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Measurement of the Dynamics of Stimulation and Inhibition of Steroidogenesis in Isolated Rat Adrenal Cells by using Column Perfusion

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Isolated adrenal cells were perfused in a small column by using Bio-Gel polyacrylamide beads as an inert supporting matrix, and the time-course of the response to various stimuli was observed by measuring fluorogenic 11-hydroxycorticosteroids in the effluent. A small but significant response was observed 1 min after stimulation with physiological concentrations of ACTH (adrenocorticotrophin), but the response did not start to build up rapidly for 3–4 min and eventually reached a plateau after 9–10 min. A similar pattern of events was observed for the decay of the steroid output on removal of ACTH. ACTH analogues, including one with a long duration of action *in vivo*, were found to produce responses with similar kinetics. However, cyclic AMP caused a more rapid increase in steroidogenesis and its effects were more short-lived after withdrawal. If, as present evidence suggests, cyclic AMP is produced rapidly after ACTH stimulation the delayed build-up of the steroidogenic response to ACTH would indicate that cyclic AMP may not be the intracellular mediator. When inhibitors were applied during ACTH stimulation, aminoglutethimide, which blocks mitochondrial conversion of cholesterol into pregnenolone (3β-hydroxypregn-5-en-20-one), caused a rapid fall in steroid output (1 min), whereas cycloheximide took longer to achieve its full effect. Nevertheless, the response had fallen by 50% in 2 min, indicating a much shorter half-life than that previously reported for the labile protein implicated in steroidogenesis. In addition the rapid response to cyclic AMP makes it unlikely that steroid production is induced as a result of initiation of protein synthesis. This suggests that the labile protein plays an obligatory but permissive role in the development of the response. Column perfusion has proved to be a simple technique which can readily yield accurate data on responses of cells to stimulants and inhibitors.

Investigations of the mechanism of action of ACTH* on the adrenal cortex have been hampered by failure to produce a cell-free system in which the effects of the hormone can be studied by a conventional biochemical approach, e.g. isolation of vital intermediates from an homogenate.

However, a number of informative studies have been carried out by using intact adrenals *in vivo* (Beaven *et al.*, 1964; Garren *et al.*, 1965) or superfused adrenals or adrenal quarters *in vitro* (Schulster *et al.*, 1970; Grahame-Smith *et al.*, 1967; Pearlmutter *et al.*, 1973). For example, Grahame-Smith *et al.* (1967) reported that ACTH caused an increase in cyclic AMP concentrations in adrenal quarters after 1 min, whereas an increased steroid output was apparent after 2 min. This, taken in conjunction with earlier observations (Haynes *et al.*, 1959; Imura *et al.*, 1965) that exogenous cyclic AMP stimulates corticosteroid production *in vitro* and *in vivo*, led to the proposal that cyclic AMP was the intracellular mediator of the action of ACTH on the adrenal cortex.

Further insight into the mechanism of action of ACTH has been obtained with inhibitors of protein synthesis (Garren *et al.*, 1971). The steroidogenic response is blocked by inhibitors of mRNA translation (cycloheximide and puromycin) (Garren *et al.*, 1965; Ferguson, 1963), but not by actinomycin D, which inhibits the transcription of DNA to mRNA (Garren *et al.*, 1965). *In vivo*, when cycloheximide is administered during ACTH stimulation the steroid response decays with a half-life of approx. 8 min, indicating that a protein with a short half-life may be essential for steroidogenesis.

Submaximal concentrations of cycloheximide produce equal percentage falls in steroid output and protein synthesis (Schulster *et al.*, 1972), which suggests that the inhibition is specific and that the amount of the protein is rate determining.

It is not clear whether cyclic AMP and ACTH act by inducing the translation of mRNA to yield a labile protein or whether a protein is continually synthesized which must be present for the response to develop.

Several workers (Grahame-Smith *et al.*, 1967; Schulster *et al.*, 1972) have reported a 3 min time-lag between the addition of ACTH and the onset of steroidogenesis. It has been suggested that this might

* Abbreviation: ACTH, adrenocorticotrophin.

be the time taken for synthesis of a protein in response to ACTH, and there is some evidence (Schulster *et al.*, 1972) that the time-lag may increase in the presence of submaximal doses of cycloheximide, which would be consistent with this hypothesis.

