Localization of an Adrenocorticotropic Hormone Receptor on Bovine Adrenal Cortical Membranes*

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SUMMARY

The preparation of a subcellular fraction from bovine adrenal cortex is described. Characterization by electron microscopy, electron microscopic cytochemistry, and enzymatic assay indicates that this fraction is enriched plasma membranes. The preparation binds the steroidogenically active [Glu'] [D-Phe7] corticotropin-1-24 amide. The specificity of this binding is demonstrated by competitive binding studies with pig corticotropin as well as adrenocorticotropic hormone (ACTH) fragments and analogs. The membrane preparation contains an adenylate cyclase sensitive to both sodium fluoride and corticotropin-2-18. Corticotropin-1-20 amide binds to the membranes but fails to activate adenylate cyclase. This membrane preparation provides a means to correlate peptide structure and in vivo corticotrophic activity with selective binding and adenylate cyclase activation. The observation that corticotropin-1-20 amide binds to the membranes but does not activate the adenylate cyclase supports the previous hypothesis that major binding sites of the ACTH molecule reside in this portion of the sequence. Two peptides exhibiting significantly higher in vivo adrenocorticotropic activity than ACTH from natural sources were only weakly active in stimulating the membrane adenylate cyclase. The experimental results strongly suggest that the membrane preparation contains an ACTH receptor.

It is generally accepted that the initial event in the action of adrenocorticotropic hormone, as well as other peptide hormones, involves attachment to and activation of a hormone specific receptor in or on the target cell. The activated receptor molecule then triggers a series of reactions which culminate, in the case of ACTH, in enhanced production of corticosteroids. Graham-Smith et al. (1) and others have presented evidence to support the hypothesis that cyclic adenosine 3',5'-monophosphate is an intermediate (second messenger) between receptor activation and the characteristic physiological response. The specific role of cyclic AMP in this sequence of events is by no means clear; however, production of the cyclic nucleotide is stimulated by ACTH and this effect of the hormone precedes steroidogenesis (1).

Historically, the effects of ACTH on the adrenal cortex were demonstrable only in perfused adrenals (2) or adrenal quarters (3) or slices (4). Kloppeuborg et al. (5) and Giordano and Skowers (6) have recently shown that isolated cells from rat adrenals are very sensitive to ACTH. Furthermore, Schimmer et al. (7) have demonstrated that mouse adrenal tumor cells are capable of responding to ACTH even when the hormone is attached to a carrier, such as CM-cellulose, which by virtue of its size, presumably cannot enter the cell. These findings indicated that an ACTH receptor is located on the cell surface. Adenylate cyclase, the enzyme system responsible for the conversion of ATP to cyclic AMP, also appears to reside in the cell membrane. The receptor serves to activate this enzyme system in response to a specific signal, namely binding of the hormone. It has not been clearly established whether the two functions, binding of the hormone and formation of cyclic AMP, reside in the same or different molecules. Furthermore, the possibility that several, as yet unidentified, reactions may intervene between receptor activation and production of cyclic AMP cannot be excluded.

In view of these uncertainties we felt that the isolation and characterization of the ACTH receptor would be of fundamental importance for understanding the mechanism of action of this hormone. In order to achieve this goal, however, large amounts of starting material, i.e. plasma membranes from adrenal cortical cells must be readily available. This paper describes a reproducible procedure for the isolation of a preparation from bovine adrenal cortex enriched in plasma membranes as judged by biochemical and morphological techniques. This preparation binds ACTH and fragments and analogs of the hormone and contains an ACTH sensitive adenylate cyclase.

EXPERIMENTAL PROCEDURE

Materials—Pyruvate kinase (rabbit skeletal muscle, type II, 380 units per mg); hexokinase (type F-300, yeast); zwenchenferment (type XV); horse heart cytochrome c, (type II); bovine

