CHARACTERIZATION OF ACTH RECEPTORS IN BRAIN AND ADRENAL

by

© Zhen-guo Li

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Therapeutics
University of Manitoba, Faculty of Medicine
Winnipeg, Manitoba

ISBN 0-315-76682-4

CHARACTERIZATION OF ACTH RECEPTORS IN BRAIN AND ADRENAL

BY

ZHEN-GUO LI

A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
of the degree of

DOCTOR OF PHILOSOPHY

TABLE OF CONTENTS

ABSTRACT

Adrenocorticotropin (ACTH) receptors in brain and adrenal were characterized at levels of specific binding and post-receptor events. The high affinity binding of ^{125}I-ACTH$_{1-24}$ to membrane from bovine adrenal cortex was inhibited by vasoactive intestinal peptide (VIP), growth hormone releasing factor (GRF) and dynorphin$_{1-13}$ (DYN), but not by other peptides tested. These same peptides each enhanced steroidogenesis in isolated bovine adrenal cortical cells in a dose-dependent manner. ACTH competed for ^{125}I-VIP binding in membranes from rat brain and bovine adrenal cortex. The combination of VIP and ACTH, each at submaximally effective concentration, was additive in stimulating cortisol production, whereas each combined at a maximally effective concentration caused no additive response. These results, in conjunction with previous findings that the high affinity binding of ^{125}I-ACTH$_{1-24}$ to membrane from rat brain was potently inhibited by VIP, GRF and DYN, indicate that these peptides interact with a common species of receptor for ACTH.

Maximally effective concentrations of ACTH$_{1-10}$ and ACTH$_{11-24}$ each elicited cortisol secretion in isolated or cultured bovine adrenocortical cells that was only about one-half that by ACTH$_{1-24}$. The combination of ACTH$_{1-10}$ and ACTH$_{11-24}$ promoted cortisol release to the maximal level elicited by ACTH$_{1-24}$. Maximal cortisol release by ACTH$_{11-24}$, but not by ACTH$_{1-10}$ or ACTH$_{1-24}$, was enhanced by forskolin. Vanadium in a dose-dependent fashion inhibited cortisol secretion elicited by ACTH$_{1-10}$ and ACTH$_{1-24}$, but not by ACTH$_{11-24}$. In bovine adrenal membranes, both ACTH$_{1-10}$ and ACTH$_{1-24}$ induced cAMP formation, whereas ACTH$_{11-24}$ was inactive. These observations indicate that there are at least two classes of receptors mediating ACTH$_{1-24}$ dependent steroid secretion, one recognizing ACTH$_{1-10}$ and coupled with the cAMP messenger pathway, and the other

recognizing $ACTH_{11-24}$ and linked to a non-cAMP messenger system.

Maximal cortisol production in response to VIP, GRF and DYN was only about one-half that by $ACTH_{1-24}$. There was an additive steroidogenic effect of VIP plus $ACTH_{1-10}$ but not VIP plus $ACTH_{11-24}$. Specific binding of $^{125}I\text{-}ACTH_{11-24}$ in adrenal membranes was inhibited by unlabelled VIP, GRF and DYN, but not by $ACTH_{1-10}$, peptide T (PEP T) and other peptides, e.g. thyrotropin releasing hormone (TRH), α-melanocyte stimulating hormone (α-MSH) and β-endorphins (β-END). These binding and functional studies, together with the existence of homologous amino acid sequences, indicate that VIP, GRF and DYN compete for a subpopulation of ACTH receptors that recognizes a moiety within the 11-24 sequence of the ACTH molecule.

Introduction

The previous finding in this laboratory that high affinity specific binding of ^{125}I-ACTH$_{1-24}$ to rat brain membrane was potently inhibited by VIP, GRF and DYN raised the question as to whether these binding sites represent receptors for ACTH?. To resolve this question, and as an extension of the first successful demonstration achieved by our laboratory of high affinity specific ^{125}I-ACTH binding sites in rat brain, the ACTH receptor has been further investigated in terms of 1) interaction of ACTH binding sites with other neuropeptides mentioned above, and 2) the cellular effects mediated by ACTH binding sites.

Interaction of ACTH, VIP, GRF and DYN for binding sites

Although high affinity binding sites have been described in brain for most of neuropeptides, those for ACTH binding in brain have been elusive, probably due to an extraordinary property of ACTH to adhere to surfaces and the loss of biological activity during iodination of ACTH molecule. After siliconizing all labware with SurfaSil, adding BSA to ACTH preparations, and using a biologically active radioligand, ^{125}I-[Phe^2,Nle^4]ACTH$_{1-24}$, two specific high affinity ^{125}I-ACTH binding sites were revealed with dissociation equilibrium constants (K_D) of 0.65 ± 0.47 nM and maximal binding capacity (B_{max}) of 21 ± 14 fmol/mg; and low affinity site with K_D of 97 ± 48 nM and B_{max} of 3.6 ± 1.8 pmol/mg (Hnatowich et al., in press).

VIP was discovered in V. Mutt's laboratory in Stockholm from one of the peptide fractions which remained after the purification of secretin from hog upper intestinal tissue (Said and Mutt, 1970, 1972). Porcine VIP is a highly basic peptide with 28 amino acid residues and belongs to

a family of peptides composed of secretin, glucagon, gastric inhibitory peptide (GIP), growth hormone releasing hormone (GRF), peptide with N-terminal histidine and C-terminal isoleucine amide (PHI), human peptide with N-terminal histidine and C-terminal methionine (PHM), corticotropin-releasing factor (CRF), sauvagine, urotensin I, and helodermin (Said, 1986). VIP has been paid more attention in this study because of its stimulation of steroidogenic and cAMP formation. Binding of VIP to brain membranes was first reported in guinea pig by Robberecht et al. (1978). Two classes of binding sites were shown, one with an apparent K_D of 36 nM and B_{max} of 4 pmol/mg protein and a second with an apparent K_D of 285 nM and B_{max} of 20 pmol/mg protein. The specificity of the VIP receptor was examined by the same group in 1979. Among the nine peptides tested, only VIP, [Gln^9,Asn^{15}] $secretin_{5-27}$, [Val^5] secretin and secretin were shown to inhibit the binding of ^{125}I-VIP, and VIP was at least 1000-fold more potent than the three other peptides. Binding of ^{125}I-VIP to membrane from rat brain and the distribution of the binding sites in the rat brain were reported by Taylor and Pert (1979). A single class of VIP binding was suggested with a K_D of 1.0 nM and a B_{max} of 2.2 pmol/g of wet tissue. Staun-Olsen et al. (1982) reported the specific binding of ^{125}I-VIP to isolated synaptosomes from rat cerebral cortex. Two classes of VIP binding sites were revealed. K_D values were 4.1 and 45 nM, which were smaller than what Robberecht et al. reported. This discrepancy may be due to the difference in animal species. Binding sites for VIP were also characterized in dispersed pineal gland cells (Kaku et al., 1983).

GRF was discovered in pancreatic tumors obtained from two patients (Guillemin et al., 1982; River et al., 1982). It is of interest that GRF interacts with VIP receptor in rat and human intestinal epithelial cell

membranes (Laburthe et al., 1983), guinea pig pancreatice acini (Pandol et al., 1984), rat pancreatic plasma membranes (Waelbroeck et al., 1985), and, like VIP, stimulates adenylate cyclase activity in these preparations. There are no reports on an interaction of DYN with ACTH/VIP receptors

There are several ways to analyse binding data, such as Scatchard, Rosenthal, double-reciprocal (analogous to the Lineweaver-Burk plot) and Hill plots. With these plots, the binding parameters (K_D and B_{max}) are obtained. A more sophisticated, although conceptually similar, method is the nonlinear curve-fitting program, LIGAND, which was developed by Munson and Rodbard (1984). This program resolves more complex systems by iteratively determining two or more fits to the primary binding data. One first assumes a single high-affinity receptor site model and allows the program to find the best fit. Then one assumes a two receptor site model and allows the computer to attempt to fit. Indicators of goodness of fit, including residual variance and a runs test, are calculated by the program. Eventually the 'right' model will be judged according to these statistical indicators. The LIGAND program can readily resolve a single high affinity binding site, a binding system containing two binding sites, and multiple binding components.