Thus kinetic studies of steroid output have played a crucial role in the development of our understanding of the mechanism of action of ACTH. However, the techniques that have been used to date, suffer from certain drawbacks and sometimes produce conflicting evidence.

For instance, although it is possible to collect fractions frequently from superfused adrenals and to regulate the application of test substances rapidly and accurately, the tissue only responds to non-physiological concentrations of ACTH. In addition, when ACTH is washed out or when the response is blocked with cycloheximide the decay rate is much slower than that observed *in vivo*. It seems likely that diffusion artifacts have been introduced, a fact that also casts doubt on values obtained for time-lags for the onset of the response.

In vivo it is more difficult to control the concentrations of test substance reaching the adrenal, and higher plasma background concentrations prevent accurate measurement of the low concentrations of steroids released at the very start of the response.

To investigate the dynamics of the steroidogenic response thoroughly we have developed a method of perfusing isolated adrenal cells supported in a small bed of an inert packing material.

The cells respond rapidly to specific stimuli and the system can therefore be used to study the time-course of the response to cyclic AMP and ACTH accurately.

Materials and Methods

Materials

[[$^3H_2$]Tyr^{23}]Corticotrophin-(1–24)-tetracosapeptide (46Ci/mmol) was synthesized in these laboratories (Brundish & Wade, 1973). This peptide had full biological activity. The following synthetic peptides were kindly supplied by Dr. W. Rittel and Dr. B. Riniker, CIBA-GEIGY, Basle: human corticotrophin (revised sequence, Riniker *et al.*, 1973), corticotrophin-(1–24)-tetracosapeptide (SYNACTHEN) and [D-Ser^1, Lys^{17,18}]corticotrophin-(1–18)-octadecapeptide amide. Bovine serum albumin (crystallized), collagenase (type 1), cyclic AMP and cycloheximide were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Lima-bean trypsin inhibitor and trypsin were from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Cristamycin was from Glaxo Laboratories Ltd., Greenford, Middx., U.K. Nylon gauze (100μm mesh for straining cells, 20μm mesh for cell column) was from Swiss Nybolt, Zurich, Switzerland, Portex Plastic pp25 polythene cannula were from Portland Plastics Ltd., Hythe, Kent, U.K., the 2ml Gillette Scimitar syringe was from Gillette Surgical, Isleworth, Middx., U.K., and aminoglutethimide was from CIBA Laboratories, Horsham, Sussex, U.K.

Preparation of the cell column

Isolated rat adrenal cells (from male Wistar rats, 300g) were prepared from 10–16 adrenals by treatment with trypsin and mechanical dispersal in a manner similar to that of Sayers *et al.* (1971), which has been described in detail elsewhere (Lowry *et al.*, 1973). The cells were handled entirely with plastic apparatus. They were resuspended after the second spin in 1ml of Krebs–Ringer bicarbonate buffer containing 5mg of albumin/ml, 0.5mg of lima-bean trypsin inhibitor/ml, 25munits of penicillin/ml, 25μg of streptomycin/ml and 0.2% glucose. This buffer was used throughout as perfusion medium. After straining through nylon gauze (pore size 100μm), the cells were mixed with 0.5g of moist Bio-Gel P-2 (200–400 mesh), which had been swollen in 0.9% NaCl and washed in the medium. The slurry was then drawn into the 2ml syringe, which served as a column as shown in Fig. 1. The syringe was immediately clamped vertically, nozzle uppermost, connected to the pump and the cells and Bio-Gel were packed down under a flow of 1ml of perfusion medium/min. After 2 or 3 min the syringe was disconnected and the plunger pushed in to raise the bed to a point when only a small volume (0.1–0.2ml) of liquid lay between it and the syringe nozzle. The syringe was then reconnected and placed in a water bath at 37°C as shown in Fig. 1. Before test substances were applied the column was treated with a short pulse (1ng/ml for 6s) of the corticotrophin analogues to be used in the experiments and then allowed to settle for 1h. The perfusion medium was equilibrated at 37°C for 1h before use, but even so gas bubbles tended to form on top of the column unless the degassing arrangement shown in Fig. 1 was used.

