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IONIC DEPENDENCE OF ADRENAL STEROIDOGENESIS AND ACTH-INDUCED CHANGES IN THE MEMBRANE POTENTIAL OF ADRENOCORTICAL CELLS

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SUMMARY

1. The effects of changes of ionic environment upon corticosteroid production by rabbit adrenal glands have been investigated *in vitro* using a superfusion technique and on-line steroid analysis by an automated fluorescence method. In some experiments micro-electrode recordings of adrenocortical transmembrane potentials were made concomitantly with measurement of steroid output.

2. Adrenocorticotrophic hormone (ACTH), 10 m-u./ml., induced a sevenfold increase in corticosteroid production rate in normal Krebs solution.

3. The steroidogenic response to ACTH was not impaired after omission of $[K]_o$ for 1 hr but was inhibited following exposure to K^+-free medium for 3 hr. Increase of $[K]_o$ tenfold to 47 mM increased the basal but not the ACTH-stimulated output of corticosteroid whereas raising $[K]_o$ twenty-fold to 94 mM enhanced both the basal and ACTH-stimulated steroid production rate. In K^+-free solution the adrenocortical cells hyperpolarized from -67 to -86 mV; subsequently on addition of ACTH they depolarized. Reintroduction of K^+ restored the membrane potential.

4. Omission of Ca^{2+} partially depolarized the cells but only affected the steroidogenic response to ACTH in the presence of EDTA. A threefold increase of $[Ca]_o$, to 7·68 mM, had no effect on either membrane potentials or steroid formation, but increasing $[Ca]_o$ tenfold to 25·6 mM partially blocked ACTH action. Increasing $[Mg]_o$ twentyfold to 22·6 mM had little effect on ACTH-stimulated corticosteroid output and Sr 2·56 mM, in

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substitution for Ca^{2+}, supported ACTH action, but La, 0·25 mM, completely blocked the steroidogenic effect of ACTH.

5. Replacement of NaCl, 118 mM by choline chloride, 118 mM, was without effect on ACTH-induced steroidogenesis, whereas LiCl, 118 mM, reduced it by 50 %. NaF, 1 and 10 mM, inhibited ACTH-induced steroidogenesis by approximately 60 %.

6. Nupercaine, 10^{-4} M, inhibited the steroid response to ACTH with no effect upon membrane potentials: increasing the nupercaine concentration to 10^{-3} M inhibited the steroid response and depolarized the cells. Ouabain, 10^{-5} M, induced complete depolarization and suppression of the steroidogenic response to ACTH.

7. Action-potential-like changes in membrane potential appeared in cells exposed to ACTH in a K^+-free medium. The amplitude of the action potentials ranged from 10 to 60 mV according to cell, with a frequency up to 36/min; the frequency tended to increase with time. Tetrodotoxin, 10^{-6} g/ml., did not inhibit ACTH-induced action potentials in K^+-free medium.

8. These observations are discussed in relation to the ionic requirements for the steroidogenic action of ACTH. The results further emphasize the dissociation of membrane polarization and the secretion of steroid. The mechanism of output of steroid hormone from the adrenocortical cell may thus differ fundamentally from the secretory mechanisms in other, particle-storing cells.

INTRODUCTION

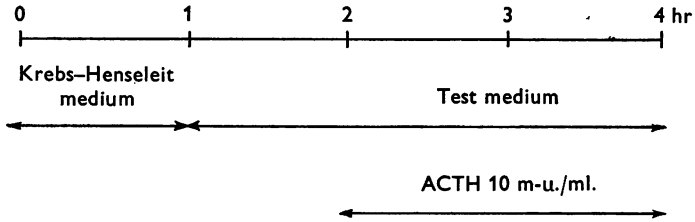
The ability of ACTH to stimulate steroid production in adrenocortical cells is not impaired by concentrations of $[K]_o$ sufficient to bring about an extensive change in cell membrane permeability (Matthews & Saffran, 1967). Other extracellular ions, e.g. Ca^{2+} and Na^+, are known to have important effects upon cell membrane structure and function in all mammalian cells, but their ability to influence intracellular metabolic events, particularly in steroid producing cells, is less clearly defined. To obtain information about the ionic dependence of corticosteroid production we have explored the effects of a variety of mono- and polyvalent ions on the steroid forming capacity of rabbit adrenal tissue. This information together with results of a further study of the relationship between the electrical properties of the adrenocortical cell membrane and the action of ACTH has given valuable clues as to the mode of output of steroid hormones from the adrenocortical cell. A preliminary account of some of these observations has been published (Matthews & Saffran, 1968).

METHODS

Adrenal tissue was obtained from neo-natal rabbits between 1 and 20 days old and superfused as described by Matthews & Saffran (1967). The superfusate was analysed continuously for corticosterone by the automated fluorescence method of Saffran, Ford, Matthews, Kraml & Garbaczewska (1967).

The Krebs–Henseleit solution used had the following composition (mM): NaCl 118, KCl 4·7, $CaCl_2$ 2·56, $MgCl_2$ 1·13, $NaHCO_3$ 25, NaH_2PO_4 1·15, glucose 5·55. It was bubbled with 95% O_2 and 5% CO_2 (v/v). In experiments in which the effects of increased $[K]_o$, $[Ca]_o$ or $[Mg]_o$ were studied, the NaCl content of the medium was reduced proportionately to maintain isotonicity. When Ca^{2+} and Mg^{2+} were present in concentrations greater than 5 mM and when La^{3+} was used, the bicarbonate-CO_2 buffer system and the NaH_2PO_4 were omitted and Tris buffer, 5 mM, and 100% O_2 were substituted.

Steroid production. Unless otherwise specified, experiments for analysis of steroid production were conducted in the following way:



To facilitate comparison among experiments, the record of fluorescence representing the continuous output of corticosterone was converted to the steroid output per 10 min period (Fig. 1*B*, *D*). From these data the cumulative production of corticosterone throughout the experiment was obtained, and the secretion rates in the presence and absence of ACTH were derived from the slopes of lines drawn through the plotted points (Fig. 1*A*, *C*). The rate of steroid secretion is then expressed as ng corticosterone produced per 100 mg tissue per minute.

Membrane potentials were measured with intracellular glass micro-electrodes filled with K citrate, 1·5 M, as previously described (Matthews, 1967).

RESULTS

In some experiments steroid production alone was measured, in others it proved possible to obtain membrane potential measurements from adrenocortical cells in addition to measurements of steroid production. The results of ionic manipulations upon steroid output will be considered first.

As in previous experiments (Matthews & Saffran, 1967), the adrenal output of corticosterone in the first 30 min was high and subsequently declined to a steady basal output (Fig. 1*A* and *C*). The addition of ACTH caused a prompt increase in the rate of steroid formation (Fig. 1*C*). The mean basal production rate was 0·42 ± S.E. 0·05 ng/100 mg tissue.min.