Steroidogenesis and dual ACTH receptor

The key property of a binding site which defines it as a receptor is its association with physiological effect, i.e. only a binding site which is coupled to a response may be considered a receptor (Burt, 1978; Hollenberg & Cuatrecasas, 1979). At the present time there are no specific assay systems for examination of action of neuropeptide in the

central nervous system, probably because neuropeptides usually cause a broad spectrum of effect in CNS (see review by LaBella et al., 1985). In contrast, ACTH has been firmly established to stimulate steroid secretion from adrenal cortex. In vitro systems, Hechter (1949) first reported ACTH stimulated corticosteroid release from perfused adrenal glands of beef and sheep. Haynes and Savard (1952) first reported that ACTH induced steroid secretion from adrenal slices. Saffran et al. (1952) reported that production of corticoids was induced by ACTH from rat adrenal halves. From late 60's, the action of ACTH have been extensively studied on the isolated adrenal cells. These isolated cells were prepared by either collaganase (Kloppenborg et al., 1968; Haning et al., 1970; Rivkin & Chasin, 1971; Richardon & Schulters, 1972; Moyle et al., 1973; Falke et al., 1975; Goverde et al., 1980; Goverde & Smals, 1984) or trypsin treatment (Sayers et al., 1971; Schwyzer et al., 1971; Seelig & Sayers, 1971; Kitabchi & Sharma, 1971; Nakamura & Tanaka, 1971; Lowry et al, 1973; Fehm et al., 1973; Gewirtz et al., 1974; Liotta and Krieger, 1977) or both (Finn et al, 1976). Effects of ACTH on steroidogenesis have been shown in bovine adrenal cells in primary culture (Hornsby & Gill, 1978; Goodyer et al., 1976; Kramer et al., 1983). Therefore the steroidogenic activity can be used to investigate the post-receptor events by measuring cortisol content in the medium from isolated or cultured bovine adrenocortical cells with RIA.

VIP exerts steroidogenic activity in cultured murine adrenal tumor cells (Kowal et al., 1977; Morera et al., 1979), rat ovarian granulosa cells (Davoren and Hsueh, 1985), testicular cells (Kasson et al., 1986) and in rat (Nussdorfer and Mazzocchi, 1987; Cunningham and Holzwarth, 1988) and amphibian (Leboulenger et al., 1984) adrenal glands. In our

hand, VIP stimulates steroidogenesis in isolated and cultured bovine adrenocortical cells, although the maximal response is only about half that by ACTH. One of the explanations for this lower efficacy of VIP in stiulating steroid secretion is that VIP binds to only one class of ACTH receptors.

A few studies have proposed the existence of two classes of ACTH receptors. Early binding studies carried out in rabbit adrenal glands, mouse adrenocortical tumor cell membranes and isolated rat adrenocortical cells demonstrated two classes of binding sites for ACTH (Lefkowitz et al., 1971; Wolfsen et al, 1972; McIlhinney and Schulster, 1975). Later, Moyle et al. (1973) reported that the effect of ACTH on cAMP formation, but not on steroidogenesis, was inhibited by an 0-nitrophenyl sulfenyl derivative of ACTH, NPS-ACTH, indicating two types of receptors on the adrenal cells were involved in the effect of ACTH on cAMP formation and steroidogenesis. Yanagibashi et al. (1978) demonstrated two binding sites for ACTH in isolated adrenal cortical cells and found K_D value of ACTH effect on steroidogenesis in the presence of Ca^{++} was almost identical to that for the high affinity ACTH binding sites, while the K_D value of ACTH effect on cAMP formation was very close to that for the low affinity ACTH binding sites. They suggested that steroidogenesis or cAMP formation was linked to either one class of ACTH receptor. Bristow et al. (1980) observed that $ACTH_{6-24}$ inhibited the action of $ACTH_{1-39}$ and its full agonist, $ACTH_{5-25}$, to different extents, and pointed out that two different receptor types might be involved in the steroidogenic action of these two agonists. Schwyzer (1980) proposed that the steroidogenic action of ACTH was mediated by two receptors: receptor A which was lined to cAMP pathway and receptor B which was coupled with

non-cAMP pathway. Recently, the dual ACTH receptor concept was confirmed and extended by Kojima et al (1985) and Rasmussen et al. (1986). They provided evidence to support the concept that both types of ACTH receptors (type I and type II) on adrenal cortical cell membrane mediate steroid production via different 'second messenger pathway', i.e. receptor I was linked to calcium influx and receptor II was coupled with cAMP messenger system.

The relationship between ACTH structure and two classes of receptors has not been clarified. ACTH molecule has 39 amino acids. The 1-24 sequence is the active site of ACTH (full agonist for all effects of ACTH in vitro and in vivo) and the 25-39 sequence contains information about stability and the species label (Schwyzer, 1980). Within the 1-24 sequence, Schwyzer (1980) suggested there are three message sequences and two auxiliary sites (address site and potentiator site). The address site (the 11-18 sequence) adds receptor-specific affinity, and the potentiator (the 1-3 sequence) enhances potency and intrinsic activity. The three message sequences activate different receptors. Message I is located with N-terminal region (probably the 4/6-9 sequence, depending on animal species), which triggers receptors in adipocytes, adrenal cortex cells, melanophores and central neurons; message II is located in the centre of ACTH molecule (the 11-13 sequence), which triggers the melanophore receptor only; and message III is located in the 20-23 region and triggers a subtype of receptor on the rat adipocyte. Among three messages, only message I appears to be related to steroidogenesis. He correlated message I with two classes of receptor mediating steroidogenesis such that message I provides signal information for activating receptor B, and message I in conjunction with the adjacent

methionine residue may contribute to activation of receptor A. In contrast, two distinct centres in the ACTH molecule for steroidogenesis are predicted by dual adrenal receptor concept. Goverde and Smals (1984) reported the 11-24 sequence of ACTH molecule possesses the steroidogenic effect, thus providing evidence to support the existence of the second steroidogenic centre in ACTH molecule. In this study we tested $ACTH_{10}$ and $ACTH_{11-24}$ on steroidogenesis via different receptor mechanisms. Furthermore, we have tested whether VIP, GRF and DYN compete for one type or both types of ACTH receptors as well.

Second Messenger and Dual ACTH Receptors

The interaction of ACTH with adrenocortical cell receptors results in an activation of intracellular sigal mediators (second messenger), including cAMP and Ca^{++}.

1. Calcium

Calcium has been diversely implicated in the mechanism of action of ACTH on steroidogenesis: (a) ACTH-mediated steroid production requires Ca^{++}. Birmingham et al. (1953) reported that increased steroid production by ACTH in rat adrenal halves was abolished when calcium was absent from the incubation medium. Later, other studies showed that ACTH failed to stimulate steroidogenesis in the absence of Ca^{++} from sectioned rat adrenal (Péron and Koritz, 1958), isolated rat adrenal cells (Sayers et al., 1972) and isolated perfused cat adrenals (Jaanus et al., 1970). (b) ACTH enhances clacium uptake. Leier and Jungmann (1973) and Kojima et al. (1985) demonstrated an enhanced uptake of ^{45}Ca by rat adrenal slices and isolated calf adrenal cells incubated in the presence of ACTH;

(c) calcium can replace ACTH in triggering steroidogenesis in isolated rat adrenal cortical cells (Neher and Milani, 1978) and in rat adrenal homogenates (Koritz and Péron, 1959); (d) Activation of adenylate cyclase by ACTH requires the presence of Ca^{++} (Bär and Hetchter, 1969, Lefkowitz et al., 1970); (e) Calcium at 1 mM concentration, stimulates the conversion of cholesterol to pregnenolone, which is the rate-limiting step of adrenal steroidogenesis, from the rat adrenal mitochondria preparations (Simpson et al., 1978); and (f) Calmodulin, a calcium binding protein, plays a role in the regulation of steroid production. Hall et al. (1981) showed that when trifluoperazine (an inhibitor of calmodulin) was added to the medium, the ACTH-mediated steroidogenesis from mouse adrenal tumor cells (y-1 cell) was inhibited. Carsia et al. (1982) reported that ACTH-induced steroidogenesis in isolated rat adrenal cortical cells was inhibited by another calmodulin inhibitor, chlorpromazine. In the light of the foregoing facts, calcium has been suggested as a 'second messenger' in ACTH-mediated steroidogenesis.

