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ISOLATED ADRENAL CORTEX CELLS IN SUSPENSION: STIMULATION AND INHIBITION OF STEROIDOGENESIS BY ANALOGUES OF ACTH*

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SUMMARY

Suspensions of isolated adrenal cortex cells of the rat secrete corticosterone upon addition of ACTH and related peptides. The cells respond with a high degree of sensitivity, specificity and reproducibility. Estimates of maximum response (V_{max}) and of apparent dissociation constant (A_{50}) for a given analogue are derived from complete log concentration response curves. That the active center is located in the region $Met^4.Glu^5.His^6.Phe^7.Arg^8.Trp^9.Gly^{10}$ is supported by the observation that $ACTH_{1-39}$, $ACTH_{1-24}$, $ACTH_{1-16}$, $ACTH_{5-24}$ and $ACTH_{4-10}$ exhibit the same V_{max}; differences in A_{50}'s reflect differences in affinity of these analogues for receptor. $ACTH_{6-24}$ has a V_{max} equal to 0·4 that of $ACTH_{1-24}$, suggesting that Glu^5 contributes to, but is not essential for receptor excitation. $ACTH_{11-24}$ and $ACTH_{7-23}$ are competitive antagonists of $ACTH_{1-24}$, they induce no steroidogenesis when added alone but inhibit steroidogenesis when added together with $ACTH_{1-24}$ to the adrenal cortex cell suspensions.

INTRODUCTION

Suspensions of isolated cells, prepared from the fasciculatareticularis region of the adrenal cortex, respond to the addition of adrenocorticotropic hormone (ACTH) with secretion of corticosterone, in the case of the rat, and cortisol, in the case of the guinea-pig, dog, cat and man [1–3]. The functional capacities of the isolated cells, as measured by sensitivity to ACTH and by maximum rate of steroidogenesis, match their counterparts *in situ*. In this communication, we review our work on the application of the isolated adrenal cortex cell technic to the elucidation of the relation between chemical structure and steroidogenic potency among ACTH and a series of analogues. Particular attention is directed toward the characterization of competitive antagonists of the tropic hormone. The work has been presented in a series of communications from our laboratory [4–9]. Experimental findings on structure and function based on earlier bioassays, adrenal ascorbic acid depletion, adrenal vein steroids and adrenal quarters, have been reviewed by Hofmann[10], by Schwyzer[11] and more recently by Ramachandran[12].

RESULTS AND DISCUSSION

Details of the isolated adrenal cortex cell technic have been described [2]. Briefly, rats are sacrificed, the adrenals removed, quartered and dispersed in buffer containing trypsin; the freed cells are collected and the pellet suspended in medium. Aliquots of the suspension (0·9 ml containing 300,000 to 500,000 cells) are incubated for 60 min with ACTH or an analogue and the quantity of corticosterone produced is related to the quantity of ACTH added. The isolated adrenal cortex cell technic is as sensitive as radioimmunoassay; it is less sensitive than the redox system (Table 1). Accuracy of the isolated cell technic corresponds more to a chemical rather than to a biological assay. (Inter-animal variation is eliminated by aliquoting from a single large pool of isolated adrenal cortex cells in suspension.) As to specificity, isolated adrenal cortex cells respond to ACTH and related polypeptides but not to insulin, glucagon, vasopressin, oxytocin, angiotensin II, tryptophan or to mixtures of amino acids.

Most importantly, since complete log concentration response curves can be constructed even for analogues of low potency, the isolated adrenal cortex cell technic has provided new insight into the relation between structure and biological activity of ACTH analogues. Analogues are characterized by two parameters: first, capacity to induce a maximum biological response (V_{max}), and second, a concentration which induces one-half the maximum response (apparent dissociation constant). In general terms, the maximum response induced is a measure of capacity to excite the receptor; the concentration necessary to induce one-half the maximum response is a measure of the affinity of the analogue for the receptor. Corticosterone production is related to the quantity of ACTH by the equation $B/B_{max} = [A]/([A] + A_{50})$ where B is corticosterone production; B_{max} is maximum corticosterone production; [A] is the quantity of ACTH added and A_{50}, that quantity of ACTH which induces 50% of maximum corticosterone production (the apparent dissociation constant). The A_{50} for isolated

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Table 1. Comparison of various assays for ACTH

	Sensitivity (pg)	Accuracy (λ)
In vivo		
Adrenal ascorbic acid depletion	2000	0·15
Adrenal vein steroids	400	0·15
Adrenal content of corticosterone	200	0·15
In vitro		
Adrenal quarters	10,000	0·1
Isolated adrenal cortex cells	1·0	0·03
Redox assay	0·1	0·1
Radioimmunoassay	1·0	*

* Difficult to estimate.

