Effects of ACTH (Corticotropin) Analogues on Steroidogenesis and Cyclic AMP in Rat Adrenocortical Cells

EVIDENCE FOR TWO DIFFERENT STEROIDOGENICALLY RESPONSIVE RECEPTORS

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Comparative studies on the mechanism of action of ACTH_{1–39} and ACTH_{5–24} [corticotropin-(1–39)- and corticotropin-(5–24)-peptides] on isolated rat adrenal cells were performed. The relationship between stimulated steroidogenesis and cyclic AMP was very different, suggesting that cyclic AMP does not play the same role in mediating the action of the two agonists. Data from three separate experiments showed that the competitive antagonist ACTH_{6–24} [corticotropin-(6–24)-peptide] had mean inhibitor constants of 13.4 ± 3.1 nM against ACTH_{1–39} and 3.4 ± 1.0 nM against ACTH_{5–24}, indicating that the steroidogenic actions of these two agonists are mediated by two different receptor types. We conclude that there are two possible mechanisms for corticotropin action, only one of which involves the necessary production of cyclic AMP.

The classical involvement of cyclic AMP (Graham-Smith *et al.*, 1967) in the mechanism of action of ACTH (corticotropin) has recently been questioned. Studies using perfused isolated cat adrenals showed that increases in tissue cyclic AMP alone were not sufficient to elicit steroid release (Carchman *et al.*, 1971). Hudson & McMartin (1975) investigated the kinetics of steroidogenesis and cyclic AMP accumulation in response to ACTH and concluded that their findings did not support an obligatory role for cyclic AMP as a mediator of ACTH action on the adrenal, a conclusion that has also been reached by other authors (Moyle *et al.*, 1973; Ramachandran & Moyle, 1977). In isolated adrenal cells increased cyclic AMP concentrations were evident in response to cholera toxin 20 min before increased steroidogenesis could be discerned (Palfreyman & Schulster, 1975), thus indicating that this initial cyclic AMP stimulation was not linked to steroidogenesis. Binding studies utilizing ¹²⁵I-label-

led ACTH have provided evidence for the existence of two populations of adrenal ACTH receptor; occupation of one receptor appeared to be related to cyclic AMP production, and occupancy of the other was related to steroidogenesis (McIlhinney & Schulster, 1975). The implications of such a receptor heterogeneity have been discussed in terms of a dual receptor or compartment-guidance concept (Schwyzer, 1978), and these aspects of ACTH mechanism of action have been reviewed by Schulster & Schwyzer (1980).

We have now used ACTH and two analogues, ACTH_{5–24}, a full agonist (Seelig *et al.*, 1975), and ACTH_{6–24}, a competitive inhibitor, to investigate further the role of cyclic AMP in steroidogenesis, and whether or not an alternative mechanism is available for the stimulation of steroidogenesis.

Experimental

Preparation of isolated adrenal cells and assay of steroidogenesis

Disaggregated rat adrenal cells were prepared by collagenase digestion and corticosteroids accumulated in cells and medium were measured by the fluorescence method (Richardson & Schulster, 1972; Mackie *et al.*, 1972).

Abbreviations used: ACTH_{1–39}, corticotropin-(1–39)-peptide; ACTH_{5–24}, corticotropin-(5–24)-peptide; ACTH_{6–24}, corticotropin-(6–24)-peptide.

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Measurement of cyclic AMP

Radiolabelling *in vitro* and assay of cyclic AMP were as described by Schulster *et al.* (1978). Cells were preincubated with [3 H]adenine to produce intracellular [3 H]ATP. Incubations of adrenal cells with ACTH were stopped by placing the tubes in a boiling-water bath for 2 min and then freezing. Accumulated cyclic [3 H]AMP was assayed by sequential chromatography (Salomon *et al.*, 1974) on columns of Dowex AG-50-X4 (Bio-Rad, Richmond, CA, U.S.A.) and aluminium oxide (BDH, Poole, Dorset, U.K.).

ACTH and ACTH analogues

ACTH₁₋₃₉ was the Third International Standard, obtained from the National Institute for Biological Standards and Control, Hampstead, London N.W.3, U.K. ACTH₅₋₂₄ and ACTH₆₋₂₄ were synthesized as described by Fauchère & Petermann (1978).

Other reagents

Collagenase was obtained from Boehringer/Mannheim Corp., Lewes, Sussex, U.K. Human serum albumin was a gift from the Lister Institute, Elstree, Herts., U.K. Other chemicals were of AnalaR grade from BDH.