2. cAMP

Haynes and Bathet (1957) first reported that ACTH caused a rapid and specific activation of phosphorylase in steer adrenal slices. Haynes (1958) showed that when ACTH is added to incubation medium of bovine adrenal cortex slices, cAMP accumulates with the tissue, and when cAMP is added to the incubation medium, phosphorylase activity is increased, indicating that the activation of adrenal cortical phosphorylase by ACTH is mediated through cAMP. Later, Haynes et al. (1959) demonstrated that addition of cAMP to an incubation of slices of rat adrenal cortex

stimulated steroidogenesis, whereas addition of substances closely related to cAMP had no effect. It was proposed that ACTH acts to increase the content of cAMP within the adrenocortical cells and that cAMP, as the intracellular mediator of ACTH, elicits steroidogenesis. Since then many studies demonstrate the role of cAMP on ACTH-induced steroidogenesis (Grahame-Smith et al., 1967; Taunton et al., 1969; Schulster et al., 1970) and support the above concept. Recently, John et al. (1986) using cDNA specific for enzymes of the steroidogenic pathway indicated that ACTH stimulates steroid hydroxylase gene expression through the action of cAMP. However, it is generally recognized that there is no consistent association between ACTH-induced steroidogenesis and cAMP formation (Beall and Sayer, 1972; Mackie et al., 1972; Bowyer and Kitabchi, 1974, Sharma et al., 1976; Honn and Chavin, 1977; Ramachandran et al., 1987). The concentration of ACTH required for half-maximal activation of steroid production is smaller than that needed for half-maximal activation of adenylate cyclase in rat adrenal cortical cells. To explain this discrepancy, several hypothesis have been proposed. Ramanchandran et al. (1987) suggested the receptor-reserve model that only a small fraction of and cAMP formed in response to ACTH is needed for steroidogenesis. Another suggestion is the compartment guidance concept that an acute action of ACTH at low concentration is through a mechanism utilizing pre-existing background amounts of cAMP (Schulster and Schwyzer, 1980). Bristow et al. (1980) proposed that ACTH stimulated steroidogenesis by a pathway involved cAMP and another pathway completely independent of cAMP formation (dual receptor concept). Evidence has accumulated to indicate two types of ACTH receptors on adrenocortical cells, one coupled to Ca^{++} and one to cAMP.

In this study, cAMP formation induced by ACTH fragments has be measured to determine the correlation with specific types of ACTH receptors. The formation of cAMP from ATP may be assayed in vitro by measuring the production of radioactively labeled cyclic AMP from the substrate [α-^{32}P]ATP. Difficulty in this assay results from the fact that only a minute fraction, usually less than 0.05% of the substrate, ATP, is converted to cAMP. Assay sensitivity and reliability are therefore largely dependent upon and proportional to the efficiency with which cAMP is separated from the labeled ATP and its breakdown products. Krishna et al. (1968) reported that the separation of cAMP from ATP may be accomplished by employing chromatography on Dowex cation exchange resin, followed by teatment with $ZnSO_4$ - $Ba(OH)_2$ to remove most of the remaining ATP and its breakdown products. White and Zenser (1971) have shown that the bulk of ATP may be separated from cAMP by chromatography on aluminum oxide. However, neither of these methods resulted in the complete elimination of ^{32}P in the assay blanks. Later, Solomon et al. (1974) showed that a combination of chromatography on both Dowex cation exchange resin and aluminum oxide permitted the nearly complete elimination of radioactivity in the assay blanks. Therefore, the sequential Dowex-Aluminum oxide chromatography is used in this study to examine cAMP formation in response to ACTH fragments.

References

Bar HP and Hechter. Adenyl cyclase and hormone action III. Calcium requirement for ACTH stimulation of adenyl cyclase. Biochem. Biophys. Res. Commun. 35: 681-686, 1969.

Beall RJ and Sayer G. Isolated adrenal cells: Steroidogenesis and cyclic AMP stimulation in response to ACTH. Arch. Biochem. Biophys. 148: 70-76, 1972.

Birmingham MK, Elliott FH and Valero HLP. The need for the presence of calcium for the stimulation in vitro of rat adrenal glands by adrenocorticotrophic hormone. Endocrinol. 53: 687-689, 1953.

Bowyer F and Kitabchi AE. Dual role of calcium in steroidogenesis in the isolated adrenal cell of the rat. Biochem. Biophys. Res. Commun. 57: 100-105, 1974.

Bristow AF, Gleed C, Fauchere JL and Schwyzer R. Effects of ACTH (corticotropin) analogues on steroidogenesis and cyclic AMP in rat adrenocortical cells. Biochem. J. 186: 599-603, 1980.

Burt DR. Criteria for receptor identification. In: Yamamura HI, Enna SJ and Kuhar MJ eds. Neurotransmitter Receptor Binding. Raven Press, New York, 41-55, 1978.

Carsia RV, Moyle WR, Wolff DJ and Malamed S. Acute inhibition of corticosteroidogenesis by inhibitors of calmodulin action. Endocrinol. 111: 1456-1461, 1982.

Cunningham LA and Holzwarth MA. Vasoactive intestinal peptide stimulates adrenal aldosterone and corticosterone secretion. Endocrinol. 122: 2090-2093, 1988.

Davoren JB and Hsueh AJW. Vasoactive intestinal peptide: a novel stimulator of steroidogenesis by cultured rat granulosa cells. Biol. Reprod. 33: 37-52, 1985.

Falke HE, Degenhart HJ, Abelin GJA, Visser HKA and Croughs RJM. Studies on isolated rat adrenal cells. I. Continuous flow and batch incubation. Acta Endocrinol (Kbh). 78: 110-121, 1975.

Fehm HL, Voight KH, Lang R, Ozyol MB and Pfeiffer EF. Influence of plasma on ACTH stimulated corticosterone production of isolated adrenal cells. FEBS Lett. 36: 109-112, 1973.

Finn FM, Johns PLA, Nishi N and Hofmann K. Differential response to adrenocorticotropic hormone analogs of bovine adrenal plasma membranes and cells. J. Biol. Chem. 251: 3576-3585, 1976.

Gewirtz G, Schneider B, Krieger DT and Yallow RS. Big ACTH: conversion to biologically active ACTH by trypsin. J. Clin. Endocrinol. Metab. 38: 227-230, 1974.

Goodyer CG, Torday JS, Smoth BT and Giroud CJP. Preliminary observation of bovine adrenal fasciculator-reticularis cells in monolayer culture: steroidogenesis, effect of ACTH and cyclic AMP. Acta Endocrinol. 89: 373-385, 1976.

Goverde HJM, Pesman GJ and Benraad TJ. Improved sensitivity to drenocorticotrophin after purification and pre-incubation of isolated rat adrenal cells. Acta Endocrinol. 94: 221-228, 1980.

Goverde HJM and Smals AGH. The anomalous effect of some ACTH fragments missing the amino acid sequence 1-10 on the corticosteroidogenesis in purified isolated rat adrenal cells. FEBS Lett. 173: 23-26, 1984.

Grahame-Smith DG, Butcher RW, Ney KL and Sutherland EW. Adenosine 3',5'-monophosphate as the intracelular mediator of action of adrenocorticotrpic hormone on the adrenal cortex. J. Biol. Chem. 242: 5535-5541, 1967.

Guillemin R, Brazeau P, Bohlen P, Esch F, Ling N and Wehrenberg WB. Growth hormone-releasing factor from a human pancreatic tumour that caused acromegaly. Science 218: 585-587, 1982.

Hall PF, Osawa S and Thomasson CL. A role for calmodulin in the regulation of steroidogenesis. J. Cell. Biol. 90: 402-407, 1981.

Haning R, Tait SAS and Tait JF. In vitro effects of ACTH, angiotensins, serotonin and potassium on steroid output and conversion of corticosterone to aldosterone by isolated adrenal cells. Endocrinol. 87: 1147-1167, 1970.

Haynes RC, Jr. The activation of adrenal phsophorylase by the adrenocorticotropic hormone. J. Biol. Chem. 233: 1220-1222, 1958.

Haynes RC, Jr. and Berthet L. Studies on the mechanism of action of adrenocorticotropic hormone. J. Biol. Chem. 225: 115-124, 1957.

Haynes RC, Jr, Koritz SB and Péron FG. Influence of adenosine 3',5'-monophosphate on corticoid production by rat adrenal glands. J. Biol. Chem. 234: 1421-1423, 1959.

Haynes R and Savard K. An action of ACTH on adrenal slice. Science 116: 690-691, 1952.

Hechter O. Corticosteroid release from the isolated adrenal gland. Fed. Proc. 8: 70-71, 1949.

Hnatowich MR, Queen G, Stein D and LaBella FS. ACTH receptors in nervous tissue. High affinity binding/sequestration of ^{125}I-[Phe^{2},Nle^{4}]ACTH$_{1-24}$ in homogenates and slices from rat brain. Canad. J. Physiol. Pharmacol., in pres.

Hofmann K, Wingender W and Finn FM. Correlation of adrenocorticotropic activity of ACTH analogs with degree of binding to an adrenal cortical particulate preparation. Proc. Natl. Acad. Sci. USA 67: 829-836, 1970.

Hollenberg MD and Cuatrecasas P. Distinction of receptor from nonreceptor and interaction in binding studies. In: The Receptors (Ed. O'Brian RD) Vol 1, pp. 193-214, Plenum, New York, 1979.

Honn KV and Chavin W. In vitro temporal cAMP and cortisol responses to ACTH by the normal human adrenal gland. Acta Endocr. 85: 823-831, 1977.

Hornsby PJ and Gill GN. Characterization of adult bovine adrenocortical cells throughout their life span in tissue culture. Endocrinol. 102: 926-936, 1978.

