An Investigation of the Involvement of Adenosine 3':5'-Cyclic Monophosphate in Steroidogenesis by Using Isolated Adrenal Cell Column Perfusion

By ANNE M. HUDSON and COLIN McMARTIN
Research Centre, CIBA Laboratories, Horsham, Sussex RH12 4AB, U.K.

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The involvement of cyclic AMP in corticosteroidogenesis was investigated by using isolated adrenal cell column perfusion. Steroids were produced in response to 0.5, 1.0 and 5.0 mg of cyclic AMP/ml. Analysis of the shape of the response curves indicated an inverse relationship between rate of onset of steroid production and dose. A further increase in steroid production during the washout period after the 5 mg/ml dose was considered to indicate an intracellular inhibitory effect of cyclic AMP. Release of cyclic AMP into the perfusate only occurred in response to supramaximal steroidogenic doses of ACTH (adrenocorticotrophin). A connexion between dose and response was demonstrated over a narrow concentration range. Variation in the time-lag before cyclic AMP production and in the duration of the response was marked; further, no reproducible ratio of steroid output to cyclic AMP output was shown at any level of stimulation. These results are discussed together with those of other recent investigations. It is considered that these findings do not support an obligatory role for cyclic AMP as mediator of ACTH action in the adrenal.

Cyclic AMP has been implicated as the intracellular mediator of the steroidogenic effects of ACTH* *in vivo* and *in vitro* (Garren *et al.*, 1971). Evidence in favour of this hypothesis is provided by the observations that added cyclic AMP can stimulate steroid production in adrenal sections (Haynes *et al.*, 1959) and in isolated adrenal cells (Kitabchi & Sharma, 1971) *in vitro*, and in perfused adrenals *in vivo* (Imura *et al.*, 1965). Production of cyclic AMP after ACTH stimulation of intact adrenals *in vivo* and adrenal tissue *in vitro* has also been demonstrated (Grahame-Smith *et al.*, 1967) and after ACTH stimulation of isolated adrenal cells (Mackie & Schulster, 1973). However, this evidence is not completely conclusive, since large amounts of cyclic AMP must be used to elicit steroid production and in addition substantial increases in cellular cyclic AMP concentrations only occur when concentrations of ACTH are used which are supramaximal with respect to the steroid response.

It has not been possible to demonstrate steroidogenic effects of cyclic AMP at the subcellular level (Kahnt *et al.*, 1974) and knowledge of the role of cyclic AMP therefore depends on the use of intact cells or tissue.

One method of obtaining more information about the involvement of cyclic AMP by using intact cells or tissue is to study the time-course of the steroid response and of cyclic AMP formation.

Isolated rat adrenal cell column perfusion (Lowry & McMartin, 1974) affords an ideal system for investigating the dynamics of the steroid response. It combines the advantages of an isolated cell system, overcoming the problems of diffusion of stimulants and products associated with the use of tissue sections, with that of sensitive measurements at time-intervals shorter than those that are practicable with batch incubations of cells. In addition, the cell-column system has been demonstrated to respond reproducibly to concentrations of ACTH (50 pg/ml) in the physiological range.

Preliminary experiments of Lowry & McMartin (1974) with cyclic AMP, using the cell column system, have shown a shorter time-lag for onset of production of corticosterone, after stimulation of the cells by cyclic AMP, than after ACTH stimulation. It was decided to pursue this observation with measurements of steroid production after stimulation by cyclic AMP, and also to measure cyclic AMP output from the cells after ACTH stimulation.

Materials and Methods

Materials

Bovine serum albumin (fraction V, crystalline), cyclic AMP, 6-N,2'-O-dibutyryl adenosine 3':5'-cyclic monophosphate, theophylline and 2-mercaptoethanol were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Lima-bean trypsin inhibitor

* Abbreviation: ACTH, adrenocorticotrophin.

and trypsin were from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Cristamycin (penicillin G, streptomycin) was from Glaxo Laboratories Ltd., Greenford, Middx., U.K. Cyclic [8-³H]AMP was from The Radiochemical Centre, Amersham, Bucks., U.K. Norit GSX charcoal was obtained from Norit Clydesdale Co. Ltd., Glasgow, U.K. Corticotrophin-(1-24)-tetracosapeptide was kindly supplied by Dr. W. Rittel and Dr. B. Riniker, CIBA-GEIGY, Basle, Switzerland.