Results and Discussion

Dose-response characteristics of isolated adrenal cells for both steroidogenesis and cyclic AMP accumulation were determined for the physiological hormone ACTH₁₋₃₉ and for the fragment ACTH₅₋₂₄ (Fig. 1). The maximum rate of steroidogenesis elicited by ACTH₅₋₂₄ was the same as that elicited by ACTH₁₋₃₉, indicating that ACTH₅₋₂₄ is a full agonist in this system. Both molecules also elicited a dose-related accumulation of cyclic AMP. These findings are in agreement with those previously reported by Seelig *et al.* (1975). It can be seen, however, that the correlation between steroidogenesis and cyclic AMP accumulation for ACTH₁₋₃₉ was quite different from that for ACTH₅₋₂₄. For ACTH₅₋₂₄ the concentration required to elicit an increase in cyclic AMP output is close (within one order of magnitude) to that necessary to stimulate steroidogenesis (Fig. 1). These data are similar to those from previous studies (Mackie *et al.*, 1972) in which statistically significant increases in cyclic AMP were observed at minimally effective doses of hormone (Schulster *et al.*, 1976), in a competitive protein-binding assay for cyclic AMP. This conclusion has been confirmed by measurements of intracellular receptor-bound cyclic AMP, which have shown an even closer correlation with steroidogenesis (Podesta *et al.*, 1979). However, for ACTH₅₋₂₄ there is a marked discrepancy between these parameters. It is clearly evident that

Fig. 1. Steroidogenesis and accumulation of cyclic AMP in response to ACTH₁₋₃₉ and ACTH₅₋₂₄: dose-response characteristics

Isolated rat adrenal cells were incubated with ACTH₁₋₃₉ and ACTH₅₋₂₄ for 1 h. Accumulation of cyclic AMP in response to ACTH₁₋₃₉ (●) and ACTH₅₋₂₄ (▲) and also steroidogenesis in response to ACTH₁₋₃₉ (O) and ACTH₅₋₂₄ (Δ) were measured. The error bars are the standard errors of the means of triplicate incubations. (a) and (b) are data from two typical experiments.

steroidogenesis is stimulated by several hormone concentrations at which no cyclic AMP increases can be discerned (Fig. 1), and cyclic AMP accumulation only occurs at a peptide-hormone concentration more than 100-fold higher than that required for stimulation of steroidogenesis. Moreover, steroidogenesis is stimulated half-maximally by 0.1 nM-ACTH₁₋₃₉, as opposed to 10 nM-ACTH₅₋₂₄ (a 100-fold difference), but for comparable cyclic AMP responses there is a 3000-fold difference in peptide concentration. There is thus a vast discrepancy between the changes in the two parameters elicited by the two analogues. Fig. 1 shows data from two typical experiments, and this discrepancy between the effects of ACTH₁₋₃₉ and ACTH₅₋₂₄ has been consistently observed.

Ways *et al.* (1979) reported that the fragment ACTH₆₋₃₉ competitively inhibits the steroidogenic activity of ACTH₁₋₃₉. In preliminary experiments (not shown) we have found that the 6-24 fragment of the ACTH molecule is also a potent competitive inhibitor of ACTH₁₋₃₉.

Fig. 2. Inhibition of $ACTH_{1-39}$ - and $ACTH_{5-24}$ -stimulated steroidogenesis by $ACTH_{6-24}$. Dose-response characteristics for $ACTH_{1-39}$ (—) and $ACTH_{5-24}$ (---) were determined in the presence of different concentrations of $ACTH_{6-24}$. Concentrations of $ACTH_{6-24}$ used were 0 (○), 5 nM (●), 10 nM (△), 20 nM (▲), 40 nM (□) and 100 nM (■). (a), (b) and (c) represent three different experiments. Steroid was determined after 1 h incubations and error bars are the standard errors of the means of triplicate determinations.