Steroid determination and the timing of pulses

During a perfusion experiment 1min fractions (1ml) were collected. These were assayed fluorimetrically for 11-hydroxycorticosteroids as described previously (Lowry *et al.*, 1973) by using a variant of the H_2SO_4–ethanol procedure (Silber *et al.*, 1958).

The precise time-lag of the system and the profile of test pulses was determined by applying samples of cyclic [^{3}H]AMP, [[$^3H_2$]Tyr^{23}]corticotrophin-(1–24)-tetracosapeptide or corticosterone and assaying the eluate by liquid-scintillation counting (Bennett *et al.*, 1974) or fluorimetry. Identical profiles were obtained with the different substances, the concentration reaching 50% of its maximum value in 2min, indicating a dead volume of about 2ml.

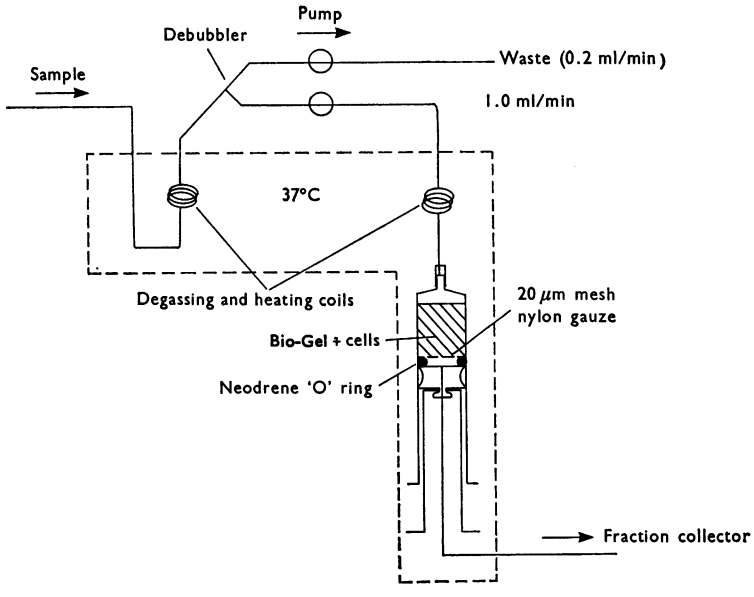

Fig. 1. *Column-perfusion apparatus*

The degassing and heating coil were made of pp25 polythene cannula. Air bubbles formed in the first heating coil pass into the upper arm of the debubbling junction and are pumped to waste.

Results

Response to ACTH

As shown in Fig. 2 the response to an 8 min pulse of ACTH rose over a period of several minutes and then flattened before returning to basal values. The 16 min pulse of corticotrophin did not produce a significantly greater maximum rate of steroid production, although it produced a longer plateau. Shorter pulses produced responses which did not reach a plateau. An 8 min pulse time was therefore used as the minimum time required for a given concentration of peptide to produce its full effect. Fig. 3 shows results obtained with different concentrations of corticotrophin-(1–24)-tetracosapeptide. The response was very dependent on concentration in the 5–10 pg/ml range, but did not increase so much when the concentration was raised further to 20 pg/ml.

Time-course of the response to different corticotrophin analogues

Fig. 4 show results obtained with three corticotrophin analogues. There were no significant differences in the rate of onset or decay of the responses.

Comparison of the time-courses of responses to cyclic AMP and corticotrophin-(1–24)-tetracosapeptide

To compare the effects of cyclic AMP and ACTH the results of several experiments were combined for statistical analysis. Because the outputs varied from day to day the results from each experiment were corrected by subtraction of the base-line secretion rate and were then normalized by multiplying by a factor so that the peak value of the response was 1 (i.e. at 10 min for ACTH, at 6 min for cyclic AMP). The mean and standard error of the responses at different times were then calculated and in this way a measure of the variability of the shape of the response was obtained.

