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ACTH Antagonists

(ACTH receptor/adenylate cyclase/S-peptide-S-protein system/peptide hormones/adrenal cortical plasma membranes)

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ABSTRACT **Structural modifications within the active site of the ACTH molecule have produced analogs that inhibit the hormone sensitive adenylate cyclase system of bovine adrenal cortical plasma membranes. It is demonstrated that the tryptophan residue of the ACTH molecule is essential for stimulation of the enzyme. Substitution of tryptophan by phenylalanine or by N^{α}-methyltryptophan as in [Gln^5, Phe^9]corticotropin$_{1-20}$ amide or [N^{α}-Metrp^9]corticotropin$_{1-24}$ provides ACTH analogs that exhibit high affinity for the ACTH receptor(s) but fail to activate the adenylate cyclase system. It is concluded that affinity for the receptors alone is not sufficient for expression of hormonal activity. The observation that adrenal cortical adenylate cyclase activated by fluoride ion is not inhibited by the antagonists indicates that hormonal and fluoride activation proceed via different mechanisms.**

The discovery of competitive inhibitors of polypeptide hormones is of considerable importance from a fundamental as well as from a practical point of view. Not only are compounds exhibiting such properties valuable tools for probing the mechanism of action of polypeptide hormones but they may have clinical importance in controlling specific endocrinopathies. Considerable efforts are presently directed toward discovery of such compounds. Knowledge of the essential structural features (active site) of the parent hormone may provide clues for the development of competitive antagonists.

We have regarded the S-peptide-S-protein system* (1) as a model for the mode of action of peptide hormones (2). In this system two enzymically inactive components, S-peptide and S-protein, combine in a highly specific manner to form the enzymically fully active ribonuclease S. In the model, S-protein is analogous to the receptor; S-peptide represents a peptide hormone. Structure-function studies (3) have demonstrated that [His^{12}] is the active site of S-peptide and that the rest of the molecule provides the vehicle to bring this residue into the correct stereochemical position on S-protein to form the active site of ribonuclease S. Since mechanistic studies (4) had shown that the pK of the imidazole ring of [His^{12}] in S-peptide is of prime importance for enzymic activity, it was a logical step to replace this histidine with an isosteric amino acid exhibiting a different pK from histidine in order to develop an S-peptide antagonist. [β-Pyrazolyl(3)alanine^{12}] S-peptide$_{1-14}$([Pyr(3)Ala^{12}]S-peptide$_{1-14}$) was synthesized and proved to be a potent competitive inhibitor of S-peptide (5).

It has now become evident that many peptide hormones combine with receptors on the cell surface and bring about stimulation of hormone sensitive adenylate cyclase(s) (6). This activation appears to provide the key to physiological function. Details of the mechanisms involved are missing but peptide hormones, like S-peptide, do indeed function as enzyme activators.

The sequence -His-Phe-Arg-Trp-Gly- (positions 6–10 in Fig. 1) was postulated to be the essential structural feature (active site) of the ACTH molecule (2) and structure-function studies based on *in vivo* assays have largely confirmed this hypothesis (7). Based on observations with the S-peptide-S-protein system, we speculated that amino acid substitutions in the active site of the ACTH molecule would produce competitive antagonists. Experimental results presented in this communication support this concept.