Nylon gauze (100 μ m mesh for straining cells, 20 μ m mesh for cell column) was from Swiss Nybolt, Zurich, Switzerland. Portex plastic pp25 polythene cannula was from Portland Plastics Ltd., Hythe, Kent, U.K., and the 2ml Gillette scimitar syringe was from Gillette Surgical, Isleworth, Middx., U.K. Conical glass centrifuge tubes were from MSE, London S.W.1, U.K.

Methods

Preparation of the cell column. Isolated rat adrenal cells were prepared by trypsin disaggregation of the adrenals of ten male Wistar albino rats (300g approx.) as described by Lowry *et al.* (1973). The isolated cells were washed and resuspended in perfusion medium (Krebs-Ringer bicarbonate buffer containing 5mg of albumin/ml, 0.5mg of lima-bean trypsin inhibitor/ml, 25 i.u. of penicillin G/ml, 25 μ g of streptomycin/ml and 0.2% glucose). To this suspension was added 0.5g of P2 Bio-Gel beads (200-400 mesh) and the slurry thus obtained was packed into the column (Fig. 1). This procedure has been described

by Lowry & McMartin (1974) and the apparatus and method used here represents a slight modification of this original method. The piston at the top of the column was removed and the slurry poured into the column and then left to bed down under perfusion (1 ml/min) with the Krebs-Ringer medium. Once the packing was complete the inflow tubing was disconnected and the upper piston lowered to decrease the volume of the medium above the column. Perfusion was then continued and the column lowered into the 37°C water jacket as shown. The cell column was then primed with a short pulse (1 ng/ml for 6s) of corticotrophin-(1-24)-tetracosapeptide and the cells were allowed to recover during continuous perfusion for 1 h.

Test substances could be applied to the column in the same manner as the perfusion medium, and fractions of perfusate were collected at 1 min intervals in a fraction collector. Pulses (8 min) of stimulants were applied and fractions were collected over a total of 20 or 30 min.

In these experiments the ACTH analogue corticotrophin-(1-24)-tetracosapeptide was used. This compound has been demonstrated to be more potent in the isolated-cell system (Lowry *et al.*, 1973) but has the same overall shape of response curve in the cell column (Lowry & McMartin, 1974). Seelig & Sayers (1973) have shown that shortening the corticotrophin chain from 1-39 to 1-16 amides does not affect the shape of the dose-response curve of adrenal cell adenylate cyclase.

Fluorimetric steroid assay. 11-Hydroxy corticosteroids in the perfusate were assayed by a modification of the H₂SO₄-ethanol procedure (Lowry *et al.*, 1973).

This assay was also used to determine the time-lag and test profile of the system by applying a test pulse of 200 ng of corticosterone/ml to the column and assaying the perfusate. The profile thus obtained was found to be identical with that obtained with cyclic [³H]AMP application and liquid-scintillation counting of the perfusate (see below).

Cyclic AMP assay. A modification of the method of Brown *et al.* (1971) was used. Cyclic AMP-binding protein from bovine adrenal cortex was prepared and diluted to the required strength. Extraction of the perfusate was carried out by a method based on that of Albano *et al.* (1974). A portion (0.5 ml) of each perfusate fraction was taken for assay and to this was added 1 ml of ethanol to precipitate the protein. After centrifugation the resulting supernatant was dried in a vacuum desiccator and redissolved in 0.2 ml of assay buffer (50 mM-Tris-HCl buffer, pH 7.4, containing 8 mM-theophylline and 6 mM-2-mercaptoethanol). This final solution was assayed for cyclic AMP. The following were mixed and incubated for 1.5 h at 4°C: 100 μ l of assay buffer; 100 μ l (approx. 16 nCi) of

Fig. 1. Cell-column-perfusion apparatus

For details see the text.

stock cyclic [^3H]AMP solution; 100 μl of binding-protein solution; 50 μl of the cyclic AMP solution to be determined, or 50 μl of standard solution (for the standard curve). After incubation 100 μl of charcoal suspension (10% in aq. 2%, w/v, solution of bovine serum albumin) was added to each tube, mixed and immediately centrifuged. Free cyclic AMP was adsorbed to the charcoal and was precipitated; bound cyclic AMP was estimated by liquid-scintillation counting of the supernatant. An estimate of total radioactivity was made by counting without addition of charcoal and percentage bound was calculated for each tube. A standard curve was plotted for the range 0–100 ng of cyclic AMP/ml.