Sensitivity is expressed here as the smallest dose of ACTH to induce a measureable response. Accuracy is expressed in terms of λ which equals deviation/slope; the lower the λ, the greater accuracy.

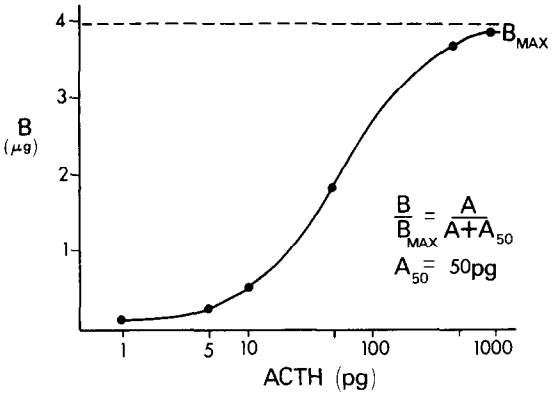

Fig. 1. A typical log concentration response curve for $ACTH_{1-24}$. Abscissa: picograms (pg) of $ACTH_{1-24}$/ml of suspension of isolated adrenal cortex cells incubated 60 min at 37°C. Ordinate: corticosterone (B) synthesized in micrograms (μg) per ml of suspension. Points are averages of corticosterone production for two aliquots at each concentration of ACTH. Curve, connecting the points, is a good fit of the rectangular hyperbola $B/B_{max} = A/(A + A_{50})$ (see text for definition of symbols).

adrenal cortex cells is 50 to 100 pg of $ACTH_{1-24}$/ml of suspension (Fig. 1).

Log concentration response curves for the natural ACTH molecule of 39 amino acids (Fig. 2) and a series of analogues shortened progressively at the carboxyl terminal are presented in Fig. 3. That for $ACTH_{1-24}$ is not shown. Per unit weight (the abscissa is in pg) $ACTH_{1-24}$ is slightly more potent than $ACTH_{1-39}$; the two are equipotent on a molar basis. The tail piece 25–39 appears to serve no role and is very likely a vestigial appendage. With the loss of the dibasic amino acids $Lys^{15}.Lys^{16}.Arg^{17}.Arg^{18}$ the log concentration response curve is shifted decidedly to the right. V_{max} i.e. B_{max} remains the same; there is a loss of affinity but no loss of capacity to excite the receptor. Remarkably, $ACTH_{1-10}$, although weak in potency, induces a V_{max} equal to that of $ACTH_{1-39}$. The V_{max} was estimated by computer from the observation points shown in Fig. 3. We have independently established that the V_{max} for $ACTH_{1-10}$ equals that of $ACTH_{1-24}$ using a more sensitive bioassay, isolated adrenal cortex cells from rats hypophysectomized two days prior to sacrifice [14].

The fragment $ACTH_{5-10}$ is active, but we cannot make an estimate of V_{max}; the highest concentration employed (Fig. 3) is about the limit of solubility of this peptide.

Curves for $ACTH_{1-24}$, $ACTH_{4-23}$, $ACTH_{5-24}$ and $ACTH_{6-24}$ are shown in Fig. 4. Note the increase in apparent dissociation constant (that is loss of affinity) as the polypeptide is shortened. Note particularly the reduction in V_{max} for the transition, $ACTH_{5-24}$ to $ACTH_{6-24}$. We interpret this reduction in V_{max} to mean that glutamic acid at position 5 of the ACTH molecule is not essential but contributes to the excitation of the receptor. $ACTH_{7-23}$ and $ACTH_{11-24}$ were tested over a wide range of concentrations and found to be inactive.