MATERIALS AND METHODS

[Gln^5, Phe^9]corticotropin$_{1-20}$ amide (VIII) (8), corticotropin$_{1-23}$ (V) (9), [Gln^5]corticotropin$_{1-20}$ amide (VI) (10), and corticotropin$_{11-20}$ amide (X) (11), were prepared in our laboratories. [Lys^8]corticotropin$_{1-24}$ (12), corticotropin$_{1-24}$ (I) (13) and [N^{α}-Metrp^9]corticotropin$_{1-24}$ (II) (14) were gifts from Dr. W. Rittel of Ciba-Geigy Corp., Basel, Switzerland. Pig ACTH was obtained from Dr. Joseph D. Fisher of Armour Pharmaceutical Co., Kankakee, Ill. Bovine adrenal cortical plasma membranes were prepared as described (15). For the inhibition studies, a membrane concentrate was obtained by diluting membrane rich fractions from the zonal centrifugation with two volumes of 0.001 M sodium bicarbonate (pH 7.5) and subjecting the suspension to centrifugation at 25,000 $\times$ *g*. The clear supernatant was removed and the pellet was resuspended in 0.001 M sodium bicarbonate to a concentration of 4 mg of protein per ml in a Dounce homogenizer with a loosely fitting pestle. Adenylate cyclase assays were initiated by addition of suitable aliquots of the membrane suspension to the incubation mixture. Adenosine 3′:5′-cyclic monophosphate (cAMP) was determined as described (15).

RESULTS AND DISCUSSION

On the basis of preliminary structure-function studies with α-MSH and ACTH, it was postulated (2) that the sequence -His-Phe-Arg-Trp-Gly-(positions 6–10) is the functional region (active site) of these hormones and that the cationic sequence -Lys-Lys-Arg-Arg- (positions 15–18) represents a region of the ACTH molecule that is concerned with binding to the cell receptor(s) (binding site). As has been pointed out, a large body of information derived from structure-function studies with *in vivo* and *in vitro* systems has confirmed these

* Ribonuclease S = subtilisin-modified bovine ribonuclease A; S-peptide = the eicosapeptide obtained from ribonuclease S; S-protein = the protein component obtained from ribonuclease S.

Peptide No.

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

(II) H-Ser----------Arg-CO-N-Trp----------Pro-OH
CH_2

(III) H-Ser----------Orn----------Pro-OH

(IV) H-Ser----------*Glu-His-Phe-Arg*-CO-N-*Trp*----------Pro-OH
H

(V) H-Ser----------Tyr-OH

(VI) H-Ser----------Gln----------$Val-NH_2$

(VII) H-Ser----------Gln-Pyr----------$Val-NH_2$

(VIII) H-Ser----------Gln----------Phe----------$Val-NH_2$

(IX) H-β-Ala----------*Phe*----------Orn----Arg-NH_2

(X) H-Lys----------$Val-NH_2$

(XI) H-Lys----------Pro-OH

FIG. 1. Amino-acid sequences of ACTH (corticotropin$_{1-39}$) analogs. (*I*) Corticotropin$_{1-24}$; (*II*) [N^{α}-Metrp^9]corticotropin$_{1-24}$; (*III*) [Orn^8]corticotropin$_{1-24}$; (*IV*) [D-Glu^5, D-His,^{6} D-Phe^7, D-Arg^8, D-Trp^9]corticotropin$_{1-24}$; (*V*) corticotropin$_{1-23}$; (*VI*) [Gln^5]corticotropin$_{1-20}$ amide; (*VII*) [Gln^5, Pyr(3)Ala^6] corticotropin$_{1-20}$ amide; (*VIII*) [Gln^5, Phe^9]corticotropin$_{1-20}$ amide; (*IX*) [β-Ala^1, D-Phe^7, Orn^{15}]corticotropin$_{1-18}$ amide; (*X*) corticotropin$_{11-20}$ amide; (*XI*) corticotropin$_{11-24}$.

early postulates. Final proof to substantiate this concept had to await the discovery of a system or systems capable of discriminating between binding and function of ACTH peptides. Such a system was found in bovine adrenal cortical plasma membranes (15). These membranes bind radioactive [Gln^5]corticotropin$_{1-20}$ amide (radioactive ACTH (VI), and certain nonradioactive fragments and analogs of ACTH displace the labeled peptide (16). The ability to displace radioactivity, with few exceptions, coordinates remarkably well with the *in vivo* adrenocorticotropic activity of the peptides tested. One exception is [Gln^5, Phe^9]corticotropin$_{1-20}$ amide (VIII in Fig. 1). This peptide exhibits a low level of *in vivo* biological potency (8) but displaces radioactive ACTH as well as the biologically highly potent corticotropin$_{1-24}$ (I in Fig. 1) from the membranes. Originally it was assumed that the low biological activity of (VIII) was the result of poor affinity for the cell receptor and a binding role was assigned to the tryptophan residue. The results of binding experiments clearly indicated that this is not the case (16).