The results are shown in Fig. 5. Zero time is taken as the time when the pulse of test substance reached 50% of its plateau value. There was a large difference between responses to 5 pg of corticotrophin-(1–24)-tetracosapeptide/ml and 0.5 mg of cyclic AMP/ml, both of which are submaximal.

With cyclic AMP the response rose and fell much more rapidly and although no values were obtained at 1 min the value of 0.55 obtained at 2 min suggests that the response must have started to build up rapidly as soon as the cyclic AMP was applied. With ACTH at the low concentration there was a small but significant response at 2 min, but the rate of increase of the output was not maximum until 5 min, at which point the output had reached 50% of its maximum rate. Although the response to the supramaximal dose of ACTH was slightly more rapid, this is due to the fact that only 5% of the final concentration is required to produce maximal stimulation. This occurs appreciably earlier (0.8 min) than the 50% value and

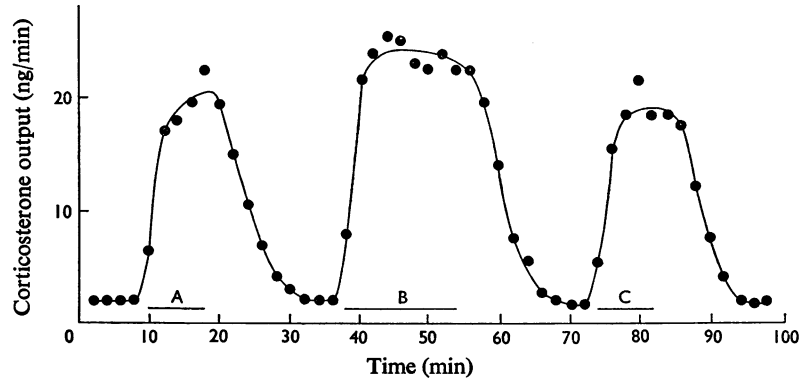

Fig. 2. *Steroid output from column-perfused adrenal cells after infusion (shown by the black bars) of* 10 *pg of corticotrophin-(1–24)-tetracosapeptide/ml*

Two 8 min and one 16 min pulses are shown. The position of the bars is adjusted for the time-lag due to the dead volume of the system. The flow rate was 1 ml/min and 1 min fractions were collected.

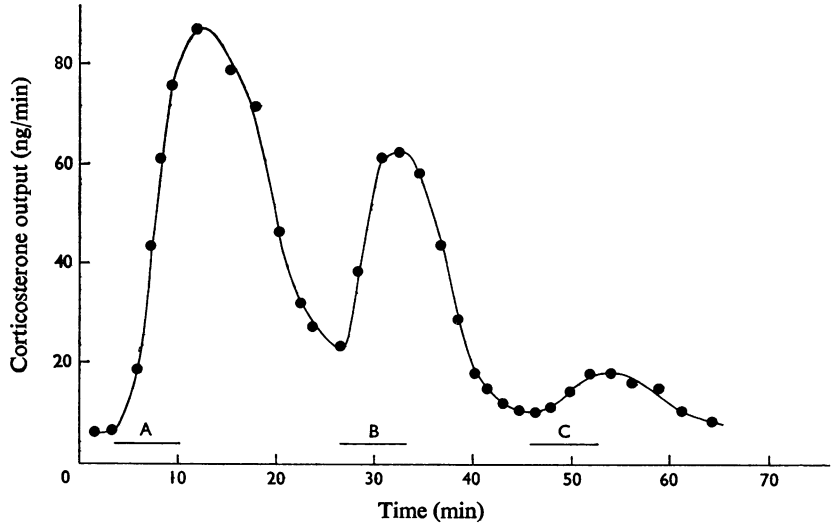

Fig. 3. *Response of column-perfused adrenal cells to different concentrations of corticotrophin-(1–24)-tetracosapeptide*

Black bars show when cells are exposed to the pulse of ACTH: A, 20 pg/ml; B, 10 pg/ml; C, 5 pg/ml. An 8 min pulse of each concentration was applied followed by a 22 min wash-out period.

fully accounts for the slight difference in the time-courses.