Radioactivity counting. Radioactivity was measured in a Packard Tri-Carb model 3003 liquid-scintillation counting system. Up to 0.5 ml aqueous samples were added to vials containing 15 ml of scintillator [containing 30 g of 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)-thiophen and 340 g of naphthalene in a mixture of 2.5 litres of toluene and 1.7 litres of methylCello-solve].

Results

Stimulatory effect of cyclic AMP. Three doses of cyclic AMP were used, 0.5, 1.0 and 5.0 mg/ml, and these were compared with the stimulatory effect of 20 pg of corticotrophin-(1–24)-tetracosapeptide/ml, a dose that normally gave about 70% of the maximum steroid response.

Fig. 2 shows that the output of steroid is related to the dose of cyclic AMP. For comparison of the results obtained on different days (when steroid output may vary considerably according to the number of cells packed on the column, among other factors), each curve was normalized by multiplying the results by a factor so that the value of the response at 8 min was 1. Thus it was possible to combine, and evaluate statistically, results from several experiments (Fig. 3).

This procedure also allowed the shapes of the response curves for different cyclic AMP concentrations to be compared. If the responses gave the same kinetic pattern at each dose, the normalized curves would be expected to overlaid each other. This is clearly not the case; a lower rate of onset of steroid production is observed with higher doses of cyclic AMP, although all doses tested gave a more rapid response than corticotrophin-(1–24)-tetracosapeptide. At 5 mg of cyclic AMP/ml, steroid output continued increasing after the applied cyclic AMP was washed out. This effect was not observed at any other dose or with corticotrophin-(1–24)-tetracosapeptide. Fig. 4 shows a comparison of this effect with the response to a continuous infusion for 20 min of 5 mg of cyclic AMP/ml, which maintained a plateau value from 8 min onwards. Addition of

Fig. 2. Steroid output from the column after stimulation with cyclic AMP and corticotrophin-(1–24)-tetracosapeptide

Results of two typical experiments are shown. Hatched area represents the profile of an 8 min test pulse of corticosterone as it emerges from the column. ●, 0.05 mg of cyclic AMP/ml; ▼, 1.0 mg of cyclic AMP/ml; ◆, 5.0 mg of cyclic AMP/ml; □, 20 pg of corticotrophin-(1–24)-tetracosapeptide/ml.

aminogluthethimide (3 $\mu\text{g}/\text{ml}$) during the first 10 min of the washing-out period resulted in steroid output immediately being decreased to about 20% of its value at 8 min. It remained at this concentration during the pulse, then rose again rapidly as aminogluthethimide was washed out. The shape of the steroid response before addition and after washing out aminogluthethimide was identical with that obtained in previous experiments in response to 5 mg of cyclic AMP/ml.

Stimulatory effect of dibutyryl cyclic AMP

Dibutyryl cyclic AMP is known to stimulate steroidogenesis at a lower concentration than cyclic AMP. At 10 $\mu\text{g}/\text{ml}$ no stimulation was detected, but 50 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$ had a marked effect. On

Fig. 3. Normalized time-courses of the responses to cyclic AMP and corticotrophin-(1-24)-tetracosapeptide

The hatched area represents the test profile. The procedure for normalizing is described in the text. Three responses to cyclic AMP are shown, $\pm$ s.e. ($n=4$). $\bullet$, 0.5mg of cyclic AMP/ml; $\blacktriangledown$, 1mg of cyclic AMP/ml; $\blacklozenge$, 5mg of cyclic AMP/ml. s.e. values are not shown for 20 pg of corticotrophin-(1-24)-tetracosapeptide ($\square$), but each point represents the mean of four values.

Fig. 4. Comparison of response to single 8min pulse of cyclic AMP with that to 20min infusion of same concentration

The hatched area represents the test profile. Responses $\pm$ s.e. are shown, for 5mg of cyclic AMP/ml ($\blacklozenge$, 8min pulse, $n=4$; $\diamond$, 20min pulse, $n=3$).

normalizing the results obtained at 100 μ g/ml, a curve was obtained which lay almost exactly on top of the curve for 20pg of corticotrophin-(1-24)-tetracosapeptide/ml. The results at 50 μ g/ml were not statistically evaluated, but appeared very similar.

