong evidence for exocytosis as the mechanism of secretion. On the other hand, the data presented in this investigation do at least suggest that the action of calcium in regulating the secretory process might be a more general effect which transcends the means by which secretion from a given organ is initiated.

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