From the totality of observations on isolated cells we have arrived at the following conception of hormone–receptor interaction. The hormone is attracted by the receptor and molded into a state of secondary structure dictated by the nature and the topology of complementary sites on the receptor. A signal is generated in the region 5–10, the active center of the ACTH molecule, and this signal traverses the plasma membrane to active adenylate cyclase. Cyclic

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

NH_2
|
Asp-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39

Fig. 2. Primary structure of α porcine $ACTH_{1-39}$. From Rimiber *et al.* (1972).

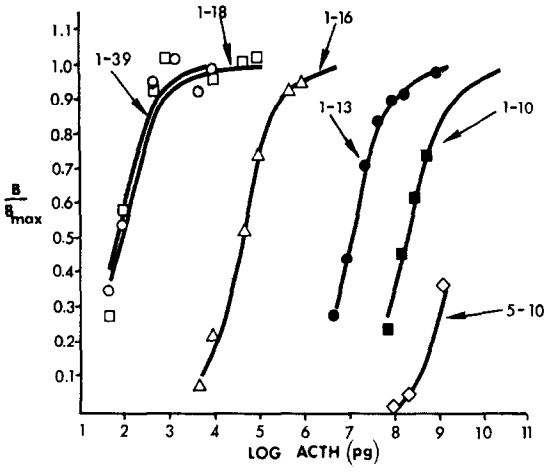

Fig. 3. Log concentration response curves for $ACTH_{1-39}$ and peptides shortened at the C terminal; a partial curve for $ACTH_{5-10}$. Abscissa: picograms (pg), the quantity added to each ml aliquot of cell suspension. Ordinate: B/B_{max}. Points are averages for two aliquots at each dose of peptide. Curves fitted by computer [13].

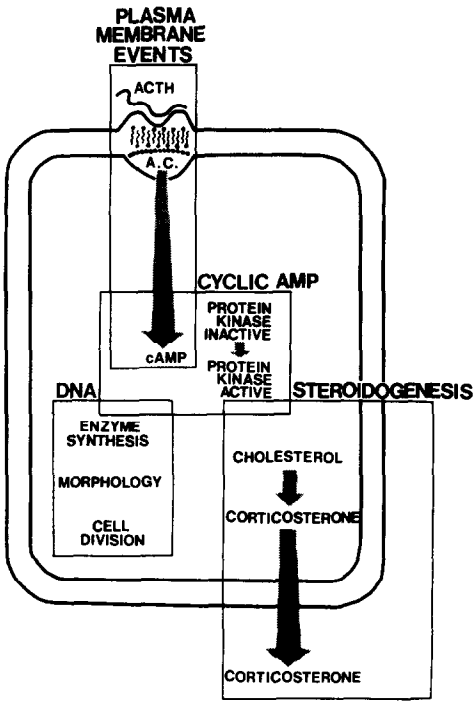

Fig. 5. A highly schematized drawing of a cell of the adrenal cortex. For convenience the sequence of events initiated by interaction of ACTH molecules with receptors on the cell surface and ending (in the case of the rat adrenal cortex cells) with corticosterone secretion has been depicted in terms of four categories: (1) Plasma Membrane Events; (2) Cyclic AMP; (3) Steroidogenesis; and (4) DNA.
(1) *Plasma Membrane Events.* ACTH molecules interact with receptors on the surface of plasma membrane, a signal is generated, amplified and transduced as it traverses the plasma membrane to activate adenylate cyclase confined to a compartment on the inner surface of the plasma membrane. Cyclic AMP production is enhanced.
(2) *Cyclic AMP.* Cyclic AMP converts a protein kinase from an inactive to an active form. A protein or proteins is phosphorylated together with as yet ill-defined processes setting in motion an increase in the rate of steroidogenesis by accelerating the conversion of cholesterol to pregnenolone.
(3) *Steroidogenesis.* Once the rate limiting step, cholesterol to pregnenolone, has been accelerated, the subsequent biosynthetic steps, pregnenolone to corticosterone proceed rapidly. The conversions of precursors to products involve hydroxylating enzymes, the P-450 system, NADPH and NADP.
(4) *DNA* is acted upon by cyclic AMP (or more likely a product of the actions of the cyclic nucleotide) with continued but varied rates of syntheses of enzymes (e.g. hydroxylating enzymes, components of the P-450 system), with syntheses of the components of cell organelles (e.g. smooth endoplasmic reticulum, mitochondria) and with cell division.