ACTH (10 m-u./ml.) caused a sevenfold increase in the output rate (Table 1). A higher dose of ACTH induced a still greater increase in secretion rate (Table 1).

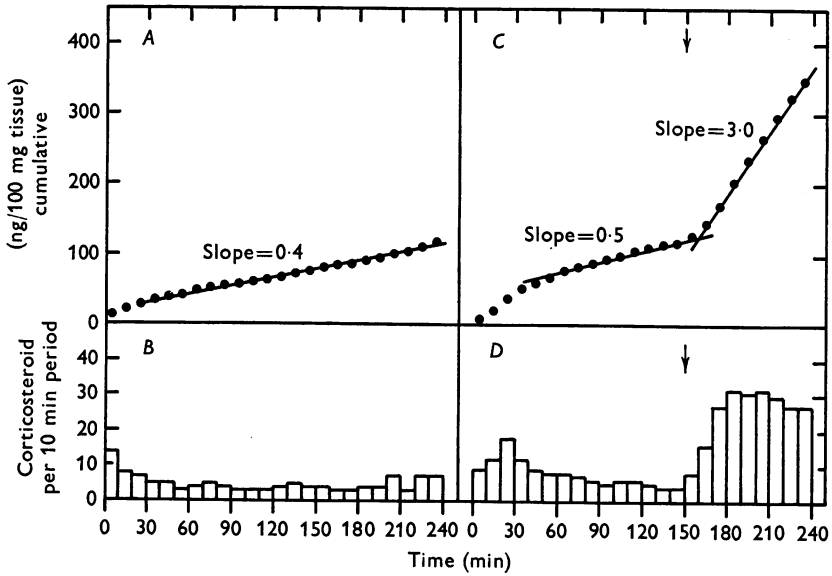


Fig. 1. Corticosterone production by superfused rabbit adrenal tissue. In *B* and *D* the corticosterone output is plotted as ng/100 mg tissue. 10 min period. This data is replotted in *A* and *C* as the cumulative output in ng/100 mg tissue. ACTH 10 m-u./ml. is introduced for the duration of the experiment at the time indicated by the arrows in *C* and *D*. The slopes of the lines drawn through the plotted points in *A* and *C* yield the production rates of corticosterone in ng/100 mg tissue.min.

TABLE 1. Rate of corticosterone production before and after ACTH

ACTH	No. of experiments	Corticosterone production (ng/100 mg tissue.min) Mean ± S.E.	Range
None	10	0·42 ± 0·05	0·1–0·6
10 m-u./ml.	9	2·94 ± 0·14	2·2–3·6
20 m-u./ml.	1	4·10	

Effect of polyvalent cations

In many endocrine and neuroendocrine cells the Ca^{2+} ion plays a crucial role in the process of hormone release (Matthews, 1970). Furthermore, the $[Ca]_o/[Mg]_o$ ratio is often of critical importance in controlling hormone secretion. We have therefore examined the effects of these and other divalent and trivalent cations on the steroidogenic process.

Calcium. The omission of Ca^{2+} or of Ca^{2+} and Mg^{2+} from the medium was without effect on either the basal or ACTH-stimulated output (Table 2). However, addition of 3 mM-EDTA in the absence of Ca^{2+} and Mg^{2+} completely obliterated the increased rate of steroid production normally induced by ACTH; the basal output was unaffected. Concentrations of Ca^{2+} ranging from 1·28 to 11·8 mM; i.e. one half to five times the normal concentrations, had no effect on either basal or stimulated steroid production rates. A further increase in Ca^{2+} to 25·6 mM did, however, reduce significantly both the stimulated ($P < 0·05$) and the basal ($P < 0·05$) rate of production.

TABLE 2. Effect of polyvalent cations on the rate of corticosterone production before and after ACTH 10 m-u./ml. The basal rate is that measured in the 60 min exposure period to test medium before addition of ACTH

Experimental procedure	No. of experiments	Corticosterone production (ng/100 mg tissue.min)	
		Basal	ACTH stimulated
Normal Krebs	9	0·42 ± 0·05*	2·94 ± 0·14
Tris 5 mM	1	0·4	3·3
Ca-free; Mg-free; EDTA 3 mM	1	0·3	0·3
Ca-free; Mg-free	1	0·2	2·1
Ca-free	1	0·6	2·4
Ca 1·28 mM	1	0·2	2·8
Ca 5·12 mM	1	0·2	2·4
Ca 7·68 mM	1	0·5	3·1
Ca 12·80 mM†	2	0·2	2·3
Ca 25·6 mM†	4	0·2 ± 0·02	1·1 ± 0·42
Sr 2·56 mM	3	0·3 ± 0·10	2·3 ± 0·39
La 0·25 mM	2	0·4	0·6
Mg 11·3 mM†	2	0·2	2·2
Mg 22·6 mM†	2	0·2	2·1

* Mean of ten experiments. † With a Tris 5 mM buffer system.

Strontium. Total replacement of the normal $[Ca]_o$ by Sr^{2+} appeared to have no influence on either the basal ($P < 0·05$) or the stimulated rates ($P < 0·05$) of steroidogenesis. This accords with previous observations in rat (Peron & Koritz, 1958) and cat adrenal tissue (Jaanus, Rosenstein & Rubin, 1970).

Magnesium. Although an increase in $[Mg]_o$ markedly inhibits secretion in many endocrine cells, both basal and ACTH-stimulated corticosteroid production remained within the normal range in the presence of $[Mg]_o$ as high as 10 or 20 times the normal, i.e. 11·3 and 22·6 mM (Table 2).

Lanthanum. In a concentration of 0·25 mM (i.e. one tenth that of $[Ca]_o$), the trivalent La^{3+} ion almost completely inhibited the stimulant action of

TABLE 3. Effect of monovalent ions on the rate of corticosterone production before and after ACTH 10 m-u./ml. The basal rate is that measured in the 60 min exposure period to test medium before addition of ACTH

Experimental procedure	No. of experiments	Corticosterone production (ng/100 mg tissue.min)	
		Basal	ACTH stimulated
Normal Krebs	9	0·42 ± 0·05*	2·94 ± 0·14
Choline, 118 mM	1	0·3	2·7
Li, 118 mM	2	0·4	1·3
NaF			
1 mM	1	0·2	1·0
10 mM	1	0·5	1·2
K-free (1 hr)	3	0·5	—
	2	—	2·5
K-free (3 hr)	1	0·1	0·5
K-free; Mg-free (1 hr)	1	0·3	3·3
K, 47 mM	3	0·9 ± 0·20	2·9 ± 0·44
K, 94 mM	1	1·2	4·8

* Mean of ten experiments.