Jaanus SD, Rosenstein MJ and Rubin RP. On the mode of action of ACTH on the isolated perfused adrenal gland. J. Physiol. 209: 539-556, 1970.

John ME, John MC, Boggaram V, Simpson ER and Waterman MR. Transcriptional regulation of steroid hydroxylase genes by corticotropin. Proc. Natl. Acad. Sci. USA 83: 4715-4719, 1986.

Kaku K, Isoue Y, Matsutani A, Okubo M, Hatac K, Kaneko T and Yanaihara N. Receptors for vasoactive intestinal polypeptide on rat dispersed pineal cells. Biomed. Res. 4: 321-328, 1983.

Kasson BG, Lim P and Hsueh AJW. Vasoactive intestinal peptide stimulates androgen biosynthesis by cultured neuronatal testicular cells. Mol. Cell. Endocrinol. 48: 21-29, 1986.

Kitabachi AED and Sharma RK. Corticosteroidogenesis in isolated adrenal cells of rats. I. Effect of corticotropins and 3',5'-cyclic nucleotides on corticosterone production. Endocrinol. 88: 1109-1116, 1971.

Kloppenborg PWC, Island DP, Liddle GW, Michelakis AM and Nicholson WE. A method of preparing adrenal cell suspensions and its applicability to the in vitro study of adrenal metabolism. Endocrinol. 82: 1053-1058, 1968.

Kojima I, Kojima K and Rasmussen H. Role of cAMP in the action of adrenocorticotropin in aldosterone secretion. J. Biol. Chem. 260: 4248-4256, 1985.

Koritz SB and Péron FG. The stimulation in vitro by Ca^{++}, freezing and proteolysis of corticoid production by rat adrenal tissue. J. Biol. Chem. 234: 3122-3125, 1959.

Kowal J, Horst J, Pensky J and Alfonzo M. A comparison of effects of ACTH, vasoactive intestinal peptide and cholera toxin on adrenal cAMP and steroid synthesis. Ann. N.Y. Acad Sci. 247: 314-328, 1977.

Kramer RE, McCarthy JL, Simpson ER and Waterrman M. Effects of ACTH on steroidogenesis in bovine adrenocortical cells in primary culture-increased secretion of 17α-hydroxylated steroid associated with a refractoriness in total steroid output. J. Steroid Biochem. 18: 715-723, 1983.

Krishna G, Weiss B and Brodie BB. A simple sensitive method for the assay of adenyl cyclase. J. Pharmacol. Exp. Ther. 163: 379-385, 1968.

LaBella FS, Geiger JD and Glavin G. Administered peptides inhibit the degradation of endogenous peptides. The dilemma of distinguishing direct from indirect effects. Peptides, 6: 645-660, 1985.

Laburthe M, Amiranoff B, Boige N, Rouyer-Fessard C, Tatemoto K and Moroder L. Interaction of GRF with VIP receptors and stimulation of adenylate cyclase in rat and human itnestinal epithelial membranes. Comparison with DHI and secretion. FEBS Lett. 159: 89-921, 1983.

Leboulenger F, Perroteau I, Netchitalile P, Liharmann I, Leroux P, Delarue C, Coy DH and Vaudry H. Action of vasoactive intestinal peptide (VIP) on amphibian adrenocortical function in vitro. Peptides 4: 299-303, 1984.

Lefkowitz RJ, Roth J and Pastan I. Effect of calcium on ACTH stimulation of the adrenal: separation of hormone binding from adenyl cyclase activation. Nature 228: 864-866, 1970.

Leier DJ and Jungmann RA. Adrenocorticotropic hormone and dibutyryl adenosine cyclic monophosphate-mediated Ca^{++} uptake by rat adrenal glands. Biochim. Biophys. Acta 329: 196-210, 1973.

Liotta A and Krieger D. An improved bioassay for ACTH determination. Endocr. Res. Commun. 4: 159-169, 1977.

Lowry RJ, McMastin C and Peters J. Properties of a simplified bioassay for adrenocorticotrophic activity using the steroidogenic response of isolated adrenal cells. J. Endocrinol. 59: 43-55, 1973.

Mackie C, Richardon MC and Schulster D. Kinetics and dose-response characteristics of adenosine 3',5'-monophosphate production by isolated rat adrenal cells stimulated with adrenocorticotrophic hormone. FEBS Lett. 23: 345-348, 1972.

McIlhinney RAJ and Schulster D. Studies on the binding of ^{125}I-labelled corticotropin to isolated rat adrenocortical cells. J. Endocr. 64: 175-184, 1975.

Morera AM, Cathiard AM, Laburthe M and Saez JM. Interaction of vasoactive peptide (VIP) with a mouse adrenal cell line (y-1): specific binding and biological effects. Biochem. Biophys. Res. Commun. 90: 78-85, 1979.

Moyle WR, Kong YC and Ramachandran J. Steroidogenesis and cyclic adenosine 3',5'-monophosphate accumulation in rat adrenal cells: divergent effects of adrenocorticotropin and its 0-nitrophenyl sulfenyl derivative. J. Biol. Chem. 248: 2409-2417, 1973.

Munson PJ and Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107: 220-239, 1984.

Nakamura M and Tanaka A. A simple method for the preparation of ACTH responsive isolated cells from rat adrenals. Endocrinol. Jpn. 18: 291-299, 1971.

Neher R and Milani A. Steroidogenesis in isolated adrenal cells: excitation by calcium. Mol. Cell. Endocrinol. 9: 243-253, 1978.

Nussdorfer GG and Mazzocchi G. Vasoactive intestinal peptide stimulates aldosterone secretion by rat adrenal gland in vivo. J. Steroid Biochem. 26: 203-205, 1987.

Pandol SJ, Seifert H, Thomas MW, Rivier J. and Vale W. Growth hormone-releasing factor stimulates pancreatic enzyme secretion. Science 225: 326-328, 1984.

Péron FG and Koritz SB. On the exogenous requirements for the action of ACTH in vitro on rat adrenal glands. J. Biol. Chem. 233: 256-259, 1958.

Ramachandran J, Tsubokawa M and Gohil K. Corticotropin receptors. Ann. N.Y. Acad. Sci. 512: 415-425, 1987.

Rasmussen H. Cell communication, calcium ion, and cyclic adenosine monophosphate. Science 170: 404-412, 1970.

Rasmussen H, Kojima I, Apfeldorf W and Barrett P. Messenger function of calcium and cyclic AMP. Kidney International 29: 90-97, 1986.

Richardson MC and Schulster D. Corticosteroidogenesis in isolated adrenal cells: effect of adrenocorticotrophic hormmone, adenosine 3',5'-monophosphate and B^{1-24} adrenocorticotrophic hormone diazotized to polyacrylamide. J. Endocrinol. 55: 127-139, 1972.

Rivier J, Spiess J, Thorner M and Vale W. Characterization of a growth hormone-releasing factor from a human pancreatic islet tumour. Nature 300: 276-278, 1982.

Rivkin I and Chasin M. Nucleotide specificity of the steroidogenic response of rat adrenal cell suspension prepared by collagenase digestion. Endocrinol. 88: 664-670, 1971.

Robberecht P, Neef PD, Lammens M, Deschodt-Lanckman M and Christophe JP. Specific binding of vasoactive intestinal peptide to brain membranes from the guinea pig. Eur. J. Biochem. 90: 147-154, 1978.

Robberecht P, Konig W, Deschodt-Lanckman M, Neef PD and Christophe J. Specificity of receptors to vasoactive intestinal peptide in guinea pig brain. Life Sci. 25: 879-884, 1979.

Saffran M, Grad B and Bayliss MJ. Production of corticoids by rat adrenals in vitro. Endocrinol. 50: 639, 1952.

Said SI. Vasoactive intestinal peptide. J. Endocrinol. Invest. 9: 191-200, 1986.

Said SI and Mutt V. Polypeptide with broad biological activity: Isolation from small intestine. Science 169: 1217-1218, 1970.

Said SI and Mutt V. Isolation from porcine intestinal wall of a vasoactive octacosapeptide related to secretion and glucagon. Eur. J. Biochem. 28: 199-204, 1972.

Sayers G, Beall RJ and Seelig S. Isolated adrenal cells: adrenocorticotropic hormone, calcium, steroidogenesis, and cyclic adenosine monophosphate. Science 175: 1131-1135, 1972.

Sayers G, Swallow RI and Giordano ND. An improved technique for the preparation of isolated rat adrenal cells. A sensitive, accurate and specific method for the assay of ACTH. Endocrinol. 88: 1063-1068, 1971.

Schulster D and Schwyzer R. ACTH receptors. In: Schulster D and Levitzki A eds. Cellular Receptors for Hormones and Neurotransmitters. John Wiley & Sons. London, 197-217, 1980.