The plasma membranes contain an ACTH sensitive adenylate cyclase system that responds to stimulation by pig corticotropin, corticotropin$_{1-24}$ (I), corticotropin$_{1-23}$ (V), [Gln^5] corticotropin$_{1-20}$ amide (VI) (Fig. 2), and other biologically active ACTH analogs (15). Of interest is the observation that the natural hormone activates the enzyme to a lesser extent than peptides (I), (V), and (VI). At present we have no explanation for this finding. Comparison of the relative steroidogenic activities of corticotropin$_{1-24}$ (I) and pig ACTH determined by different techniques is of interest in this connection. In the *in vivo* steroidogenic assay (17), peptide (I) exhibits approximately 50% of the potency of the natural hormone on a molar basis but in the *in vitro* steroidogenic assay (isolated rat cells), it is 1.4 times as active as ACTH in stimulating corticosterone synthesis (18).

The analog [Gln^5, Phe^9]corticotropin$_{1-20}$ amide (VIII) fails to stimulate the cyclase system; on the contrary this peptide amide is a potent antagonist of corticotropin$_{1-24}$ (Fig. 3) with a 50% inhibition ratio (peptide VIII to peptide I) of approximately 5 to 1. The results presented in Fig. 4

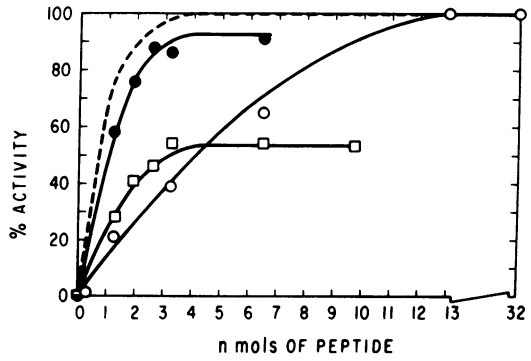

FIG. 2. Stimulation of bovine adrenal cortical plasma membrane adenylate cyclase by ACTH and analogs. – – –, corticotropin$_{1-24}$ (I); ●, corticotropin$_{1-23}$ (V); ○, [Gln^5]corticotropin$_{1-20}$ amide (VI); □, pig ACTH. Full activation above basal level (100% activity) corresponds on an average to 382 pmol of cAMP per mg of protein per 15 min. Values are composites of several experiments.

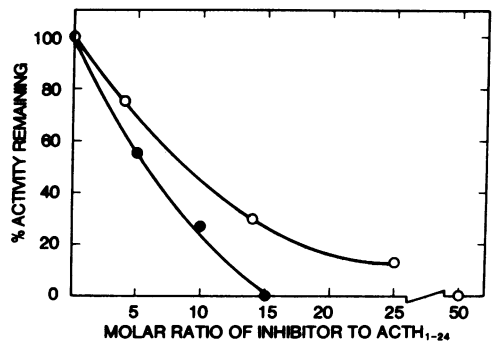

FIG. 3. Inhibition of corticotropin$_{1-24}$ activated adenylate cyclase by (○), [N^{α}-Metrp^9]corticotropin$_{1-24}$ (II) and ●, [Gln^5, Phe^9]corticotropin $_{1-20}$ amide VIII. Full activation above basal level (100% activity) corresponds on an average of 263 pmol of cAMP per mg of protein per 15 min. Concentration of corticotropin $_{1-24}$, 1.95 nmol per assay mixture. Values are composites of several experiments.