ACTH. This may result from an effect upon adrenal Ca^{2+} exchange mechanisms because in many cellular systems La^{3+} ions are known to block membrane calcium flux (Van Breemen & Van Breemen, 1969; Goodman & Weiss, 1969; Van Breemen & De Weer, 1970).

Effect of monovalent ions

Sodium. Complete replacement of the NaCl content of the medium, 118 mM by choline chloride had no detectable action on steroidogenesis. In contrast, when the NaCl was replaced by LiCl, stimulation of steroid production by ACTH was reduced by about 50 % (Table 3).

Fluoride. The F^- ion is a potent stimulator of adenyl cyclase activity in many cell-free preparations (Sutherland, Rall & Menon, 1962). In adrenal homogenates, NaF, 10 mM, markedly increases cyclic AMP formation (Grahame-Smith, Butcher, Ney & Sutherland, 1967; Wolfe & Jones, 1970), and cyclic AMP is supposed to mediate the steroidogenic effect of ACTH (Garren, Gill, Masui & Walton, 1971). The ability of F^- to stimulate steroidogenesis was therefore tested in intact adrenal cells. In fact, not only did F^- fail to stimulate, but in a concentration of 1–10 mM it inhibited ACTH-induced steroidogenesis by about 60 % (Table 3). Thus, although, adenyl cyclase activation in the intact adrenal cell cannot be discounted, its effects are probably negated by other membrane and metabolic actions of the F^- ion (Eckel, 1954; Davson, 1941; Whittam, 1964; Mahler & Cordes, 1966).

Potassium. Omission of K^+ or of K^+ and Mg^{2+} from the medium for 1 hr before addition of ACTH had no effect upon the stimulation of steroidogenesis. However, when the tissue was superfused with K^+-free medium for 3 hr, the subsequent response to ACTH was markedly reduced. Increases of $[K]_o$ to 10 and 20 times the normal concentration, 47 and 94 mM, increased the basal rates of steroid production by approximately twofold and threefold respectively. At 47 mM there was no effect upon ACTH action but raising $[K]_o$ to 94 mM did lead to some increase in the secretion rate after ACTH (Table 3).

Membrane potentials and steroid production

In the present series of experiments the mean resting potential of rabbit adrenocortical cells in normal Krebs–Henseleit solution was $-67{\cdot}0$ mV $\pm$ 0·59 S.E. (n = 136), measured in the period 50–60 min after placing the tissue in the bath; this is in agreement with our previous determinations, i.e. $-66{\cdot}2$ mV $\pm$ 0·35 S.E. (Matthews, 1967).

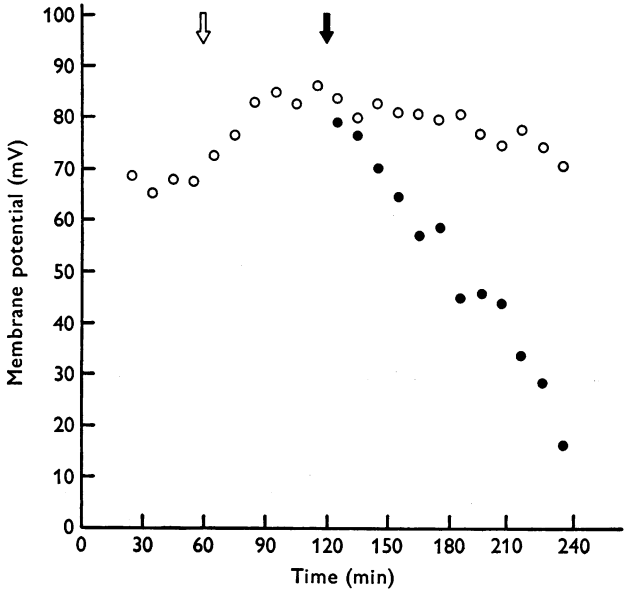


Fig. 2. Membrane potentials of rabbit adrenocortical cells in K^+-free medium in the presence and absence of ACTH. At the open arrow superfusion with K^+-free Krebs–Henseleit solution was begun. The filled arrow indicates the introduction of ACTH 10 m-u./ml. into the K^+-free medium. Filled circles denote membrane potentials measured in the presence of ACTH; open circles, in its absence. Each plotted point is the mean membrane potential derived from between twelve and seventy-two impalements in up to sixteen different experiments.

An increase in extracellular K^+ concentration depolarizes the adrenocortical cell without affecting the steroidogenic response to ACTH (Matthews & Saffran, 1967). Furthermore, ACTH does not appear to influence the membrane potential, either in normal medium or in the presence of a raised extracellular K^+ concentration (Matthews & Saffran, 1967). However, in the absence of K^+, tested in the present experiments, ACTH appeared to exert some effect upon membrane permeability. This is shown in Fig. 2, which illustrates the effect of omission of K^+ from the medium upon membrane potential measurements and the subsequent effects of ACTH. Replacement of the Krebs–Henseleit medium by the K^+-free solution resulted in a significant hyperpolarization of the cells, from $-67{\cdot}2$ mV $\pm$ 0·82 S.E. to a mean value of $-86{\cdot}0$ mV $\pm$ 1·22 S.E. after 50 min ($P < 0{\cdot}001$). Subsequently, the membrane potential declined to $-70{\cdot}3$ mV $\pm$ 2·52 S.E. after 170 min exposure to K^+-free medium.

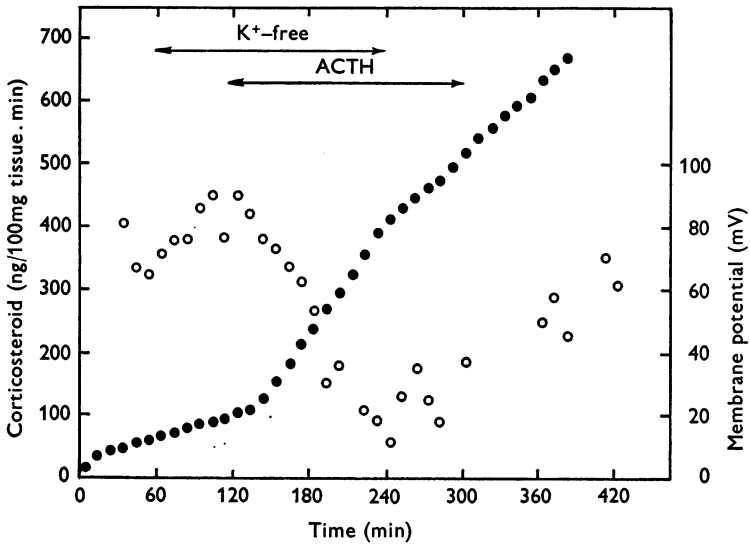


Fig. 3. Cumulative corticosteroid production (●) and membrane potentials (○) in adrenocortical cells: effect of the absence of K^+ with and without ACTH (10 m-u./ml.). The open circles are the means of membrane potentials measured during successive 10 min periods.