Schulster D, Tait SAS, Tait JF and Mrotek J. Production of steroids by in vitro superfusion of endocrine tissue III. Corticosterone output from rat adrenals stimulated by adrenocorticotropin or cyclic 3',5'-adenosine monophosphate and the inhibitory effect of cycloheximide. Endocrinol. 86: 487-502, 1970.

Schwyzer R. Organization and transduction of peptide information. Trends Pharmacol. Sci. 1: 327-331, 1980.

Schwyzer R. Structure and function in neuropeptide. Proc. Roy. Soc. London B 210: 5-20, 1980.

Schwyzer R, Schiller P, Seelig S and Sayers G. Isolated adrenal cells: log dose response curves for steroidogenesis induced by $ACTH_{1-24}$, $ACTH_{1-10}$, $ACTH_{4-10}$ and $ACTH_{5-10}$. FEBS Lett. 19: 229-231, 1971.

Seelig S and Sayers G. Structure activity relationship among ACTH. Fed. Proc. 30: 698, 1971.

Simpson ZR, McCarthy JL and Petersen JA. Evidence that cycloheximide-sensitive site of adrenocorticotropic hormone action is in the mitochondrion. J. Biol. Chem. 253: 3135-3139, 1978.

Sharma RK, Ahmed NK and Shanker G. Metabolic regulation of steroidogenesis in isolated adrenal cells of the rat. Eur. J. Biochem. 70: 427-433, 1976.

Solomon Y, London C and Rodbell M. A highly sensitive adenylate cyclase assay. Anal. Biochem. 58: 541-548, 1974.

Staun-Olsen P, Ottesen B, Bartels PD, Nielsen MH, Gammeltoft S and Fahrenkrug J. Receptors for vasoactive intestinal polypeptide on isolated synaptosomes from rat cerebral cortex. Heterogeneity of binding and desensitization of receptors. J. Neurochem. 39: 1242-1251, 1982.

Taunton OD, Roth J and Pastan I. Studies on the adrenocorticotropic hormone-activated adenyl cyclase of a functional adrenal cortex. J. Bio. Chem. 244: 247-243, 1969.

Taylor DP and Pert CB. Vasoactive intestinal polypeptide. Specific binding to rat brain membranes. Proc. Natl. Acad. Sci. USA. 76: 660-664, 1979.

Waelbroeck, M., Robberecht P, Coy DM, Camus JC, Neef PD and Chrostophe J. Interaction of growth hormone-releasing factor and 14 GRF analogs with VIP receptors of rat pancreas. Discovery of (N-Ac-Tyr^1,D-Phe^2) GRF (1-29)-NH_2 as a vasoactive intestinal peptide antagonist. Endocrinol. 116: 2643-2649, 1985.

White AA and Zenser TV. Separation of cyclic 3',5'-nucleotide monophosphate from other nucleotides on aluminum oxide columns. Analyt. Biochem. 41: 372-396, 1971.

Wolfsen AR, McIntyre MB and Odell WD. Adrenocorticotropin measurement by competitive binding receptor assay. J. Clin. Endocr. 34: 684-689, 1972.

Yanagibashi K, Kamiya N, Lin G and Matsuha M. Studies on adrenocorticotropic hormone receptor using isolated rat adrenocortical cells. Endocrinol. Jap. 25: 545-551, 1978.

$ACTH_{1-10}$ AND $ACTH_{11-24}$ PROMOTE ADRENAL STEROIDOGENESIS BY DIFFERENT MECHANISMS

Zhen-guo Li, Donald Park and Frank S. LaBella

University of Manitoba, Faculty of Medicine
Department of Pharmacology and Therapeutics
770 Bannatyne Avenue
Winnipeg, Manitoba, Canada R3E 0W3

Endocrinology, in press.

Key Words: ACTH, adrenal cortex, cyclic AMP, cortisol, cell cultures, peptides.

Corresponding Author: F.S. LaBella
University of Manitoba
Faculty of Medicine
Department of Pharmacology & Therapeutics
770 Bannatyne Avenue
Winnipeg, Manitoba, Canada R3E 0W3

FOOTNOTES

Abbreviations: ACTH, adrenocorticotropin; AII, angiotensin II; BSA, bovine serum albumin; INS, insulin; Met-Enk, methionine enkephalin; SP, substance P; TRF, thyrotropin releasing hormone.

ABSTRACT

$ACTH_{1-10}$ and $ACTH_{11-24}$ each elicit cortisol secretion submaximally in freshly dispersed or cultured beef adrenal cortical cells. The combination of $ACTH_{1-10}$ and $ACTH_{11-24}$ promotes cortisol release to the maximal level elicited by $ACTH_{1-24}$. Maximal cortisol release by $ACTH_{11-24}$, but not by $ACTH_{1-24}$ or $ACTH_{1-10}$, was enhanced by forskolin. Calcium channel blockers, nifedipine and verapamil, inhibited cortisol release by $ACTH_{1-10}$, $ACTH_{1-24}$ or $ACTH_{11-24}$, suggesting calcium influx to be essential for steroid secretion irrespective of the secretogogue. Vanadium in a dose-dependent manner inhibited cortisol secretion elicited by $ACTH_{1-24}$ and $ACTH_{1-10}$ but not by $ACTH_{11-24}$. These results suggest that there are at least two receptors mediating $ACTH_{1-24}$- dependent steroid secretion. One class of receptor recognizes $ACTH_{1-10}$ but not $ACTH_{11-24}$ and is linked to the cAMP messenger pathway.

INTRODUCTION

ACTH stimulates steroidogenesis in adrenocortical cells by at least two distinct mechanisms: both appear to involve specific cell membrane receptors, but only one class of receptor recruits the formation of cyclic AMP in the signal transduction process (1-6). Several laboratories have proposed that mobilization of intracellular calcium mediates steroidogenesis in response to a receptor species not linked to adenyl cyclase (4,5,7-9). Furthermore, an amino acid sequence containing the N-terminus seems to be essential for evoking steroidogenesis (10-12), and Bristow et al. (1) concluded that there are at least two different sequences involved, each utilizing a distinct receptor/effector mechanism. We now present evidence that the N-terminus sequence $ACTH_{1-10}$ stimulates steroidogenesis through the cAMP-linked receptor and $ACTH_{11-24}$ through activation of a receptor linked to another effector system.

EXPERIMENTAL

Materials. $ACTH_{1-24}$ and $ACTH_{1-10}$ were generously provided by Organon (W. Orange, NJ), $ACTH_{11-24}$ was purchased from Bachem (Torrence, CA); Collagenase Ia from Sigma (St. Louis, MO), DNase from Boehringer Mannheim Corp. (Dorval, Quebec), cortisol antiserum from Endocrine Science (Tarzana, CA), ^3H-cortisol from New England Nuclear (Boston, MA) and Ham's F-12 medium and Dulbeco's modified Eagle medium from Flow Laboratories (McLean, VA).

Preparation of dispersed adrenocortical cells. Fresh bovine adrenal glands were obtained from a local slaughterhouse and placed in Hank's Balanced Salt Solution (HBSS), pH 7.4, containing penicillin G 1000

IU/ml, streptomycin 1 mg/ml and gentamycin 40 ug/ml. Upon arrival at the laboratory the medium was changed. Adrenal glands were stripped of fat, bisected longitudinally, the medulla removed and the cortical portion dissociated from the capsule. Cortical tissue was minced with scissors, washed once with HBSS and incubated in HBSS containing sodium bicarbonate (2.0 g/l), HEPES (6.51 g/l), BSA (5 g/l), collagenase (2 mg/ml) and DNase (25 ug/ml) for 60 min at 37°C on a gyratory shaker. The undigested fragments were allowed to settle and the mixture decanted through four layers of sterile cheesecloth. The cells were obtained by centrifugation at 100 x g for 10 min and washed 3 times. The final pellet was resuspended in a mixture of Ham's F-12 medium and Dulbecco's modified Eagles medium (1:1) containing fetal calf serum (FCS) (10%), penicillin G (100 IU/ml), streptomycin (100 ug/ml), gentamycin (10 ug/ml) and fungizone (0.25 ug/ml) and preincubated overnight in a humid incubator at 37°C.

Overnight pre-incubated cell suspensions were washed twice with culture medium without FCS and antibiotics. The final pellet was resuspended with culture medium containing BSA (0.5%). Viability of free cells was determined with trypan blue and cell number counted with a hemocytometer. Aliquots of cell suspension containing about 0.5×10^6 cells/ml were incubated in the presence or absence of ACTH fragments or other treatment for 1 hour at 37°C under 95% O_2:5% CO_2. The cell cultures were rinsed three times with the same medium and incubated with/without treatment for 3 hours at 37°C in the CO_2 incubator. After incubation, the cell suspension was collected and centrifuged at 1000 x g for 10 min. Aliquots of the supernatant were analyzed for cortisol by radioimmunoassay.