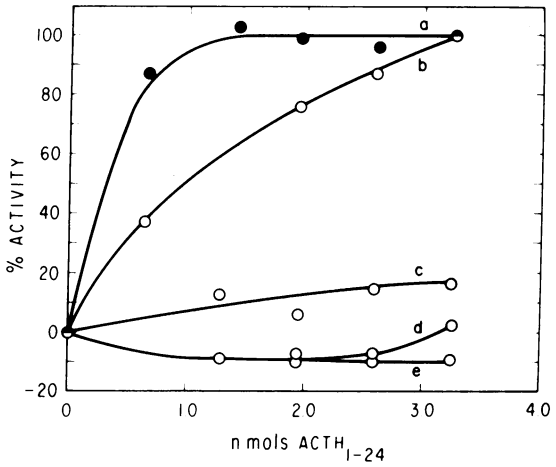

FIG. 4. Competitive inhibition of corticotropin$_{1-24}$ (I) stimulated adenylate cyclase by various concentrations of [Gln^5, Phe^9] corticotropin$_{1-20}$ amide (VIII). Concentration of inhibitor: curve *a*, none; curve *b*, 9.77 nmol; curve *c*, 32.5 nmol; curve *d*, 65.0 nmol; curve *e*, 162 nmol. Full activation above basal level (100% activity) corresponds on an average to 333 pmol of cAMP per mg of protein per 15 min. Values are composites of several experiments.

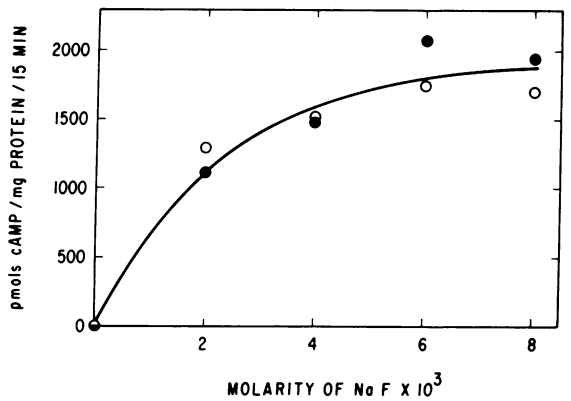

FIG. 5. Sodium fluoride stimulation of adenylate cyclase in the presence ● or absence ○ of [Gln^5, Phe^9]corticotropin$_{1-20}$ amide (VIII) (13 nmol). A basal level of 156 pmol of cAMP per mg of protein per 15 min has been subtracted from each value. The numbers on the abscissa have been multiplied by 10^3.

illustrate the competitive nature of this antagonism. At high inhibitor-peptide (I) ratios, adenylate cyclase activity falls below the basal level. We attribute this behavior to displacement of endogenous ACTH bound to the adrenal cells. It should be remembered that the adrenals are obtained from stressed animals and it appears likely that some receptor bound ACTH survives the isolation procedure and is present in the membrane preparation.

It follows from these results that the indole sidechain of [Trp^9] is intimately concerned with a major if not the only direct function of ACTH, namely stimulation of an ACTH specific adenylate cyclase system.

The adrenal cortical adenylate cyclase system shares with other hormone sensitive adenylate cyclases (19) the ability to be stimulated by fluoride ion. That this stimulation operates by a mechanism different from ACTH activation follows from the results shown in Fig. 5 where adenylate cyclase is stimulated by fluoride ion to the same degree in the presence or absence of the inhibitor (VIII). ACTH and fluoride ion stimulation have been shown to be nonadditive in the adenylate cyclase of fat cell ghosts (20) and it has been previously postulated that the two activators operate by different mechanisms. The present finding supports this view. The exact role of the tryptophan residue for adenylate cyclase activation is far from clear but it has become apparent that occupation of the receptor site by an analog does not necessarily result in expression of hormonal activity.

Substitution of tryptophan by N^{α}-methyltryptophan as in [N^{α}-Metrp^9]corticotropin$_{1-24}$ (II) provides another analog exhibiting low *in vivo* adrenocorticotropic activity (14). This analog, with a 50% inhibition ratio of approximately 10 to 1, also is a potent antagonist of corticotropin$_{1-24}$ (I) in activating the adenylate cyclase system (Fig. 3). Substitution of the α-peptide hydrogen of [Trp^9] by a methyl group may alter the ability of the peptide to assume a functional hormone-receptor complex because of a conformational change. Indeed, receptor affinity is somewhat impaired since peptide (II) binds less firmly to the membranes than peptides (I) or (VIII). Equal binding of peptides (I) and (II) at a molar ratio of 1 to 1 would be expected to produce 50% inhibition.