Addition of ACTH to the K^+-free solution accelerated the decline in membrane potential more than fourfold. After 170 min the mean membrane potential had fallen to $-16{\cdot}2$ mV $\pm$ 1·84 S.E. (Fig. 2), significantly below the corresponding potential of $-70{\cdot}3$ mV in the absence of ACTH ($P < 0{\cdot}001$).

Illustrated in Fig. 3 is an experiment in which membrane potentials and production of corticosterone were measured simultaneously on the same

sample of tissue. The tissue was first superfused for 60 min in normal Krebs–Henseleit medium before changeover to K^+-free medium. While the omission of K^+ caused no discernible change in basal steroid production, the membrane potentials rose to values well above those found in normal medium. The addition of ACTH (10 m-u./ml.) in the continued absence of K^+ caused a progressive depolarization of the adrenocortical cells but the steroidogenic response to ACTH was unaffected under these conditions. The output of steroid increased from 0·55 to 2·9 ng/100 mg tissue.min, identical to the increase seen in normal medium (Table 1). Reintroduction of K^+ with the continued presence of ACTH had little effect on the ACTH-stimulated rate of steroid production but led to a recovery of the membrane potentials towards normal values of -60 to -70 mV.

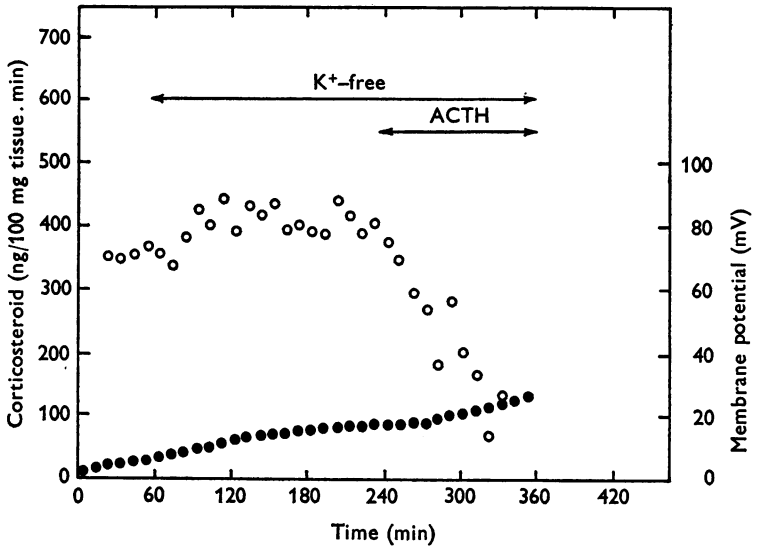


Fig. 4. Cumulative corticosteroid production (●) and membrane potentials (○) in adrenocortical cells: effect of the absence of K^+ with and without ACTH (10 m-u./ml.). Open circles are the means of membrane potentials measured in successive 10 min periods. Note that in this experiment the cells were exposed to K^+-free medium for 3 hr before the introduction of ACTH. Compare with Fig. 2.

In contrast to the results depicted in Fig. 3, longer exposure to K^+-free medium (i.e. 180 *vs.* 60 min) inhibited the steroidogenic response to ACTH (i.e. 0·53 *vs.* 2·9 ng/100 mg tissue.min), but ACTH still induced a marked depolarization of the cells (Fig. 4).

In one experiment the tissue was placed immediately in K^+-free medium after excision from the animal. Instead of hyperpolarizing as in previous experiments (e.g. Fig. 2), under these conditions the membrane potentials

fell progressively to reach a value of −25 mV at 120 min (Fig. 5). It seems probable that this effect results from residual circulating ACTH to which the gland was exposed *in vivo* immediately before its excision because on restoration of K^+, the membrane potentials recovered to −50 mV. Removal of K^+ at this point caused the cells to hyperpolarize rapidly to −80 mV. Subsequent introduction of ACTH was now followed by the characteristic depolarization produced by ACTH in K^+-free medium (Figs. 2 to 4), although the steroid output in response to ACTH (1·0 ng/100 mg tissue.min) was less than expected.

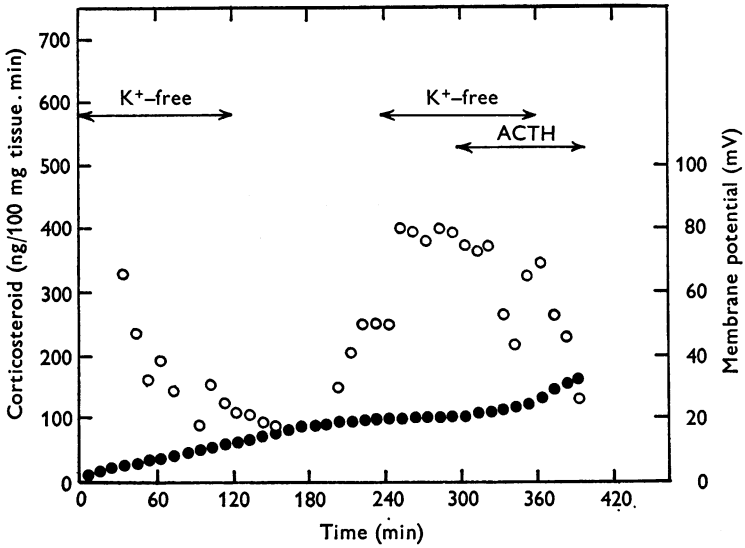


Fig. 5. Cumulative corticosteroid production (●) and membrane potentials (○) in adrenocortical cells: effect of immediate exposure to K^+-free medium on the responses to ACTH (10 m-u./ml.). Open circles are the means of membrane potentials in successive 10 min periods.

In contrast to the effect of K^+ removal (Figs. 2 to 4), the omission of Ca^{2+} from the medium resulted in a partial depolarization of the adrenocortical cells (Fig. 6), but again there appeared to be no immediate impairment of the steroidogenic response to ACTH. Reintroduction of Ca^{2+} restored the membrane potentials to normal values. Depolarization without an impaired steroidogenic response to ACTH was also seen in another experiment (not illustrated) in which *both* Ca^{2+} and Mg^{2+} were omitted for 1 hr before the introduction of ACTH. However, the addition of 3 mM-EDTA to Ca^{2+} and Mg^{2+}-free medium led to a complete depolarization of the cells and obliteration of the steroidogenic response to ACTH (Fig. 7). The membrane potentials recovered fully, and steroid formation partially, when normal medium was reintroduced.