Effects of inhibitors and conversion of pregnenolone into corticosterone

The effects of cycloheximide and aminoglutethimide were tested during a prolonged pulse of corticotrophin as shown in Fig. 6. The onset and offset of the effect of aminoglutethimide was extremely rapid. Although the system started to respond to application or removal of cycloheximide rapidly the overall response takes some time to reach its final value.

The response to pregnenolone is also shown in Fig. 6. The response developed rapidly in the course of 1–2 min to a final value which was 3 times the

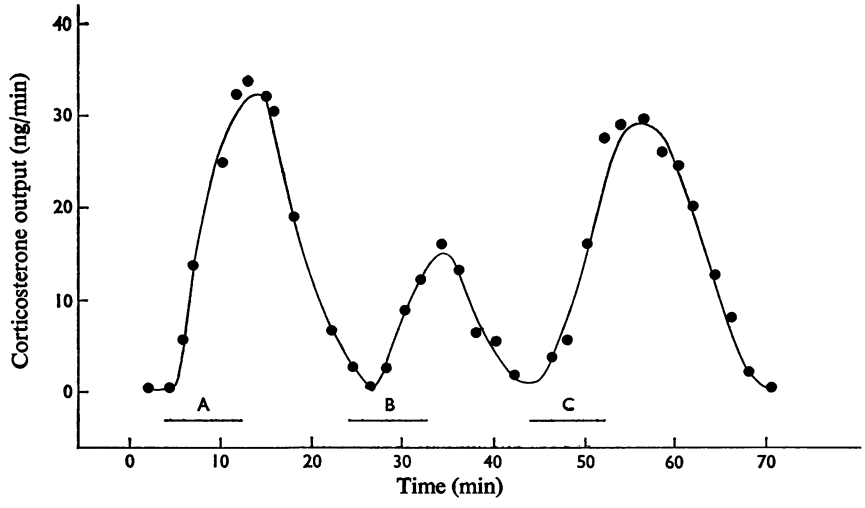

Fig. 4. *Profiles of steroid response of column-perfused cells caused by pulses of corticotrophin analogues*

A, [D-Ser[1],Lys[17,18]]Corticotrophin-(1–18)-octadecapeptide amide (50pg/ml). B, Synthetic human corticotrophin (100pg/ml). C, Corticotrophin-(1–24)-tetracosapeptide (20pg/ml). An 8min pulse of each peptide was applied and fractions were collected at 1min intervals.

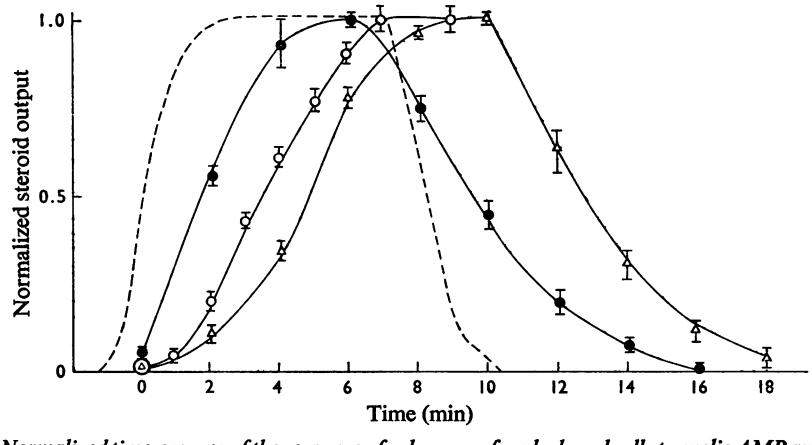

Fig. 5. *Normalized time-courses of the response of column-perfused adrenal cells to cyclic AMP and ACTH*