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The abbreviations used are: ACTH, adrenocorticotropic hormone; cyclic AMP, cyclic adenosine 3',5'-monophosphate; BSA, bovine serum albumin.
serum albumin (Fraction V); ADP (Grade I, sodium salt); P-enolpyruvate (trisodium salt, hydrate); and NADP+ (sodium salt) were purchased from Sigma Chemical Company, St. Louis, Missouri. AMP; cyclic 3'5'-AMP; ATP (disodium salt); and [3H]ATP (specific activity 20.7 Ci per mmole) were obtained from Schwarz-Mann Company, Orangeburg, New York. 2'-AMP was purchased from P-L Biochemicals, Milwaukee, Wisconsin. Theophylline was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio; AG 1-X2 (200 to 400 mesh) from Isio-Rad Laboratories, Isael, Switzerland. Peptide TV (11) corticotropin-l-16 amide (V) (10) were gifts from Dr. W. Rittel of Frankfurt, West Germany. Corticotropin-l-2:~ (II) (12), ror- 

**Table 1**

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**Biological Activity**

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**Enzyme Assays**—Cytochrome oxidase was assayed essentially as described (21). Dithionite (30 mg) was added to 50 ml of 0.1 M pH 7.4 potassium phosphate buffer containing cytochrome...
c (1 x 10^-4 m) and the solution was shaken vigorously. This solution (0.5 ml) containing reduced cytochrome c was mixed with an equal volume of suitably diluted fractions from the zonal centrifugation (see below) and the decrease in optical density at 550 nm was followed with a Cary 15 recording spectrophotometer. One enzyme unit corresponds to the oxidation of 1 mmole of reduced cytochrome c per min.

5'-Nucleotidase was determined as described (22). The inorganic phosphate liberated was measured by the method of Fiske and Subbarow (23). The reported enzyme activity represents micromoles of phosphate liberated per min.

For determination of adenylate cyclase appropriate quantities of tissue (usually corresponding to 0.1 mg of protein) were suspended in 5 ml of 0.001 M sodium bicarbonate pH 7.5 and the suspensions were centrifuged at 24,000 x g for 20 min. The supernatants were decanted, the pellets were broken up with a glass rod and the tubes were kept in an ice bath. An aqueous solution (50 mg per ml) of P-enolpyruvate (0.1 ml) and pyruvate kinase, 0.01 ml, were added followed by ATP solution (0.6 ml) and appropriate amounts of corticotropin (24) dissolved in 0.1% BSA (0.1 ml) pH 7.0. Sodium fluoride, when used, was dissolved in the ATP solution at a concentration of 10^-3 M. The suspensions were incubated in a Dubnoff shaker for 15 min at 30°C. The tubes were transferred to an ice bath, 0.1 ml of a solution of cyclic AMP (0.5 mg per ml of water) was added immediately and the tubes were then placed in boiling water for 2 min. The incubates were either stored frozen at -15° or were used immediately for determination of their cyclic AMP content.

Cell Fractionation--Operations involved in the preparation of plasma membranes were carried out at ice bath temperature. Steer adrenals (18 to 20 glands) were collected in ice cold 0.001 M sodium bicarbonate pH 7.5 (medium) within 20 min after death of the animal and were transported to the laboratory in an ice bath. The glands were defatted, bisected, demedullated, and the cortical tissue scraped from the capsule with a spatula.