Adrenal cell culture. In order to make use of the dispersed cell suspension efficiently, aliquots were plated in 5 cm petri dishes to give approximately 10 x 10^6 cells/plate for incubation. The medium was aspirated 3-4 days after plating and fresh medium with FCS and antibiotics was added. Thereafter, medium was replaced every 48 h for 5-14 days.

Radioimmunoassay of cortisol. Each centrifuge tube (1 ml) contained 50 ul of supernatant medium from incubation, 0.20 ml of the labelled antigen/antiserum mixture (12 ml of borate buffer, 0.05M, pH 8.0, 1,200,000 dpm of 1,2-^{3}H-cortisol, 0.30 ml of 2.5% human gamma globulin, 0.24 ml of 10% BSA, and 0.10 ml of gently but well mixed stock antiserum), mixed well and incubated for 1.5 hours at 37°C. Alternatively, the incubates were kept overnight at room temperature. To each tube 0.25 ml of saturated $(NH_4)_2SO_4$ was added, mixed thoroughly in a vortex shaker, and centrifuged at 3000 rpm for 10 min at room temperature. A 0.40 ml aliquot of supernatant was transferred to a counting vial containing 4 ml of scintillation fluid and mixed thoroughly. Unbound 1,2-^{3}H-cortisol was counted with a Beckman Liquid Scintillation β-counter and cortisol calculated by weighted linear regression.

RESULTS

Cortisol release from dispersed cells elicited by $ACTH_{1-10}$, $ACTH_{11-24}$ and $ACTH_{1-24}$ was compared with that from cultured cells (Table 1). Taking cortisol release induced by $ACTH_{1-24}$ as 100 per cent, there were no significant differences between the two cell preparations in response

to the ACTH fragments.

The dose-response curves for $ACTH_{1-10}$, $ACTH_{11-24}$ and $ACTH_{1-24}$-induced steroidogenesis are shown in Fig. 1. A stimulatory effect of $ACTH_{1-24}$ is detected at 0.1 nM. For $ACTH_{1-10}$ and $ACTH_{11-24}$, cortisol production was not significantly enhanced even at 1 uM peptide, but a response peak was achieved at 10 uM. The maximal responses to $ACTH_{1-10}$ and $ACTH_{11-24}$ were 52.7 and 47.8 per cent, respectively, of that to $ACTH_{1-24}$. Cortisol release was estimated in response to $ACTH_{1-24}$, $ACTH_{1-10}$ or $ACTH_{11-24}$ alone or in combination (Fig. 2). The combination of $ACTH_{1-10}$ and $ACTH_{11-24}$ significantly increased cortisol release compared to either peptide alone, and reached the same maximum as with $ACTH_{1-24}$. The combination of $ACTH_{1-24}$ plus either $ACTH_{1-10}$ or $ACTH_{11-24}$, however, resulted in an inhibition of cortisol release compared to that by $ACTH_{1-24}$ alone.

Several peptides, AII, SP, Met-Enk, TRF, INS, $ACTH_{1-10}$, $ACTH_{11-24}$, $ACTH_{1-24}$, were tested at 10 uM (Fig. 3). Only $ACTH_{1-24}$ and its fragments induced steroidogenesis significantly in adrenal cell suspensions.

Forskolin induces steroidogenesis in a dose-dependent manner, maximum response occuring with a 1 mM concentration (Fig. 4). Cortisol release by ACTH was measured in the presence and absence of forskolin (1 mM) (Fig. 5). Forskolin potentiated the steroidogenic effects of a maximally effective concentration of $ACTH_{11-24}$ but not of maximally effective concentrations of $ACTH_{1-24}$ or $ACTH_{1-10}$.

Vanadium is reported to block ACTH-, but not cAMP-mediated steroidogenesis (13). As shown in Fig. 6, vanadium inhibited $ACTH_{1-10}$ or $ACTH_{1-24}$ but not $ACTH_{11-24}$-induced cortisol release in a dose-dependent manner. An inhibitory effect of vanadium was detected at 0.1

uM.

To evaluate the role of calcium on the ACTH-induced steroid release, the effects of verapamil and nifedipine were examined. Both classes of calcium antagonist inhibited steroidogenesis evoked by $ACTH_{1-10}$, $ACTH_{1-24}$, or $ACTH_{11-24}$ (Table 2).

DISCUSSION

The present study confirms and extends the hypothesis of a dual messenger function in ACTH action on adrenal steroidogenesis. The results demonstrate that $ACTH_{1-10}$ and $ACTH_{11-24}$ each possesses steroidogenic activity, although either is much less potent than $ACTH_{1-24}$ and elicits only a fraction of the $ACTH_{1-24}$ response. Also, the additive effect of maximally effective concentrations of $ACTH_{1-10}$ and $ACTH_{11-24}$ on steroidogenesis suggests that the effects of the two fragments are mediated via separate receptors.

If actions of $ACTH_{1-10}$ and $ACTH_{11-24}$, indeed, are mediated via separate receptors, different second messenger pathways may be recruited. Rasmussen et al. (5) proposed that ACTH interacts with two species of receptors in the rat adrenocortical cell membrane. Activation of one receptor evokes steroidogenesis through cAMP production and of the other through calcium mobilization. We suggest that these two steroidogenically responsive receptors for ACTH correspond to $ACTH_{11-24}$ and $ACTH_{1-10}$ recognition sites.

Forskolin at 1 mM stimulates cAMP formation maximally in adrenal membrane preparations. Thus, enhancement by forskolin of maximal $ACTH_{11-24}$-induced cortisol release indicates that $ACTH_{11-24}$ site is not linked to cAMP formation. Conversely, that forskolin does not potentiate

maximal $ACTH_{1-10}$-induced steroidogenesis suggests that this receptor site is linked to cAMP production.

Vanadium inhibits steroid release by $ACTH_{1-24}$ or $ACTH_{1-10}$, but not $ACTH_{11-24}$ in a dose-dependent manner. According to Hayashi and Kimura (13), vanadium inhibits ACTH-stimulated formation of cAMP. Therefore, the differential effects of vanadium upon $ACTH_{1-10}$ and $ACTH_{11-24}$-induced steroidogenesis are consistent with the hypothesis that $ACTH_{1-10}$ stimulates steroidogenesis via cAMP production and $ACTH_{11-24}$ through another mechanism.

Calcium channel blockers, nifedipine and verapamil, inhibit the steroidogenesis by $ACTH_{1-10}$, $ACTH_{11-24}$ or $ACTH_{1-24}$, suggesting that calcium is required for release of steroid from adrenal cells irrespective of the receptor/effector mechanism.

Studies on the binding of I^{125}-ACTH in adrenal cells have indicated two populations of receptors (14,15). Bristow et al. (1), utilizing $ACTH_{1-39}$ and two analogues, $ACTH_{5-24}$, a full agonist and $ACTH_{6-24}$, a competitive inhibitor, reported that $ACTH_{6-24}$ antagonized to different extents the actions of $ACTH_{1-39}$ and $ACTH_{5-24}$, indicating that the steroidogenic action of the two agonists is not mediated through the same population of receptors. Schwyzer (3) proposed that the binding of ACTH to one species of adrenal receptor (receptor A) elicits steroidogenesis through the involvement of cAMP production and to a second species of receptor (receptor B) through another mechanism. $ACTH_{1-39}$ presumably interacts with both receptors, whereas $ACTH_{5-24}$ would tend to act at one receptor only. Rasmussen et al. (5) confirmed and extended the evidence in support of the dual messenger function of ACTH on adrenal steroidogenesis. They demonstrated that calcium plays a messenger role

in ACTH action and proposed that the binding of ACTH to its receptors initiates at least two events. A prompt and sustained increase in calcium influx via receptor 1 and activation of adenylate cyclase via receptor 2 gives rise to two intracellular messengers. They did not test whether or not specific ACTH fragments activate separate and distinct receptor/effector systems. Our finding that ACTH stimulates cortisol release through at least two classes of receptors is in agreement with the dual receptor concept.

Schwyzer (3) proposed three message sequences within the ACTH molecule: message I triggers receptors in adipocytes, adrenal cortex cells, melanophores and central neurons; message II triggers the melanophore receptors only; and message III triggers a subtype of receptor on the rat adipocyte. But only message I appears to be linked to steroidogenesis. Message I provides signal information for activating adrenal receptor B; and message I plus the adjacent methionine residue may be required for activation of adrenal receptor A. However, from our observations, two message sequences for steroidogenesis appear to be located more distant one from the other, and are situated in the 1-10 and 11-24 sequences, respectively. These two sequences are not restricted to the message I region of Schwyzer. The message sequence which activates receptor A seems to be located in the 1-10 region and may overlap with message I, whereas our results indicate that the message sequence which activates receptor B seems to be located in the 11-24 region. The existence of a second center within the ACTH molecule capable of inducing steroidogenesis was proposed by Goverde and Smals (16). They found a barely measurable steroidogenic activity of $ACTH_{11-24}$ or $ACTH_{11-19}$, and the potencies were comparable to that of the

$ACTH_{1-10}$ fragment, the latter finding being in agreement with us. They reported that $ACTH_{11-24}$ induced steroidogenesis with an equal efficacy to $ACTH_{1-39}$, whereas, in our hands, $ACTH_{11-24}$ evoked a partial response only. However, others failed to observe steroidogenic activity with $ACTH_{11-24}$ (17-19).