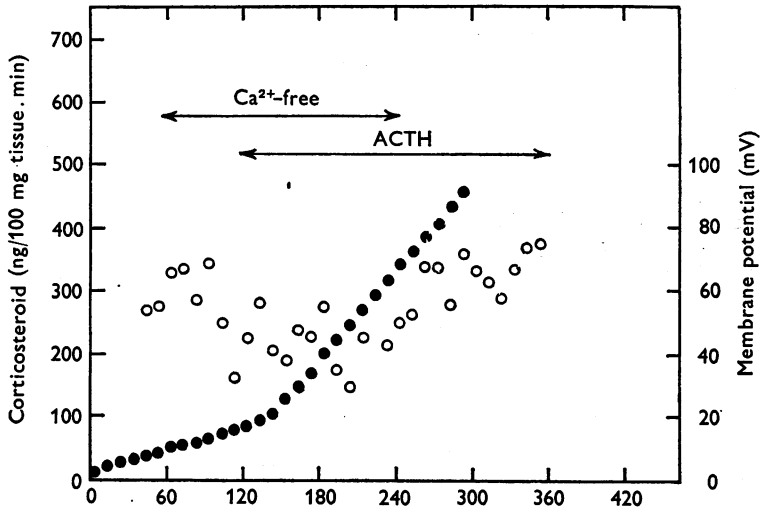


Fig. 6. Cumulative corticosteroid production (●) and membrane potentials (○) in adrenocortical cells: effect of exposure to Ca^{2+}-free medium on the response to ACTH (10 m-u./ml). Open circles are the means of membrane potentials in successive 10 min periods.

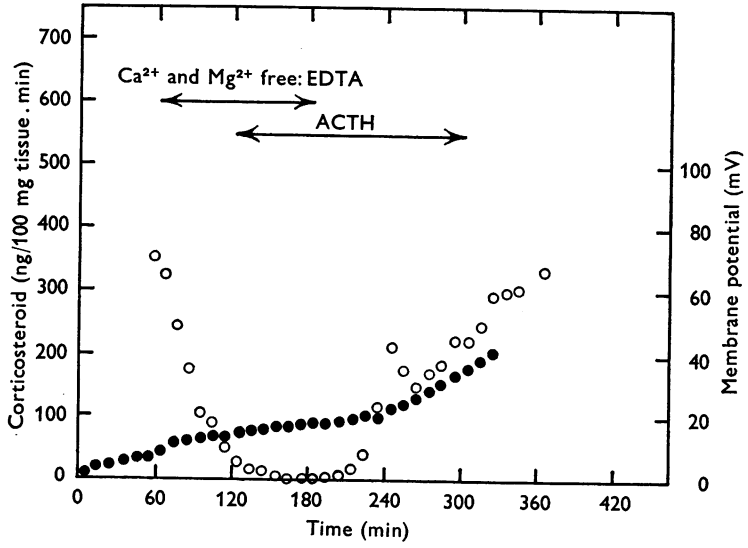


Fig. 7. Cumulative corticosteroid production (●) and membrane potentials (○) in adrenocortical cells: effect of absence of Ca^{2+} and Mg^{2+}, and addition of 3 mM EDTA, on the response to ACTH (10 m-u./ml.). Open circles are the means of membrane potentials in successive 10 min periods.

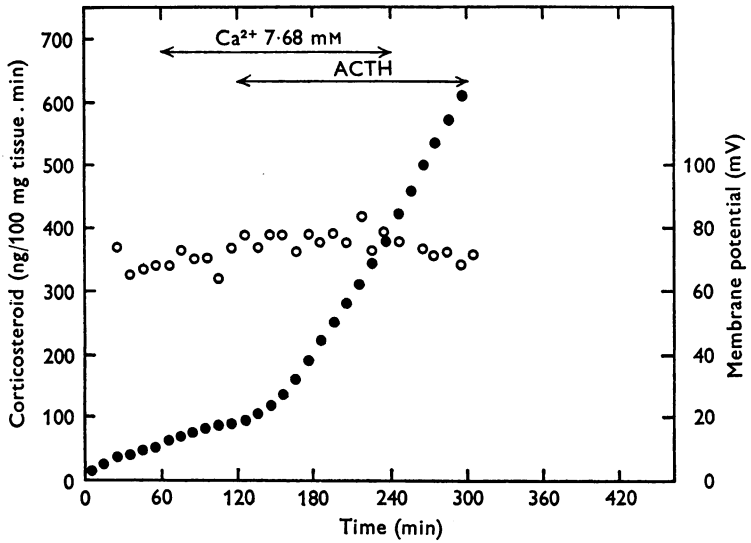


Fig. 8. Cumulative corticosteroid production (●) and membrane potentials (○) in adrenocortical cells: effect of 7·68 mM-Ca^{2+} on the response to ACTH (10 m-u./ml.). Open circles are the means of membrane potentials in successive 10 min periods.

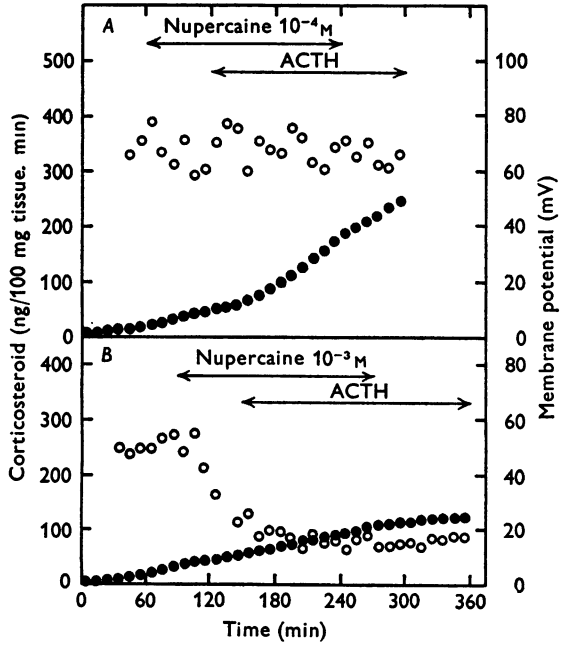


Fig. 9. Cumulative corticosteroid production (●) and membrane potentials (○) in adrenocortical cells: effect of nupercaine on the response to ACTH (10 m-u./ml.). *A*, 10^{-4} M; *B*, 10^{-3} M, nupercaine. Open circles are the means of membrane potentials in successive 10 min periods.

Medium with 5·12 or 7·68 mM, i.e. two or three times the normal concentration of Ca^{2+}, 2·56 mM, had no influence on either membrane potentials or steroid formation (Fig. 8, Table 1).