It was later found advisable to perform the dissection under 0.25 M sucrose to prevent tissue dehydration. The wet tissue (approximately 100 g) was divided into three batches and each was processed as follows: the tissue (weight) was diluted with 9 volumes of 0.25 M sucrose pH 7.5 and homogenized in a Dounce homogenizer with a loosely fitting pestle (10 strokes). The suspension was rehomogenized in a glass-glass homogenizer with a clearance of 1 mm (1 stroke). The homogenate was diluted to 1000 ml with medium and the suspension stirred for 5 min with a magnetic stirrer and filtered through two layers of 20/12 gauge. The filtrate was centrifuged (Sorvall RC2B centrifuge with GSA rotor) at 10,400 x g for 20 min and the supernatant was decanted. The pellet was resuspended in medium with the Dounce homogenizer (3 strokes) and diluted to 400 ml with medium. This suspension was centrifuged at 10,400 x g for 20 min and the supernatant aspirated and discarded. The pellets from the three batches were now combined and homogenized in medium with the Dounce homogenizer. The suspension was diluted to 300 ml with medium and 300 ml of 63% sucrose w/w were added with stirring. Six centrifuge tubes, each containing 80 ml of 45% w/w sucrose, were layered with 100 ml of the above suspension and centrifuged for 20 min at 10,400 x g. After centrifugation a top layer, a gelatinous interphase adhering to the wall, and erythrocytes at the bottom of the tube were visible. The top layer was aspirated, care being taken not to remove the interphase, the pooled supernatants were diluted with medium to 900 ml and the suspension was centrifuged in three batches in an SS-34 rotor at 25,300 x g for 20 min. The pellets were pooled, resuspended in medium with the Dounce homogenizer, diluted to 300 ml with medium, and recentrifuged at 25,300 x g for 20 min. The supernatant was aspirated, the pooled pellets remixed in 0.25 M sucrose pH 7.5 in a Teflon-glass homogenizer (A. H. Thomas, type C) and diluted with 0.25 M sucrose to a volume of 50 ml. This suspension was injected into the core of a Ti 14 zonal rotor (Beckman-Spinco) spinning at 3000 rpm and containing a linear sucrose gradient (500 ml) from 32% w/w to 45% w/w and a cushion (150 ml) of 45% w/w sucrose prepared with a Beckman High Capacity Gradient Pump, model 141. The suspension was centrifuged for 16 hours at 47,000 x g at 4°C. The centrifuge was decelerated to 3000 rpm and the rotor contents displaced with 45% w/w sucrose at a flow rate of approximately 7.5 ml per min through a tygon tube (inside diameter 1 mm). Fractions (25 ml each) were collected in plastic bottles and used immediately for protein determination and enzyme assays. Fresh or frozen (at -16°C) material was employed for the binding and displacement assays.

Electron Microscopy—Adrenal tissue was cut into 1-mm cubes and fixed in 2% glutaraldehyde, 0.1 M cacodylate buffer pH 7.2, 5 mm MgCl₂, and postfixed in the same buffer containing 1% osmium tetroxide. Membrane fractions were doubly fixed in suspension in identical buffers. The cytochemical assay for 5'-nucleotidase, and other procedures for electron microscopy have been described (24).

Binding and Displacement Studies—For the binding studies, fresh or thawed fractions from the zonal centrifugation were diluted with a 1:1 v/v mixture of medium and 37% sucrose w/w to a protein concentration of 200 μg per ml. Aliquots of these suspensions (1 ml) were added to a series of centrifuge tubes containing various proportions of [GTP]-[32P-P]-corticotropin₉₋₂₄ amide (radioactive ACTH) in water and the volume was adjusted to 2 ml with water. The tubes were shaken and kept at 0°C for 10 min; they were then centrifuged for 30 min at 20,000 x g and samples (0.5 ml each) were withdrawn and added to scintillation fluid (15 ml) for determination of radioactivity (19). A minimum of 5000 counts was collected.

The displacement studies were performed in the manner described (19).

RESULTS AND DISCUSSION

The procedure described here differs in several respects from methods which have been employed for the preparation of liver plasma membranes (25, 26). The methods of Neville and Emmelot so successfully used for the isolation of these organelles affored in our hands impure particulates in extremely low yield. Similar findings were reported by Kelly and Koritz (27). Numerous washing steps which serve to free the liver preparation from mitochondrial contamination have been eliminated because large membrane losses were incurred in these operations. The Emmelot separation is based on the observation that liver membranes, because of their high content of desmosomes, form long sheet-like structures which sediment readily in low centrifugal fields. The adrenal cortical membranes fragment into smaller particles during homogenization, presumably because of a low desmosome content (Fig. 1) and thus higher centrifugal fields are required for sedimentation. The use of higher gravitational forces results in a greater degree of mitochondrial contamination; however, equilibration of partially purified adrenal homogenates for 16 hours in a zonal rotor containing a shallow sucrose gradient allows separation of beef adrenal mitochondria from plasma membranes.

Electron micrographs of representative fields in a pellet ob-
FIGS. 1 to 5. Electron micrographs of bovine adrenal cortex, and membrane fractions obtained as described in the text.

Fig. 1. Representative field showing the plasma membranes of two adjacent cells of the zona fasciculata of the bovine adrenal gland. Note the virtual absence of desmosomes and junctional complexes. × 15,000.