Production of cyclic AMP after stimulation with corticotrophin-(1-24)-tetracosapeptide

High doses of corticotrophin-(1-24)-tetracosapeptide were required for cyclic AMP output into

Fig. 5. Cyclic AMP and steroid output from the column in response to various doses of corticotrophin-(1-24)-tetracosapeptide

—, Steroid output; ----, cyclic AMP output. Pulses of corticotrophin-(1-24)-tetracosapeptide (ACTH) for 8min were given at lines marked (1) and (2). (a) 100 pg (1) and 1ng (2) of ACTH/ml given; (b) 200 pg (1) and 300 pg (2) of ACTH/ml given.

the perfusate and all doses used were supramaximal for steroidogenesis. Fig. 5 gives an indication of the type of dose-response relationship that we have been able to demonstrate.

The difference in maximum steroid output in the two groups is probably due to different numbers of viable cells on the column in the two experiments. Other experiments have shown that maximum cyclic AMP outputs are achieved at concentrations between 500pg and 1 ng of corticotrophin-(1-24)-tetracosapeptide/ml. The ratio of cyclic AMP output to steroid output at 1 ng/ml of corticotrophin-(1-24)-tetracosapeptide was found to vary considerably [1.002 ± 0.499 (s.d., $n=8$) ng of cyclic AMP/min/ng of corticosterone/min].

Investigation of the dose-response relationship has been further complicated by an apparent tachyphylactic effect observed with repeated doses of 500pg of corticotrophin-(1-24)-tetracosapeptide/ml or higher.

Fig. 6. Tachyphylactic effect with repeated stimulation

—, Steroid output; — — —, cyclic AMP output. Pulses of corticotrophin-(1-24)-tetracosapeptide were given for 8 min at points (1) (500 pg/ml) and (2) (1 ng/ml).

Steroid output remained unchanged throughout the experiment, but cyclic AMP output was lower after a second stimulation and there was a lack of stabilization to a steady plateau value (Fig. 6).

A considerable variation in the time-lag (from 0-4 min) before appearance of cyclic AMP in the perfusate has also been observed with the same stimulatory doses of corticotrophin-(1-24)-tetracosapeptide (Hudson & McMartin, 1974). This effect was not seen with steroid production where a regular time-lag of 1-2 min was observed.

Inclusion of theophylline (5 mM) in the perfusion medium, to inhibit any phosphodiesterase activity which might have been contributing to the variability of the results, was not found to enhance cyclic AMP output and in one experiment it even appeared to inhibit output.

Discussion

The stimulatory effect of exogenous cyclic AMP on steroidogenesis is one of the main pieces of evidence cited in favour of cyclic AMP as mediator of ACTH action. Thus it was felt that a detailed investigation of the kinetics of this effect might yield new information.

Concentrations of cyclic AMP required to stimulate steroidogenesis in the cell-column system are similar to those required in batch incubations of isolated adrenal cells and superfused adrenal quarters. The concentrations are high, and this is generally attributed to difficulties in transportation across the cell membrane, but no investigation has been able to demonstrate the extent to which intact cyclic AMP enters the cell. In contrast dibutyryl cyclic AMP is active in the cell-column system at about one-tenth of the concentration of cyclic AMP, and this may be due to a greater rate of diffusion into the cell or

to the resistance of the dibutyryl derivative to hydrolysis by adrenal phosphodiesterase.

It is evident from the normalized results in Fig. 3 that the overall shape of the response to different concentrations of cyclic AMP is highly dependent on the concentration of cyclic AMP used. In this respect the kinetics of the response differ from those observed with ACTH stimulation, where the shape of the normalized curve does not depend on the concentration of ACTH (Lowry & McMartin, 1974). The results obtained with 0.5 mg/ml confirm the previous observation of a more rapid rate of onset of steroid production; however, this effect is less marked at 1 mg of cyclic AMP/ml, and even less so at 5 mg/ml, where the difference from ACTH stimulation is very marginal. In addition it can be seen that at the latter concentration steroid production continues to increase during the washout period, and the curve is steeper at this point than during initial stimulation. This is in contrast with the effect of continued stimulation with a long (20 min) infusion of 5 mg of cyclic AMP/ml, where steroid production reaches a plateau value by 8 min, as is observed with shorter pulses of the two lower concentrations.