Local anaesthetics are believed to stabilize cell membranes, interfering especially with the membrane uptake and transport of Ca^{2+} (Seeman, 1966; Kwant & Seeman, 1969); in higher concentration they may cause, by excessive molecular packing, a partial membrane disruption and non-selective permeability increase (Seeman, 1966). Nupercaine, 10^{-4} M, whilst without effect on the membrane potential, inhibited the steroidogenic

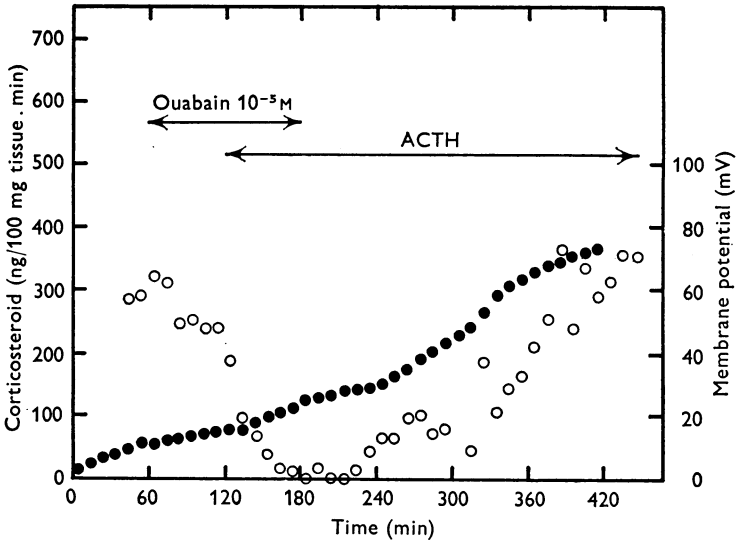


Fig. 10. Cumulative corticosteroid production (●) and membrane potentials (○) in adrenocortical cells: effect of ouabain, 10^{-5} M, on the response to ACTH (10 m-u./ml.). Open circles are the means of membrane potentials in successive 10 min periods.

response to ACTH by about 50 % (Fig. 9*A*), whereas increasing the nupercaine concentration tenfold to 10^{-3} M, led to depolarization of the cells, and a complete inhibition of the response to ACTH (Fig. 9*B*).

Exposure to the Na pump inhibitor, ouabain 10^{-5} M, induced complete depolarization of adrenocortical cells and suppressed the steroidogenic response to ACTH (Fig. 10). Both the membrane potentials and steroid response showed a slow recovery towards normal values following removal of ouabain.

Action potentials

Rapid transient potential changes resembling action potentials appeared after exposure to ACTH in K^+-free medium for periods of 30–60 min (mean 50 min) and when the adrenocortical cells had depolarized to −30

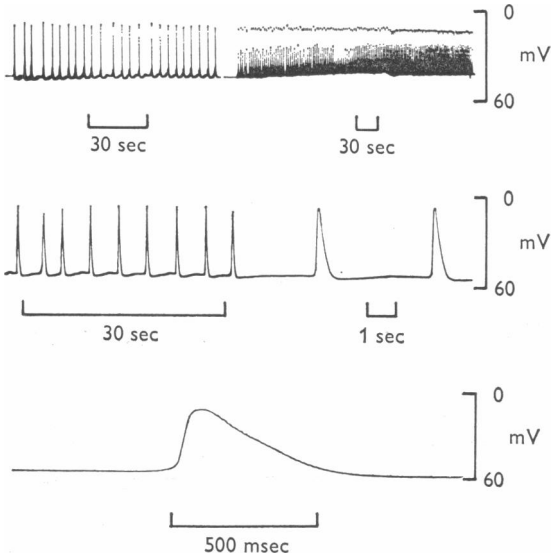


Fig. 11. Action potentials in an adrenocortical cell after exposure to ACTH (10 m-u./ml.) in K^+-free solution. Pen recording: all records are from the same cell but at different time-base amplification.

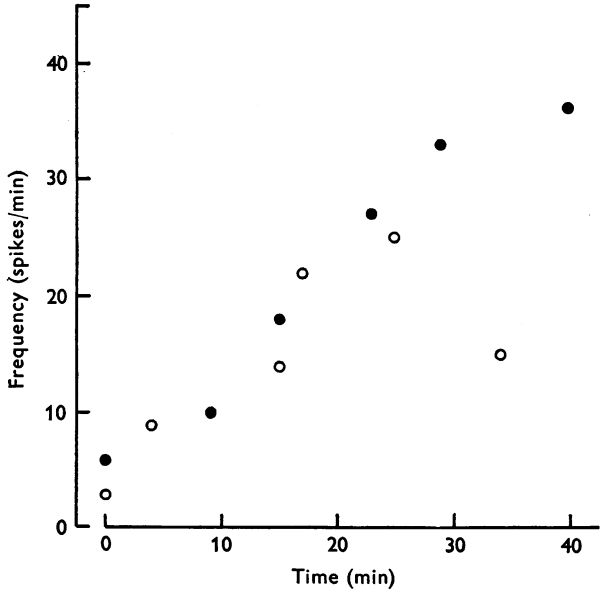


Fig. 12. Changes in action potential frequency with time. Zero time represents the time of appearance of the first action potential. Data are plotted from two separate experiments in which adrenocortical cells were exposed to K^+-free medium and ACTH (10 m-u./ml.).

to −60 mV (mean −46 mV) (Fig. 11). Sometimes these action potentials were preceded by slow low voltage fluctuations in potential (Fig. 14*B*).

The action potentials ranged from 10 to 60 mV according to cell and to time exposure to ACTH. The frequency of the spikes tended to increase for about 30–40 min after the appearance of the first spike (Fig. 12). As the spike frequency increased, the membrane potential continued to fall with the usual time course (see Fig. 2). At membrane potentials of about −20 mV, spike discharge ceased.

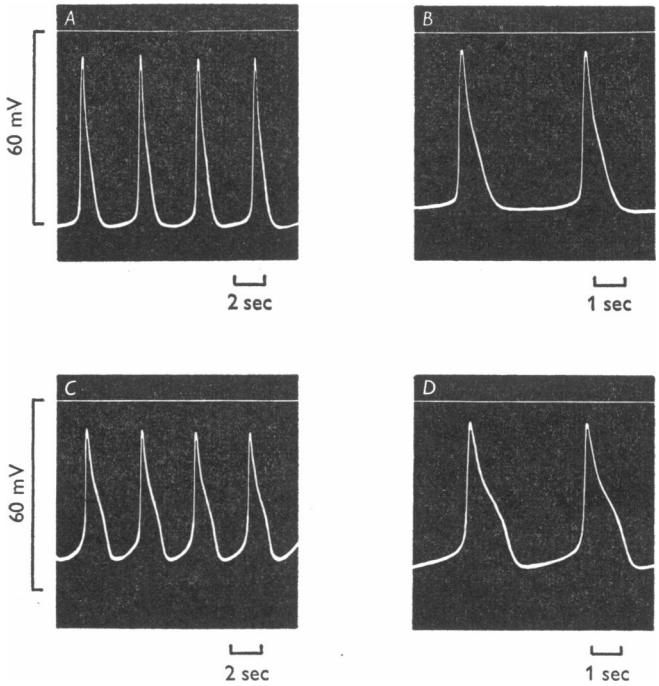


Fig. 13. Action potentials in adrenocortical cells after exposure to ACTH (10 m-u./ml.) in K^+-free solution. Oscilloscope recording: records *A* and *C* are from different cells; *B* and *D* at increased time-base amplification from *A* and *C*, respectively.