Fig. 2. Representative field of membrane Fraction 3. The preparation consists mainly of vesicles, many of which have a circumference greater than 2 μm. The fraction also contains myelin fragments (My) and partially disorganized lipofuscin granules (LG). Mitochondria and mitochondrial fragments were observed rarely. × 15,000.

The preparation contained large numbers of vesicles, together with occasional membranes which showed free ends, a feature commonly observed in isolated plasma membrane fractions. In addition, myelin fragments and partially disorganized lipofuscin granules were observed occasionally; very few mitochondria were present. The two denser fractions, 4 and 5, contained the same components, and in addition a progressively increasing proportion of mitochondria; fractions obtained from the middle or bottom of the gradient consisted of mitochondria with very few large vesicles or other membranes present.

These results suggested strongly that the lightest fractions from the gradient contained a marked enrichment of cortical

Fig. 3. The same preparation as Fig. 2, shown at higher magnification. The vesicles show the expected unit membrane structure (arrow), and occasionally membranes with free ends are observed (F). × 55,000.

Figs. 4 and 5. Membrane Fraction 4 was incubated cytochemically for 5'-nucleotidase. The reaction product was concentrated both in vesicles (Fig. 4) and in membranes with free ends (arrow, Fig. 5). Many vesicles showed no reaction product, and mitochondria were unstained (Mi, Fig. 5). × 15,000.
plasma membranes. Supporting evidence for this conclusion was obtained by studying 5'-nucleotidase activity in the fractions by an electron microscopic cytochemical procedure (24), developed for the localization of this enzyme in subcellular fractions from rat liver. 5'-Nucleotidase is concentrated in the plasma membranes of rat liver (24) and of other tissues where its activity has been determined (28-32). Figs. 4 and 5 show that when gradient Fraction 4 was incubated for 15 min in the presence of 5'-AMP and Pb (NO₃), and prepared for electron microscopy, precipitates of lead phosphate were concentrated both in large vesicles (Fig. 4) and in membranes with free ends (Fig. 5). Controls incubated with 2'-AMP showed little or no reaction product, and when other gradient fractions were examined, essentially similar staining patterns were observed.

The morphological appearance of the membrane fraction was influenced by the techniques used for preparation. The fractions described here were obtained after dissection of the glands under 0.25 M sucrose. If the dissection was carried out on an ice bath a greater proportion of long (5 to 8 μm) membranes with free ends was observed; some of these membranes had formed myelin figures, whereas others appeared intensely osmophilic at irregular intervals. This was tentatively ascribed to partial dehydration of the tissue during dissection.2

The zona fasciculata of the adrenal contains capillary endothelial cells in addition to cortical and nerve cells (33). It is almost certain that the membrane fraction described here contains plasma membranes derived from all three cell types. Attempts to subfractionate the membranes further using shallower sucrose gradients have not been successful.

The bovine adrenal cortex is extremely rich in mitochondria (Fig. 1) and these organelles are the principal constituents of homogenates of this tissue. Indeed the distribution of protein through the sucrose gradient (Fig. 6d) coincides with the distribution of the mitochondrial marker enzyme cytochrome oxidase (Fig. 6c). Determination of the plasma membrane marker enzyme 5'-nucleotidase throughout the gradient (Fig. 6b) indicates that tubes 3 to 5, the lightest fractions, contain 5'-nucleotidase of the highest specific activity. Likewise, the specific activity of fluoride stimulated adenylate cyclase (Fig. 6a) is highest in the same region of the gradient.

The location of the peak specific activity of these two enzymes varied from preparation to preparation but was always located between Fractions 3 to 5 and maximal activity of both enzymes always coincided.

The ability of the various fractions from the zonal centrifugation to bind radioactive ACTH was next investigated. The results (Fig. 7) show that this steroidogenically active compound binds more avidly to the lighter zonal fractions with a binding constant of approximately 10⁹ M⁻¹ (34, 35).

Fig. 6. Distribution of protein and marker enzymes through the gradient from the zonal centrifugation: (a) fluoride-stimulated adenylate cyclase (unit corresponds to picomoles of cyclic AMP per mg of protein/15 min); (b) 5'-nucleotidase (unit corresponds to 1 pmole of inorganic phosphate liberated per min per mg of protein); (c) cytochrome oxidase (unit corresponds to oxidation of 1 nmole of reduced cytochrome c per min per mg of protein); (d) protein concentration in milligrams per ml.