Aminogluthethimide is an inhibitor of steroid biosynthesis and the speed of response to addition and removal of this substance indicates that steroids produced after cyclic AMP stimulation can pass easily out of the cells into the perfusate. This has already been shown after ACTH stimulation in the perfusion system (Lowry & McMartin, 1974). The present findings provide clear evidence that any inhibition of steroid output from the cells by the high dose of cyclic AMP is not due to interference with transport. Thus it appears that any inhibitory effect must be occurring at an intracellular location.

It has been shown that cyclic AMP concentrations in cells may rise to a concentration as much as 10 times in excess of those at which maximum steroidogenesis is occurring (Seelig & Sayers, 1973; Mackie *et al.*, 1972). However, at least 0.5 mg of cyclic AMP/ml is required to elicit perceptible steroid production by the cells, and 5 mg/ml is obviously not very much (if at all) above that required for maximum stimulation, yet appears to be having an inhibitory effect on the cells. The discrepancy between these findings is difficult to reconcile with a theory of ACTH action in which cyclic AMP has an obligatory role. It is, of course, possible that exogenous cyclic AMP enters a different pool within the cell from that of endogenously generated cyclic AMP. However, as the latter is believed to be formed on the inner surface of the cell membrane, it is unlikely that endogenous and exogenous cyclic AMP have different sites of action.

Interpretation of changes in cyclic AMP production or concentration, in response to ACTH, is complicated by similar considerations. It might be

expected that intracellular cyclic AMP concentrations would correlate well with the steroidogenic effects of ACTH. However, Albano & Brown (1974) have shown that the elevation of intracellular cyclic AMP is transient, even in response to ACTH concentrations which would produce a prolonged steroid output. It is possible, however, that the rate of cyclic AMP output reflects diffusion from the cytoplasm, through the cell membrane, and therefore gives an indication of the free cytoplasmic concentration of cyclic AMP.

Recently, measurements have been reported of cyclic AMP and steroid output into the adrenal vein after ACTH stimulation of sheep adrenals *in vivo* (Espiner *et al.*, 1974). Cyclic AMP output could not be detected at submaximal steroidogenic doses of ACTH, but at very high doses a very rapid rise in adrenal venous cyclic AMP occurred before the corresponding rise in cortisol. At lower supramaximal doses of ACTH the time-lags for cortisol and cyclic AMP were longer and not distinct. With a second stimulation by ACTH the output of cyclic AMP was found to be lower even when a tenfold increase in ACTH concentration was used. This is similar to the tachyphylactic effect observed in the present experiments. Our experiments have also shown that output of cyclic AMP from the perfused cells only occurs at doses of corticotrophin-(1-24)-tetracosapeptide that are supramaximal for steroid production, and that the cyclic AMP output also differs from steroid production in the shape and duration of the response. This, together with the day-to-day variability (which is greater than the range of experimental error), casts further doubt on cyclic AMP as the mediator. Such discrepancies between cyclic AMP production and steroid production have been noted by other authors. Moyle *et al.* (1973) working with ACTH and its *o*-nitrophenylsulphenyl derivative have shown that although both of these elicit the same maximum steroid production in isolated rat adrenal cells, they have very different effects on cyclic AMP accumulation. Much less cyclic AMP was produced when the *o*-nitrophenylsulphenyl derivative of ACTH stimulated steroid production to 75% of the maximum concentration than when ACTH stimulated half-maximally.

Palfreyman & Schulster's (1975) results also weaken the argument for mediation by cyclic AMP. They investigated stimulation of steroidogenesis by cholera toxin which consistently caused production of cyclic AMP 20 min before increased steroidogenesis could be detected.

Thus it seems that current attempts to clarify the mechanism of action of ACTH are resulting in a growth of evidence undermining the implication of

cyclic AMP as mediator. It should be added, however, that as there is still no direct evidence, a mechanism could be proposed which includes cyclic AMP mediation. However, it would then be necessary to postulate rigid compartmentalization within the cell, so that cyclic AMP might be present and acting at high concentrations at discrete loci. This also implies that the measurements and effects of cyclic AMP which are recorded in the literature are in fact related to a separate compartment in the cell, and are thus not involved (directly at least) in the steroidogenic process. It seems that further investigation of the role, if any, of cyclic AMP must await the development of more refined techniques, or of a new approach.

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