With respect to steroidogenic activity of $ACTH_{1-10}$, here again there is controversy. In early studies by Schwyzer et al. (12), $ACTH_{1-10}$ was said to elicit maximal steroid generation equivalent to that by $ACTH_{1-24}$, but with less potency. However, in agreement with our results, Hinson and Birmingham (19) reported $ACTH_{1-10}$ to elicit only a fraction of the $ACTH_{1-24}$ response.

ACKNOWLEDGEMENTS

Supported by grants from the Medical Research Council of Canada and the Health Sciences Centre Research Foundation (Winnipeg). Z. Li was supported by Studentships from the Manitoba Health Research Council and the MRCC. F.S. LaBella is a Career Investigator of the MRCC.

REFERENCES

1. Bristow A, Gleed C, Faucherz JL, Schwyzer R, Schulster D 1980 Effects of ACTH (corticotropin) analogues on steroidogenesis and cyclic AMP in rat adrenocortical cells. Biochem J 186:599-603

2. Schwyzer R 1980 Structure and function in neuropeptides. Proc Roy Soc London B 210:5-20

3. Schwyzer R 1980 Organization and transduction of peptide information. Trends Pharmacol Sci 1:327-331

4. Kojima I, Kojima K, Rasmussen H 1985 Role of calcium and cAMP in the action of adrenocorticotropin on aldosterone secretion. J Biol Chem 260:4248-4256

5. Rasmmussen H, Kojima I, Apfeldorf W, Barrett P 1986 Cellular mechanism of hormone action in the kidney: Messenger function of calcium and cyclic AMP. Kidney International 29:90-97

6. Moyle WR, Kong YC,, Ramachandran J 1973 Steroidogenesis and cyclic adensoine 3',5'-monophosphate accumulation in rat adrenal cells. J Biol Chem 248:2409-241

7. Rasmussen H, Barrett, P 1987 Temporal and spatial events in the calcium messenger system. In: Ehrlich YH, Lenox RH, Kornecki I, Berry WO (eds) Molecular Mechanisms of Neuronal Responses. Advances in Experimental Medicine and Biology. Plenum Press, New York, vol 221:19-31

8. Yanagibashi K 1979 Calcium ion as "second messenger" in corticoidogenic action of ACTH. Endocrinol Jap 26:227-232

9. Yanagibashi K, Kamiya N, Lin G, Matsuba M 1978 Studies on adrenocorticotropic hormone receptor using isolated rat adrenocortical cells. Endocrinol Jap 25:545-551

10. Seelig S, Sayers G 1973 Isolated adrenal cortex cells: ACTH agonists, partial agonists, antagonists, cyclic AMP and corticosterone production. Arch Biochem Biophys 154:230-2311.

11. Rae PA, Tsao J, Schimmer BP 1979 Evaluation of receptor function in ACTH-responsive and ACTH-insensitive adrenal tumor cells. Can J Biochem 57:509-516

12. Schwyzer R, Schiller P, Seelig S, Sayers G 1971 Isolated adrenal cells: Log dose response curves for steroidogenesis induced by $ACTH_{1-24}$, $ACTH_{1-10}$, $ACTH_{4-10}$ and $ACTH_{5-10}$ FEBS Lett 19:229-231

13. Hayashi Y, Kimura T 1986 Vanadium inhibits ACTH-mediated but not cyclic AMP-dependent adrenal steroidogenesis. Molec Cell Endocrinol 44:17-24

14. Lefkowitz RJ, Roth J, Pastan I 1971 ACTH-receptor interaction in the adrenal: a model for the initial step in the action of hormones that stimulate adenyl cyclase. Ann N Y Acad Sci 185:195-209

15. McIlhinney J, Schulster D 1975 Studies on the binding of ^{125}I-labelled corticotrophin to isolated rat adrenocortical cells. J Endocrinol 64:175-184

16. Goverde HJM, Smals AGH 1984 The anomalous effect of some ACTH-fragments missing the amino acid sequence 1-10 on the corticosteroidogenesis in purified isolated rat adrenal cells. FEBS Lett 173:23-26

17. Ramachandran J 1973 in: C H Li (ed) Hormonal Proteins and Peptides. Academic Press, New York Vol 12:1-28

18. Seelig S, Sayers G, Schwyzer R, Schiller P 1971 Isolated adrenal cells: $ACTH_{11-24}$, a competitive antagonist of $ACTH_{1-39}$ and $ACTH_{1-10}$. FEBS Lett 19:232-234

19. Hinson J, Birmingham MK 1987 ACTH and adrenal aerobic glycolysis II: Effects of aminoterminal peptide fragments on lactic acid and steroid production by mouse adrenocortical cells. J Endocrinol 115:71-76

Table 1. Cortisol release from adrenocortical cell suspensions and cell cultures.

	$ACTH_{1-10}$	$ACTH_{11-24}$
	(% of maximum)	
Cell suspension (n = 13)	51.7 ± 4.6	47.0 ± 3.5
Cell culture (n = 5)	54.5 ± 6.0	45.9 ± 7.0

Preparation of cell suspensions and cell cultures are described in "Methods". Results are expressed as per cent of maximal activity elicited by $ACTH_{1-24}$ at 1 uM. Peptide concentration was 10 uM. Mean ± S.E.M. The number of experiments performed in triplicate is shown in parentheses.

Table 2. Inhibitory effects of calcium channel blockers on steroidogenesis induced by ACTH.

	Control	Verapamil	Control	Nifedipine
	(ng cortisol/tube)			
$ACTH_{1-10}$	0.50 ± 0.07	0.17 ± 0.04**	0.45 ± 0.01	0.32 ± 0.05*
$ACTH_{11-24}$	0.52 ± 0.09	0.08 ± 0.03**	0.41 ± 0.03	0.29 ± 0.04*
$ACTH_{1-24}$	0.75 ± 0.05	0.23 ± 0.04***	0.73 ± 0.03	0.57 ± 0.03**

$ACTH_{1-10}$ (10 uM), $ACTH_{11-24}$ (10 uM) and $ACTH_{1-24}$ (1 uM). The cell suspensions were incubated with $ACTH_{1-10}$, $ACTH_{11-24}$ and $ACTH_{1-24}$ in the presence of either verapamil (1 uM) or nifedipine (1 uM). Data are shown as the mean ± S.D. of three replicates in a single experiment.

* $P < 0.05$ compared with control.

** $P < 0.01$ compared with control.

*** $P < 0.001$ compared with control. (t test)

LEGENDS

Fig. 1. Concentration-response curves for cortisol release from cell suspensions by $ACTH_{1-24}$, $ACTH_{1-10}$ and $ACTH_{11-24}$. Data are expressed as the mean ± S.E.M. of three experiments each in triplicate.

Fig. 2. Steroidogenic response of cell suspensions to ACTH fragments. Concentration of $ACTH_{1-24}$ was 1 uM, and of $ACTH_{1-10}$ and $ACTH_{11-24}$, 10 uM. Mean ± S.E.M. of three determinations peformed in triplicate.

(a) vs $ACTH_{1-24}$, NS;

(a) vs $ACTH_{1-10}$ or $ACTH_{11-24}$, $P < 0.01$;

(b) vs $ACTH_{1-24}$, $P < 0.01$;

(c) vs $ACTH_{1-24}$, $P < 0.01$ (Tukey's multiple comparison).

Fig. 3. Steroidogenic response of cell suspensions to several peptides at 10 uM except $ACTH_{1-24}$ and $ACTH_{1-39}$(1 uM). Data are shown as the mean ± S.E.M. Brackets are the numbers of separate experiments.

Fig. 4. Dose-response curve of forskolin-induced steroidogenesis from adrenal cell cultures. The data are expressed as the mean ± S.E.M. of two experiments, each in triplicate.

Fig. 5. Effect of forskolin on steroidogenesis promoted by ACTH. Adrenocortical cell cultures were incubated with $ACTH_{1-10}$ (10 uM), $ACTH_{11-24}$ (10 uM) and $ACTH_{1-24}$ (1 uM), each alone (open bar), or in the presence of forskolin (1 mM) (hatched bar). The results are expressed as the mean ± S.E.M. of three experiments each performed in triplicate.