The broken line represents the profile of an 8min test pulse as it emerges from the column (i.e. [^{3}H]corticotrophin-(1–24)-tetracosapeptide, cyclic [^{3}H]AMP or corticosterone). Zero time is taken as the time when the applied pulse reached 50% of its plateau value. Three average responses are shown with standard errors ($n = 5$, except for 1ng of corticotrophin-(1–24)-tetracosapeptide/ml, where $n = 3$). The method for normalizing, statistical averaging and calculation of standard errors is described in the text. ●, 0.5mg of cyclic AMP/ml; ○, 1ng of corticotrophin-(1–24)-tetracosapeptide/ml; △, 5pg of corticotrophin-(1–24)-tetracosapeptide/ml. Maximum output for each curve taken as 1 unit.

maximal output obtained with corticotrophin stimulation.

Response of collagenase-prepared cells

Cells were prepared in an identical fashion with the trypsin-dispersed cells except that the 0.25% trypsin solution was replaced by 0.1% collagenase. The cells were sensitive to corticotrophin-(1–24)-tetracosapeptide, having an ED^{50} (dose producing 50% of the maximal response) of about 10pg/ml (a value comparable with that of trypsin-dispersed cells). The response profile of column-perfused cells to ACTH was similar to that obtained with the trypsin-treated cells.

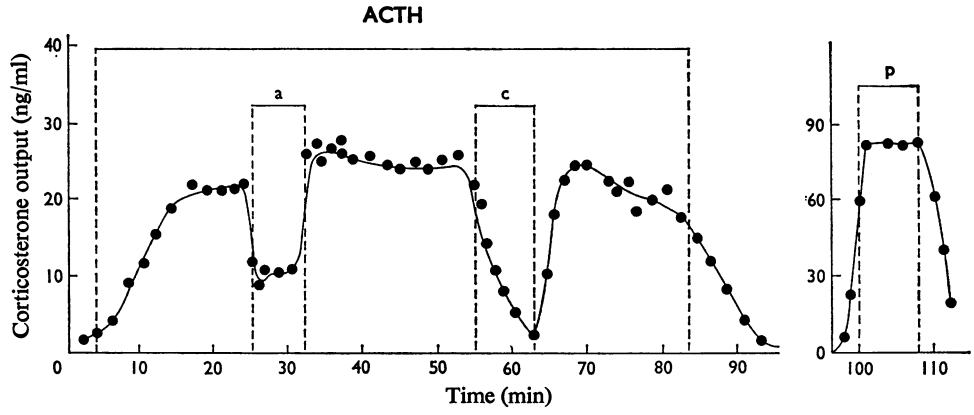

Fig. 6. *Effect of aminoglutethimide* (*a*) (3 μg/ml) *and cycloheximide* (*c*) (3 μg/ml) *applied during continuous human* (1–39) *ACTH stimulation* (500 *pg/ml*)

Broken lines show time of application and withdrawal of test substances adjusted for time-lag. The response to a pulse of pregnenolone (p) (9 μg/ml) is also shown.

Discussion

The speed of response of ACTH-stimulated column-perfused adrenal cells to addition and removal of the inhibitor of steroid biosynthesis, aminoglutethimide, demonstrates that in this perfusion system products can enter and leave the cell rapidly, and it is therefore ideal for a study of the kinetics of activation and cessation of steroidogenesis. The cells continue to respond for at least 3h and the small variations in the normalized curves of Fig. 5 show that although the steroid output varied from day to day the dynamic characteristics of the cells were remarkably constant.

The cells are sensitive to physiological concentrations of ACTH and three different ACTH analogues have identical time-courses for activation and relaxation of the steroid response. One of these, [D-Ser[1],Lys[17,18]]corticotrophin-(1–18)-octadecapeptide amide, causes a prolonged response *in vivo* (Maier *et al.*, 1971). In view of its reversible action on isolated cells it seems almost certain that the prolonged effect *in vivo* must reflect its fate in the whole animal.

The time-courses of the onset and delay of the response to corticotrophins are similar to those reported for perfused sheep adrenal transplants *in vivo* by Beaven *et al.* (1964). Other workers have reported a slightly longer delay time for the onset of steroidogenesis (3 min) *in vivo* (Garren *et al.*, 1965) and *in vitro* by using superfused rat adrenals (Schulster *et al.*, 1970; Pearlmutter *et al.*, 1973) or isolated rat adrenal cells incubated in batches for different periods of time (Mackie *et al.*, 1972).