AMP production is increased and steroidogenesis accelerated (Fig. 5).

On the basis of the observations just described we predicted that the fragment $ACTH_{11-24}$ would exhibit the properties of a competitive antagonist since it lacked the active center but contained a major fraction of the totality of affinity sites. Our prediction was confirmed in experiments in which $ACTH_{1-24}$ alone and $ACTH_{1-24}$ plus $ACTH_{11-24}$ were added in various combinations of concentration to suspensions of isolated adrenal cortex cells [5]. The fragment $ACTH_{7-23}$ is also a competitive antagonist and data on this peptide are illustrated in Fig. 6.

The *partial agonist*, $ACTH_{6-24}$, has a V_{max} equal to approximately 0·4 that of $ACTH_{1-24}$, the fragment excites the receptor but not as efficiently as the parent molecule. Observations in support of the thesis that $ACTH_{6-24}$ acts on the same receptor site as $ACTH_{1-24}$ are presented in Fig. 7.

The apparent dissociation constants and intrinsic activities of the analogues described (the competitive antagonists are assigned an intrinsic activity of zero) are listed in Table 2. In addition, two other compounds are characterized, namely, the nitrophenyl sulfenyl derivative of $ACTH_{1-24}$ and, the dinitrophenyl sulfenyl derivative of $ACTH_{5-24}$. *Note* that this latter derivative has an exceedingly low dissociation constant which means that it is a relatively potent antagonist. We are continuing our efforts to prepare new analogues and derivatives with the goal

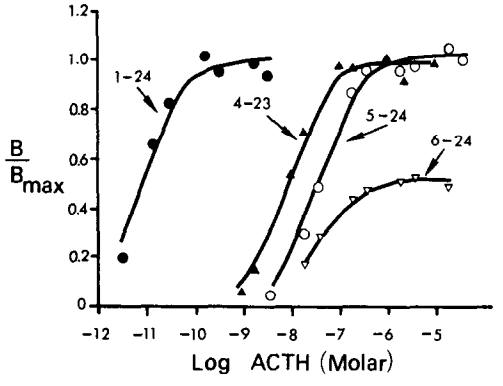

Fig. 4. Log concentration response curves for $ACTH_{1-24}$, $ACTH_{4-23}$, $ACTH_{5-24}$ and $ACTH_{6-24}$. Axes are same as for Fig. 4, except concentration of peptide (on the abscissa) is molar.

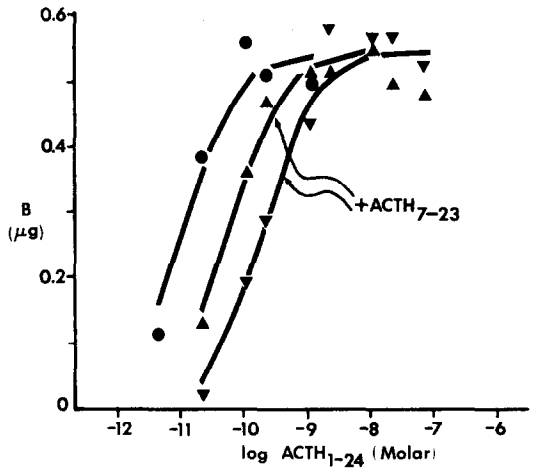

Fig. 6. The competitive antagonist, $ACTH_{7-23}$. The curve on the left depicts responses to various concentrations of $ACTH_{1-24}$ acting alone; the middle curve, responses to $ACTH_{1-24}$ in concentrations presented on the abscissa plus $ACTH_{7-23}$ in a concentration of $0{\cdot}58 \times 10^{-6}$ M; curve on right, $ACTH_{1-24}$ plus $ACTH_{7-23}$ in a concentration of $2{\cdot}32 \times 10^{-6}$ M.

of obtaining highly potent antagonists of the native ACTH. Potent antagonists of ACTH have potential value as diagnostic and therapeutic agents in man. In addition, these antagonists are of value on theoretical grounds. For example, $ACTH_{11-24}$ inhibits ongoing $ACTH_{1-24}$ induced steroidogenesis (Fig. 8) and cyclic AMP production with a time lag of less than