Binding studies with ACTH and ACTH fragments must be interpreted cautiously since these peptides have a great tendency to bind nonspecifically to many materials such as glass, talc, and BSA. The affinity of the mitochondrial fractions for radioactive ACTH may be due to contamination with plasma membrane fragments or may be another example of nonspecific binding. On the basis of available evidence (36) the presence of ACTH receptors on adrenal cortical mitochondrial membranes cannot be excluded, but it should be noted that adenylate cyclase activity is highest in the lightest fractions from the zonal centrifugation and is virtually absent from the fractions where mitochondria are present in the greatest concentration (Fig. 6).

In a previous communication (19) we have observed binding of radioactive ACTH to a crude particulate fraction from bovine adrenal cortex and the specificity of this binding was supported by competitive displacement experiments with nonradioactive ACTH fragments and analogs. As a rule, peptides exhibiting in vivo steroidogenic activity such as corticotropin₁₀,₁₈ (II) displaced radioactivity from the complex, whereas biologically inactive peptides [Gly₁₄] corticotropin₁₀,₁₈ and [Gly₁₄] [N'formyl-Lys¹] α-MSH (X) had little or no affinity for the particulate. Peptide VI was an exception as will be discussed later. Similar experiments (Fig. 8) have now been conducted using the membrane preparation with comparable results. In complete agreement with our previous findings corticotropin₁₀,₁₈ (II) displaced

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2 We are grateful to Dr. G. E. Palade for this suggestion.
Fig. 7. Binding of radioactive ACTH to various fractions from the zonal centrifugation (see text for experimental details). •, Fraction 4; ○, Fraction 7; □, Fraction 11; Δ, Fraction 15. Addition of 2.08 × 10^-4 mmoles of radioactive ACTH corresponds to 1000 counts.

Radioactivity from the complex, likewise, pig ACTH, on a molar basis, exhibited the same affinity for the membrane preparation. Structure-function studies conducted in several laboratories (9, 11, 37) have led to the discovery of ACTH fragments possessing an apparently higher in vivo corticotropic activity than the natural hormone. Peptides possessing this property are structurally modified on both the NH2 and COOH termini. Two representatives of this class of ACTH fragments (compounds III and IV) have now been tested for their ability to compete with radioactive ACTH for binding to the membrane preparation. As may be seen (Fig. 8) these peptides compete as effectively as corticotropin-24 (II) which is a considerably less active steroidogenic agent in vivo. It follows that the apparently higher biological potency of these ACTH fragments is not the result of greater affinity for the binding sites on the membrane preparation, but, as has been suggested (37), appears to reflect increased stability to cleavage by exopeptidases.

It is of interest to note that the binding sites on the plasma membranes apparently do not discriminate between the charged side chains of lysine and arginine as concerns Positions 17 and 18 (peptide III). The biological equivalence of corticotropin-24 and [diornithine-17,18] corticotropin-24 has been demonstrated by rat assay (38).

In agreement with its low in vivo corticotropic activity, [δ-Ser1] corticotropin-18 amide (V) binds less firmly to the membranes than the more active peptides. Interestingly, corticotropin-18 containing a free carboxyl group at the COOH terminus showed even less affinity for the previously described crude particulate (19). This observation suggests that the presence of a free carboxyl group at this site interferes with binding.

Using the beef adrenal particulate it was shown (19) that the steroi derogically inactive peptide VI displaces radioactive ACTH. The finding that the same peptide competes with radioactive ACTH for membrane binding sites (Fig. 8) supports the previous observation. This peptide contains the important binding site (39) -Lys-Lys-Arg-Arg- but lacks the amino acid sequence -His-Phe-Arg-Trp-Gly- necessary for function.

In order to define more specifically the importance for binding of individual lysine residues in peptide VI we synthesized peptides VII to IX and evaluated their ability to bind to the membranes (Fig. 8). In these peptides the ε-amino groups of the lysines in Positions 11, 13, and 16 are singly protected by formyl groups. All three peptides were less effective competitors than peptide VI. The marked decrease in binding which accompanies protection of the ε-amino group of lysine-11 suggests a role for this lysine in the binding process.