Much larger discrepancies appear in the delay time of the response when ACTH is washed out or when steroidogenesis is inhibited with cycloheximide during ACTH stimulation. Inhibition by cycloheximide in rats *in vivo* caused the steroid output to fall with a half-life of about 8 min (Garren *et al.*, 1965). *In vitro* with superfused adrenals (Schulster *et al.*, 1970) the equivalent value was 45 min and in the same system in the absence of cycloheximide the response fell with a half-life of about 100 min when ACTH was withdrawn. In a recent cell-superfusion procedure (Schulster, 1973) the steroid output also took a long time to return to basal values (greater than 60 min) after brief ACTH stimulation.

In contrast a half-life of 2–3 min was observed for column-perfused cells inhibited by cycloheximide during ACTH stimulation and the response fell with a similar half-life after a delay of 1–2 min after cessation of ACTH stimulation. These shorter times observed with column-perfused cells are probably due to the very efficient exchange between cells and medium referred to above.

The kinetics of responses to stimulants and inhibitors fall into three categories summarized in Fig. 7.

(1) Rapid. The response of ACTH-stimulated cells to aminoglutethimide is over within 1 min of applying or withdrawing the compound.

(2) Hyperbolic. The response starts to build up or decline almost immediately but takes some time to reach its final value (50% is reached within about 2 min). This type of response was shown by cyclic AMP and by cycloheximide administered during ACTH infusion.

(3) A delayed response. The response does not start for about 1 min and the maximum rate of change in the response occurs after 4–5 min. This type of response was shown on addition and withdrawal of corticotrophins.

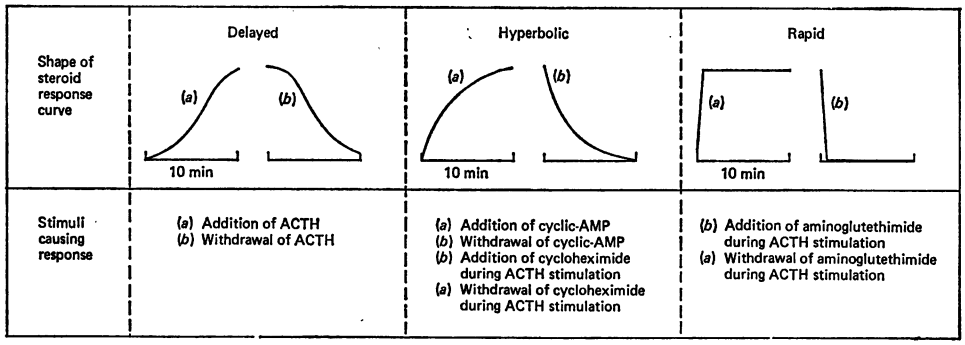

Fig. 7. *Three kinetic types of response observed with column-perfused adrenal cells and the stimuli responsible for each type*