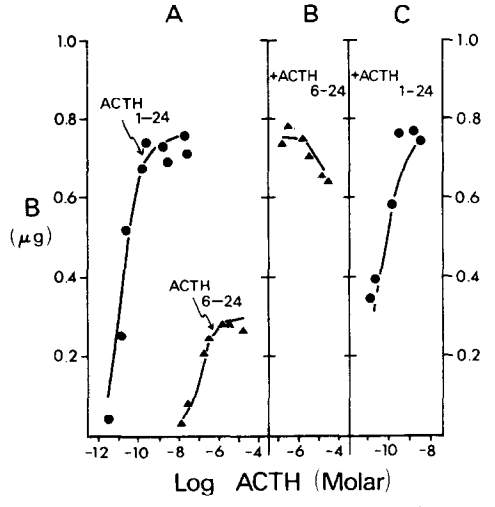

Fig. 7. Competition between the partial agonist, $ACTH_{6-24}$, and the full agonist $ACTH_{1-24}$. *Panel A*: Log concentration response curves for $ACTH_{1-24}$ and $ACTH_{6-24}$ acting alone. $ACTH_{6-24}$ is a partial agonist with an "intrinsic activity" equal to 0·4 that of $ACTH_{1-24}$. *Panel B*: When to a relatively high and fixed concentration of $ACTH_{1-24}$ ($1{\cdot}75 \times 10^{-8}$ M) increasing concentrations of $ACTH_{6-24}$ were added the V_{max} declined. (Increasing concentrations of $ACTH_{6-24}$ on abscissa). *Panel C*: When to a high and fixed concentration of $ACTH_{6-24}$ ($4{\cdot}35 \times 10^{-7}$ M) increasing concentrations of $ACTM_{1-24}$ were added, the V_{max} increased and approached that of $ACTH_{1-24}$ acting alone. (Increasing concentrations of $ACTH_{1-24}$ on abscissa).

Table 2. Molar dissociation constants and intrinsic activities for ACTH analogues

Analogue	Dissociation constant	Intrinsic activity
$ACTH_{1-24}$	$2{\cdot}6 \times 10^{-11}$	1·0
$ACTH_{6-24}$	$8{\cdot}9 \times 10^{-8}$	0·4
$ACTH_{7-23}$	$1{\cdot}2 \times 10^{-7}$	0
$ACTH_{11-24}$	$8{\cdot}5 \times 10^{-6}$	0
$[Trp(Nps)^9]ACTH_{1-24}$	$2{\cdot}3 \times 10^{-9}$	0·77
$[Trp(Dnps)^9]ACTH_{5-24}$	$6{\cdot}5 \times 10^{-8}$	0·1

one minute, indicating that $ACTH_{1-24}$ is rapidly dissociated from the receptor site on the plasma membrane [15].

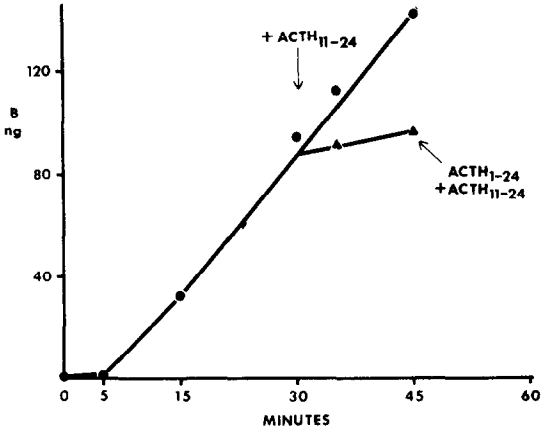

Fig. 8. "Offset" kinetics for ACTH induced steroidogenesis using a competitive antagonist. To all aliquots of the adrenal cortex cell suspension $ACTH_{1-24}$ was added in an amount equal to 25 pg. After 30 min of incubation the aliquots were divided into two groups. To the aliquots of one group no further addition, to the aliquots of the other group 100 μg $ACTH_{11-24}$ was added. Note the rapidity with which $ACTH_{11-24}$ inhibits $ACTH_{1-24}$ induced steroidogenesis [15]. $ACTH_{1-24}$ induced cyclic AMP production also quickly ceases upon addition of $ACTH_{11-24}$.

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