The action potentials varied in wave-form characteristics (Figs. 13, 14). Initially, they displayed no marked after negativity (Figs. 11, 13*A*); later a pronounced negative after-potential or plateau appeared (Figs. 13*D*, 14*A*). The spike rise time was 120–200 msec and spike duration varied from 600 to 1800 msec in different cells. Often, the action potentials were immediately preceded by well-defined slow potential changes or pre-potentials (Fig. 13*C*, *D*). Occasionally, cells were impaled which displayed action potential overshoot beyond the zero potential (Fig. 14*C*). At first

the spikes tended to be of rather uniform amplitude (e.g. Fig. 11) but in later impalements, spikes of two or more populations were sometimes recorded (Fig. 14*D*).

Tetrodotoxin, 10^{-6} g/ml., did not prevent the appearance of ACTH-induced spike activity (Fig. 15).

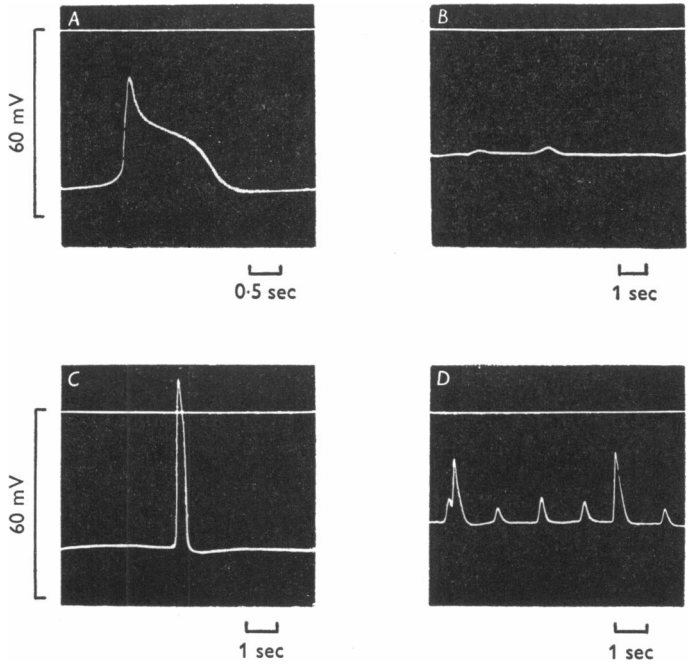


Fig. 14. Adrenocortical cells: intracellular records after exposure to ACTH (10 m-u./ml.) in K^+-free solution. Each oscilloscope recording is from a different cell in separate experiments. Illustrated are: *A*, spike with pronounced after negativity; *B*, slow wave activity; C, action potential overshoot beyond zero potential; *D*, spikes of different size in the same cell.

DISCUSSION

In the absence of extracellular Ca^{2+} the steroidogenic action of ACTH is reduced (Birmingham, Elliott & Valere, 1953), but in our experiments and in those of Carchman, Jaanus & Rubin (1971) a Ca chelating agent was required to block completely the steroidogenic response to ACTH. This suggests the retention of Ca^{2+} in an intracellular compartment of high capacity or affinity, resistant to depletion by removal of extracellular Ca, and which remains functionally intact unless a chelating agent is present.

Increasing $[Ca]_o$ to 25·6 mM also inhibited the steroidogenic response to ACTH as well as affecting the basal output (Table 2), but the response to

ACTH in the presence of increased $[Mg]_o$ showed no pronounced inhibition. A small decrease of about 35 % in the ACTH-evoked corticosteroid output was observed in the perfused cat adrenal by Jaanus *et al.* (1970) with $[Mg]_o$, 20 mM, but none at 10 mM. These effects of high extracellular concentrations of divalent cation probably reflect, in our experiments and those of Jaanus *et al.* (1970), a stabilization of the membrane by excessive Ca^{2+} or Mg^{2+} binding rather than a more fundamental effect on the production of corticosteroid.

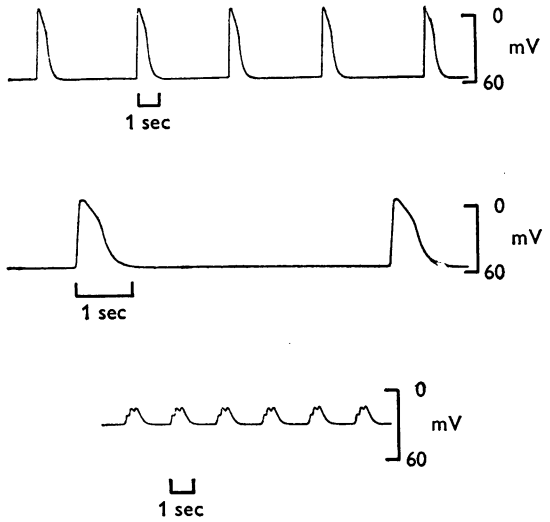


Fig. 15. Action potentials in an adrenocortical cell after exposure to ACTH (10 m-u./ml.) in K^+-free solution containing tetrodotoxin (10^{-6} g/ml.). Pen recording: the two upper records are from the same cell but with different time-base amplification.

Of the monovalent cations, choline chloride replaced $[Na]_o$ without affecting steroidogenesis whereas substitution with an equimolar amount of LiCl inhibited ACTH action. In the presence of $[Li]_o$ the adrenocortical cells will accumulate Li^+ ions, which enter passively, and be unable to remove them via the Na^+ pump (Keynes & Swan, 1959); $[Li]_i$ therefore steadily rises. An analogous increase in $[Na]_i$ occurs in the presence of ouabain due to inactivation of the membrane Na-K pump. This also leads to inhibition of ACTH action. In fact, not only will $[Li]_o$ and ouabain induce an increase in $[Li]_i$ or $[Na]_i$ but both may cause a potassium efflux and depletion of $[K]_i$. All three factors will exert significant metabolic effects (Wyatt, 1964; Bygrave, 1967). Glycolytic enzymes, essential for a rapid turnover of ATP and NADPH during steroidogenesis, are activated by K^+ and inhibited by Na^+ and Li^+ (Mahler & Cordes, 1966). It is already

evident that prolonged exposure to a K^+-free solution, with a consequent lowering of $[K]_i$, will block ACTH action (Table 3).