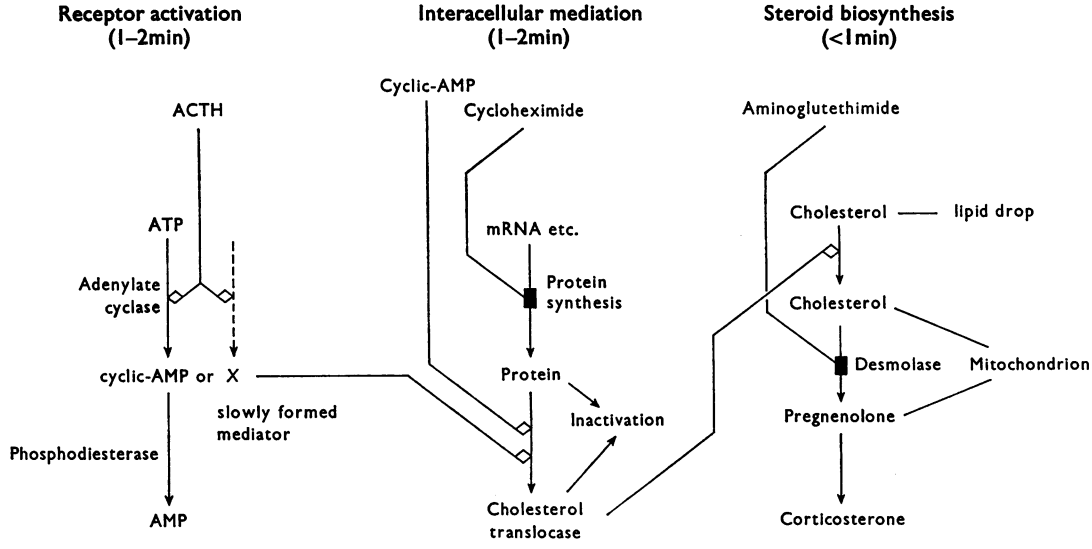

Fig. 8. *Hypothetical representation of the three different stages in the activation of steroidogenesis showing the points at which various stimulants and inhibitors act*

——◇, Stimulation; ——■, inhibition.

There is a striking symmetry in the onset and decay of the response to any particular stimulant or inhibitor.

These findings enable us to delineate three causally linked stages in the development of the steroidogenic response to corticotrophins as illustrated in Fig. 8.

The rapid response to aminoglutethimide, which inhibits mitochondrial conversion of cholesterol into pregnenolone (Cash *et al.*, 1967), shows that there is no accumulation of steroid intermediates after this step, so that time-delays of the slower responses must be a true reflexion of the rates of processes occurring before side-chain cleavage of cholesterol. Because ACTH and cycloheximide do not alter the rate of conversion of exogenous pregnenolone into corticosterone (Garren *et al.*, 1965) or the total capacity of the desmolase enzyme system in Ca^{2+}-treated mitochondria to convert cholesterol into pregnenolone (Koritz & Kumar, 1970), it seems likely that they affect the rate of transfer to the mitochondrion. The situation is in fact more complicated than that depicted in Fig. 8 because the bulk of the cholesterol in the lipid droplets is esterified, and although ACTH alone results in a decrease of cholesterol ester content with free cholesterol concentrations remaining almost unchanged, ACTH in combination with

cycloheximide causes an increase in free cholesterol corresponding to a decrease in esterified sterol.

Turning next to the events immediately preceding the conversion of lipid-droplet cholesterol into pregnenolone, the rapid onset of the action of cycloheximide shows that a short-lived protein must be closely involved in the regulation of steroid synthesis. Since cyclic AMP also appears to accelerate steroid production as soon as it is added it does not seem likely that it can be initiating protein synthesis, and indeed it has not been demonstrated that ACTH causes a significant increase in the rate of protein synthesis in the adrenal. A tentative suggestion shown in Fig. 8 is that cyclic AMP may cause the conversion of a labile precursor into an active but unstable product, 'cholesterol translocase'.

The kinetics of the decay after withdrawal of cyclic AMP or the addition of cycloheximide indicate that the half-lives of these protein components must be of the order of 2min, a value much shorter than that suggested by earlier studies (8min; Garren *et al.*, 1965).

One of the surprising results to emerge from our investigation is the difference in the time-course of responses to ACTH and cyclic AMP. The delayed response to ACTH suggests that if ACTH is acting via cyclic AMP there must be a time-delay in its production. This explanation seems unlikely, first because large doses of ACTH should rapidly produce excess amounts of cyclic AMP and secondly because in adrenal quarters it has been shown that ACTH does cause a rapid rise in cyclic AMP concentrations (Grahame-Smith *et al.*, 1967). We consider that these results suggest that there may be a second mediator for ACTH, and this possibility has been indicated in Fig. 8.

Column-perfused adrenal cells have been shown to provide clear-cut information on the kinetics of changes in steroidogenic responses obtained with a variety of stimuli. It is hoped that this technique will prove useful for similar studies on different cell types and test systems.

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