The analog [β-Ala^1, D-Phe^7, Orn^{15}]corticotropin$_{1-18}$ amide (IX) antagonizes the corresponding L-Phe peptide and natural ACTH in an adenylate cyclase preparation of rat adrenal particulates. (21). Here again, a conformational alteration appears to prevent formation of a functional hormone-receptor complex. Another such example is the analog [D-Glu^5, D-His^6, D-Phe^7, D-Arg^8, D-Trp^9]corticotropin$_{1-24}$ (IV) that is reported to inhibit the action of ACTH on fat cell ghosts (20). In all these instances antagonists are produced by alterations of amino-acid residues located in the functionally essential sequence -His-Phe-Arg-Trp-Gly-.

Based on results obtained with *in vivo* studies, it may be concluded that [Arg^8] and [Trp^9] are essential for steroidogenic activity. Replacement of [His^6] by [Pyr(3)Ala] as in peptide VII lowers but does not eliminate steroidogenic potency (8), whereas substitution of [Arg^8] by Lys (12) or Orn (22) and replacement of [Trp^9] by Phe or N^{α}-Metrp produces compounds exhibiting a very low order of activity. It could be argued that a true competitive antagonist of ACTH should exhibit no *in vivo* activity. However, most of the inhibitors discussed in this communication exhibit some, albeit low, *in vivo* steroidogenic activity (0.5–1.0% that of the natural hormone). Mobilization of stored hormone or redistribution of nonspecifically bound hormone within the organism by the inhibitor may account for this apparent discrepancy.

It is clear from the results presented in this communication that the tryptophan residue is essential for adenylate cyclase activation. The molecular basis for the low *in vivo* activity of [Orn^8]corticotropin$_{1-24}$ (III) (22) remains to be established.†

The results obtained with the three analogs of ACTH clearly indicate that binding to the receptor(s) is not sufficient for activation of the adrenal cortical adenylate cyclase system and that certain amino-acid residues [Trp^9] have a key function in this process. Similar observations have been reported with the glucagon sensitive adenylate cyclase system of rat liver plasma membranes. Des-His-glucagon does not activate the cyclase and antagonizes glucagon stimulation (23).

† *Footnote Added in Proof.* Since submission of this paper, we have observed that [Lys^8] corticotropin$_{1-24}$ (12) is an antagonist of corticotropin$_{1-24}$ in the adenylate cyclase system with a 50% inhibition ratio of approximately 50:1.

Corticotropin$_{11-20}$ amide (X), a steroidogenically inactive peptide, was shown to displace radioactive ACTH from beef adrenal cortical membranes (15) and particulates (16) although less effectively than peptides (I), (V), (VI), and (VIII). Seelig *et al.* (24), using isolated rat adrenal cells, observed that corticotropin$_{11-24}$ (XI) antagonized the steroidogenic activity of natural ACTH although large proportions of this peptide were required to demonstrate inhibition. Peptide (XI) like peptide (X) contains the major binding sequence -Lys-Lys-Arg-Arg- of the ACTH molecule; hence this result was predictable on the basis of the binding results with peptide (X).

Structure-function studies *in vivo* and *in vitro* have provided clues as to areas of the ACTH molecule involved in function (active site) and those important for binding to the receptor(s) (binding site). They have also provided detailed understanding of the role of individual amino acid residues within the functional region -His-Phe-Arg-Trp-Gly-. Structural modifications within this sequence have led to the logical development of potent antagonists of the ACTH sensitive adenylate cyclase. Experiences gained with the S-peptide-S-protein model have been applied successfully to the much more complex ACTH adenylate cyclase system and the similarity between the two appears to be more than a mere coincidence.

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