In contrast, increasing $[K]_o$ to 47 and 94 mM, concentrations depolarizing adrenocortical cells by 44 and 54 mV, respectively (Matthews, 1967), enhanced the production of fluorogenic steroids. However, it is unlikely that this small increase in output represents corticosterone produced in response to depolarization *per se* because depolarization evoked by other ionic manipulations (q.v.) and by lower concentrations of $[K]_o$, i.e. 23·5 mM (Matthews & Saffran, 1967), fails to increase steroid output.

Adrenocortical membrane potentials and the mechanism of steroid secretion

The measured membrane potential, E_m, of adrenocortical cells depends largely upon the concentration gradient for K^+ across the cell membrane (Matthews, 1967). In consequence exposure to K^+-free solutions will cause the cells to loose intracellular K^+ and gain Na^+ due to sodium pump inactivation (Garraham & Glynn, 1967). Predictably, therefore, the cells hyperpolarize and then depolarize with a rate constant dependent on the relative Na^+ permeability of the membrane. The immediate acceleration in depolarization rate from 5 to 23 mV hr^{-1} induced by ACTH may therefore be explicable as an increase in membrane Na^+ or Ca^{2+} permeability.

The membrane potential of rabbit adrenocortical cells proved to be more sensitive to a reduced $[Ca]_o$ than was steroidogenesis. In view of the known requirement of Ca^{2+} for maintenance of normal membrane structure and permeability (Maizels, 1959; Adelman & Moore, 1961; Morill, Kaback & Robbins, 1964) the depolarizing effect of a low $[Ca]_o$ is not unexpected. On the other hand, the local anaesthetic, nupercaine inhibited the steroidogenic response to ACTH at a concentration that did not depolarize the cells. As already pointed out, local anaesthetic molecules stabilize cell membranes and interfere with the membrane uptake and transport Ca^{2+} (Seeman, 1966; Kwant & Seeman, 1969). Ca is believed to be a mediator of ACTH action (Farese, 1971). An effect of nupercaine on the adrenal uptake and intracellular distribution of Ca^{2+} would thus account for its inhibition of steroidogenesis.

Ouabain inhibits the membrane Na pump (Skou, 1957; Dunham & Glynn, 1961). Thus $[Na]_i$ rises, $[K]_i$ falls, and the cells depolarize. It is, however, the change in $[Na]_i/[K]_i$ that probably accounts for the inhibition of ACTH-induced steroidogenesis by ouabain (see above) because depolarization *per se* clearly does not (Matthews & Saffran, 1967).

Taken together the electrophysiological results and those on the ionic dependence of steroidogenesis emphasize the dissociation of membrane

polarization and the secretion of corticosteroid. The mechanism of output of steroid hormone from the adrenocortical cell thus appears to differ quite fundamentally from the secretory mechanisms in other, particle-storing, cells (Douglas, 1968; Matthews, 1970), especially as it concerns the role of Ca. Indeed, in the adrenal cortex the role of Ca may differ from that in other secretory cells precisely because the secretory product is *not* stored in any particulate form but is released from the adrenocortical cell immediately after formation (Vogt, 1943; Holzbauer, 1957). Thus Ca deprivation by omission of $[Ca]_o$ has comparatively little immediate effect on the action of ACTH but rapidly inhibits the stimulated secretion of adrenal medullary (Douglas & Rubin, 1961) and other secretory cells, which store their secretory product in the form of membrane bound particles, e.g. endocrine and exocrine pancreas (Curry, Bennett & Grodsky, 1968; Hales & Milner, 1968; Hokin, 1966), neurohypophysis (Douglas & Poisner, 1964), adenohypophysis (Vale, Burgus & Guillemin, 1967; Vale & Guillemin, 1967; Samli & Geschwind, 1968), cholinergic nerve (Harvey & MacIntosh, 1940; Katz & Miledi, 1967) and adrenergic nerve terminals (Kirkepar & Misu, 1967).

Small increases of $[Mg]_o$ above normal inhibit release in particle-storing cells; but even in high concentrations Mg has little or no effect on ACTH-evoked steroidogenesis. Stimuli which depolarize particle-storing cells invariably cause marked secretion; no such correlation is seen in adrenocortical cells (Matthews & Saffran, 1967). Finally both ouabain and prolonged K^+ omission, which inhibit steroidogenesis in the adrenal cortex, actually evoke secretion in particle-storing cells by promoting Na^+ or Ca^{2+} entry. The entry of Ca can therefore be seen as vital in particle-storing secretory cells for participation in storage granule membrane-cell membrane interaction, fusion and exocytosis (Douglas, 1968; Matthews, 1970), but in the adrenocortical cells, where no such particulate storage-ejection system operates, there may be a limiting requirement for a quite different metabolic purpose, e.g. that of protein synthesis (Farese, 1971). Our evidence thus argues strongly against 'exocytosis' as the mechanism of corticosteroid secretion.

Action potentials

The action potentials induced by ACTH in K^+-free medium are unlikely to arise simply as a consequence of depolarization *per se* for ouabain and high $[K]_o$ both markedly depolarized adrenocortical cells without generating electrical activity.

The first action potentials to appear after ACTH administration were discrete spikes with no pronounced after negativity. Subsequent impalements yielded spikes with a definite plateau, resembling cardiac action

potentials (Brady & Woodbury, 1960). The long duration of the spikes indicates a prolonged Na^+ or Na^+ and Ca^{2+} influx, a delayed outward K^+ current, or an impaired repolarization mechanism.

Some cells also showed populations of action potentials of mixed amplitude. It seems quite possible that the smaller spikes represent a spread of attentuated signals originating in cells in the vicinity of the impaled cell because low resistance pathways are known to exist among many non-neuronal cells (Horridge, 1968) and extensive cell contacts resembling septate junctions have recently been described between adjacent adrenocortical cells (Friend & Gilula, 1972).

It is not yet clear how far the adrenocortical action potential is due to Na^+ carrying inward current across the membrane. Tetrodotoxin 10^{-6} g/ml., a concentration sufficient in other cells to inhibit Na^+-dependent action potential genesis (Kao, 1966), failed to block ACTH-induced action potentials in adrenocortical cells. They may therefore be mediated, at least in part, by Ca entry.

Finally, it must be emphasized that the induction of depolarization and electrical activity in adrenocortical cells by ACTH was disclosed only by exposing the cells to a K^+-free environment. Any effect of ACTH upon the membrane potential and permeability of its target cell in normal extracellular fluid is therefore likely to be extremely small and may well be masked by the presence of normal extracellular K.

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