We reasoned that if $ACTH_{1-39}$ and $ACTH_{5-24}$ act via different molecular mechanisms, it is possible that they bind, predominantly or exclusively, to different cell-surface receptors. To investigate this possibility, the inhibitory actions of $ACTH_{6-24}$ on the dose-response curves for the two agonists were compared. Dose-response curves for steroidogenesis in response to $ACTH_{1-39}$ and $ACTH_{5-24}$ were determined in the presence of fixed concentrations of $ACTH_{6-24}$ between 100 and 10 nM (Fig. 2). The dose-response curves were shifted progressively to the right, to higher median effective dose (ED_{50}) values of hormone, by increasing concentrations of the inhibitor. The inhibitor constant, K_d , for $ACTH_{6-24}$ against each of the two agonists was determined by using Schild (1947) plots. The dose ratio is most easily measured as the ED_{50} for the inhibited curve divided by the ED_{50} for the uninhibited curve; when $\log_{10}(\text{dose ratio} - 1)$ is plotted against $\log_{10}(\text{inhibitor concentration})$, the intercept with the x-axis gives the inhibitor constant, K_d (i.e. the inhibitor concentration producing a 2-fold shift in dose-response curve). From the Schild plots (Fig. 3) straight lines were obtained for both agonists, with gradients close to 1.0 (Table 1), indicating that $ACTH_{6-24}$ inhibits both agonists competitively. However, the K_d values for the two agonists were different. The values calculated from the Schild-plot data from three separate experiments are shown in Table 1. Mean K_d values were 13.4 nM against $ACTH_{1-39}$ and 3.4 nM against $ACTH_{5-24}$, with the former being 4.4 times greater than the latter.

Previous studies on the binding of radiolabelled ACTH have been reviewed (Schulster & Schwyzer, 1980) and have indicated two populations of receptors. However, the physiological significance of these binding components remains unknown. In the present study, $ACTH_{6-24}$ inhibited to a different extent the actions of $ACTH_{1-39}$ and $ACTH_{5-24}$, providing evidence that the steroidogenic action of

Table 1. Inhibitor constants (K_d) for the inhibition of $ACTH_{1-39}$ and $ACTH_{5-24}$ by $ACTH_{6-24}$. From the Schild plots presented in Fig. 3, K_d (1-39) and K_d (5-24) (the respective inhibitor constants for the inhibition of $ACTH_{1-39}$ and $ACTH_{5-24}$ by $ACTH_{6-24}$) were calculated for each experiment. These values, together with the gradients, were calculated by linear-regression analysis. The ratio of the inhibitor constants, K_d (1-39)/ K_d (5-24), is also shown.

Figure	Inhibition of $ACTH_{1-39}$ by $ACTH_{6-24}$		Inhibition of $ACTH_{5-24}$ by $ACTH_{6-24}$		Inhibitor-constant ratio K_d (1-39)/ K_d (5-24)
	Schild-plot gradient	K_d (1-39) (nM)	Schild-plot gradient	K_d (5-24) (nM)	
3(a)	1.31	12.6	1.16	3.79	3.32
3(b)	1.16	8.57	0.89	1.44	5.95
3(c)	1.23	19.1	1.27	4.98	3.83
Mean $\pm$ S.E.M.		13.4 $\pm$ 3.06		3.40 $\pm$ 1.04	4.37 $\pm$ 0.80

Fig. 3. Schild plots of the data in Fig. 2

The dose ratio (see Results and Discussion) was determined for each ACTH_{6-24} -inhibited dose-response curve presented in Fig. 2, and Schild plots for ACTH_{1-39} (O) and ACTH_{5-24} (●) are shown. (a), (b) and (c) represent the data in Figs. 2(a), 2(b) and 2(c) respectively.

the two agonists is not mediated through the same population of receptors. This evidence, when considered with the data in Fig. 1, which indicate that the role played by cyclic AMP in mediating the action of the two agonists is not the same in each case, clearly indicates the complexity of the mechanism of action of ACTH. We propose that ACTH_{1-39} , the physiological hormone, can act via either of two receptors. Binding to one receptor elicits steroidogenesis through the involvement of cyclic AMP production; binding to the other receptor elicits steroidogenesis through some other mechanism. The details of this other mechanism are as yet unknown, but one may speculate that it might involve Ca^{2+} and/or cyclic GMP and perhaps explain the current controversy regarding the involvement or non-involvement of cyclic GMP in this response (Perchellet *et al.*, 1978; Hayashi *et al.*, 1979). In this model, ACTH_{1-39} would tend to work via cyclic AMP and ACTH_{5-24} would tend to work via the other mechanism. That various hormones and neurotransmitters can act via different receptor mechanisms is well established, and in this respect parallels with the known mechanisms of action of catecholamines on α - and β -adrenergic receptors may be drawn.

With the classical second-messenger hypothesis increasingly being called into question, this modification of the role of cyclic AMP may be widely applicable to peptide hormones, and perhaps pro-

vide an answer for some of the current criticisms raised against the theory.

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