Comparison of adenylate cyclase stimulation by corticotropin-24 and sodium fluoride

Table II

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<th>Additions</th>
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<td>None</td>
<td>83</td>
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<tr>
<td>Corticotropin-24 (10 μg)</td>
<td>213</td>
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<tr>
<td>NaF (0.01 M)</td>
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* Tissue boiled for 2 min prior to incubation. See text for details of assay.
The finding that \([\text{Gln5}]\) \([\text{Tetra Nf-formyl-Lys11~1S~16~z1}]\) N-acetyl discovery of the high biological activity of corticotropin-20 binding sites occupy the segment comprising Positions 14 to 23. NH2 terminal region spanning Positions 1 to 13 and important ACTH (39). We subdivided the ACTH molecule into active enzyme activation mechanism to explain the mode of action of is approximately lo+ moles per liter.

VI. Methionine is a likely candidate since it is known (45) that oxidation of the thioether to the sulfoxide drastically reduces the 21 from further consideration. The presence of additional bind-ments on the basis of adenylate cyclase assays.

At an early stage in our studies with ACTH analogs (4, 42), it was indeed surprising to find (Fig. 9) that peptides III and IV whose in v ivo activity is significantly higher than that of corticotropin-13-23 were consistently less active in activating adenylate cyclase. The high binding capacity of these peptides to the membranes makes this result difficult to explain. Further investigation of this very interesting observation is warranted, however, the results suggest that caution should be exercised in attempts to predict in v ivo potency of ACTH analogs and fragments on the basis of adenylate cyclase assays.

At an early stage in our studies with ACTH analogs (41, 42), we observed that shortening the peptide chain of corticotropin-13-23 (II) resulted in a gradual decrease in adrenocorticotropin activity reaching very low, but reproducible, levels with corticotropin-12. Based on these admittedly preliminary data we proposed an enzyme activation mechanism to explain the mode of action of ACTH (39). We subdivided the ACTH molecule into active sites and binding sites. The active sites were located in the NH2 terminal region spanning Positions 1 to 13 and important binding sites occupy the segment comprising Positions 14 to 23. The finding that \([\text{Gln5}]\) [Tetra \(N^+\)-formyl-Lys]-[13,14,16,22] \(N^+\)-acetyl corticotropin-12 amide possessed an adrenocorticotropin activity (12, 43) focused attention on the lysine side chains and particularly on the sequence \(\text{Lys-Lys-Arg-Arg}\) located in positions 15 to 18 in the ACTH molecule as likely binding sites. The discovery of the high biological activity of corticotropin-15-23 amide (44) and of still shorter fragments (9, 11) eliminated lysine-21 from further consideration. The presence of additional binding-sites in the region spanning positions 1 to 10 is indicated by comparison of the relative binding capacities of peptides II and VI. Methionine is a likely candidate since it is known (45) that oxidation of the thioether to the sulfoxide drastically reduces the steroidogenic potency of ACTH. The sulfur atom cannot be essential for function since the replacement of methionine by \(\alpha\)-amino-n-butyric acid (13) or norleucine (37) provides biologically active analogs.

A large body of information has since accumulated and the results are in excellent agreement with these early postulates. Invariably, certain amino acid replacements in the active site or substitutions of basic amino acids in the binding site by residues containing uncharged side chains abolished or drastically reduced adrenocorticotropin potency in vivo. These results are based on biological assays of the various analogs using intact animals or quartered adrenals. Although this type of evaluation may answer the question whether or not an analog exhibits biological activity, it is not adequate to distinguish whether the inactivity of a given analog is the result of poor binding to the specific receptor or lack of function or possibly both of these. The readily accessible membrane preparation described in this communication provides a means to correlate peptide structure and in vivo corticotropin activity with selective binding and adenylate cyclase activation. Using this ACTH receptor preparation, it should be possible to delineate those structural elements of ACTH involved in binding and those essential for adenylate cyclase activation.

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REFERENCES


\footnote{Footnote  Added in Proof—The apparent \(K_a\) for this peptide is approximately \(10^{-3}\) moles per liter.}
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