(b) vs (a), $P < 0.01$;

(b) vs $ACTH_{1-24}$, NS;

(c) vs (a), NS;

(d) vs (a) $P < 0.01$;

(d) vs $ACTH_{11-24}$, $P < 0.01$ (Tukey's multiple comparison).

Fig. 6. Effect of sodium vanadate on steroidogenesis. Cell cultures were incubated with $ACTH_{1-24}$, $ACTH_{1-10}$ and $ACTH_{11-24}$ in the presence of various concentrations of sodium vanadate. Mean ± S.E.M. of two experiments, each in triplicate.

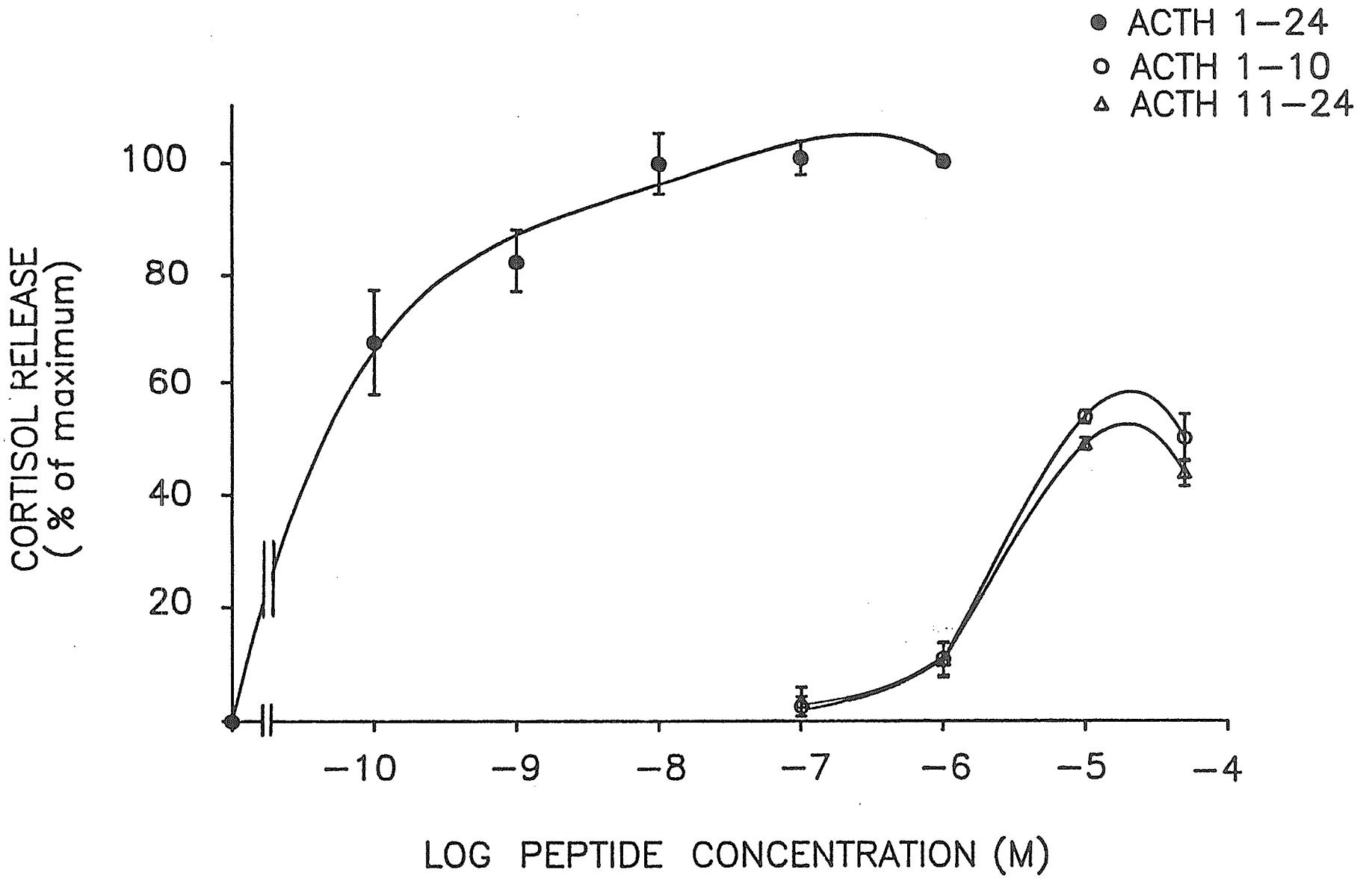

ACTH 1–24
ACTH 1–10
ACTH 11–24
CORTISOL RELEASE
(% of maximum)
100
80
60
40
20
–10
–9
–8
–7
–6
–5
–4
LOG PEPTIDE CONCENTRATION (M)

Fig. 1

Fig. 2

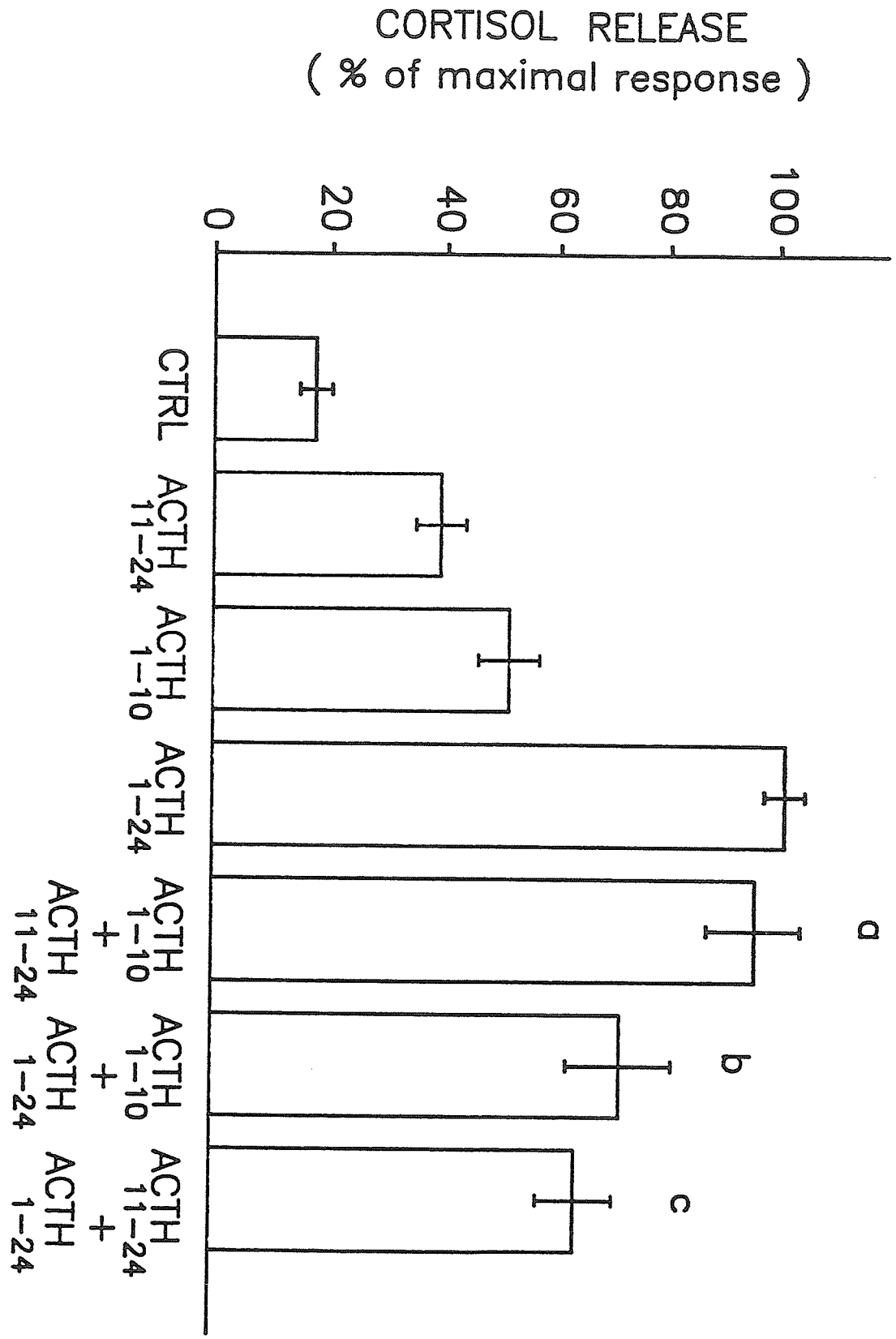

CORTISOL RELEASE
(% of maximal response)
0
20
40
60
80
100
CTRL
ACTH 11–24
ACTH 1–10
ACTH 1–24
ACTH 1–10 + ACTH 11–24
ACTH 1–10 + ACTH 1–24
ACTH 11–24 + ACTH 1–24
a
b
c

Fig. 3

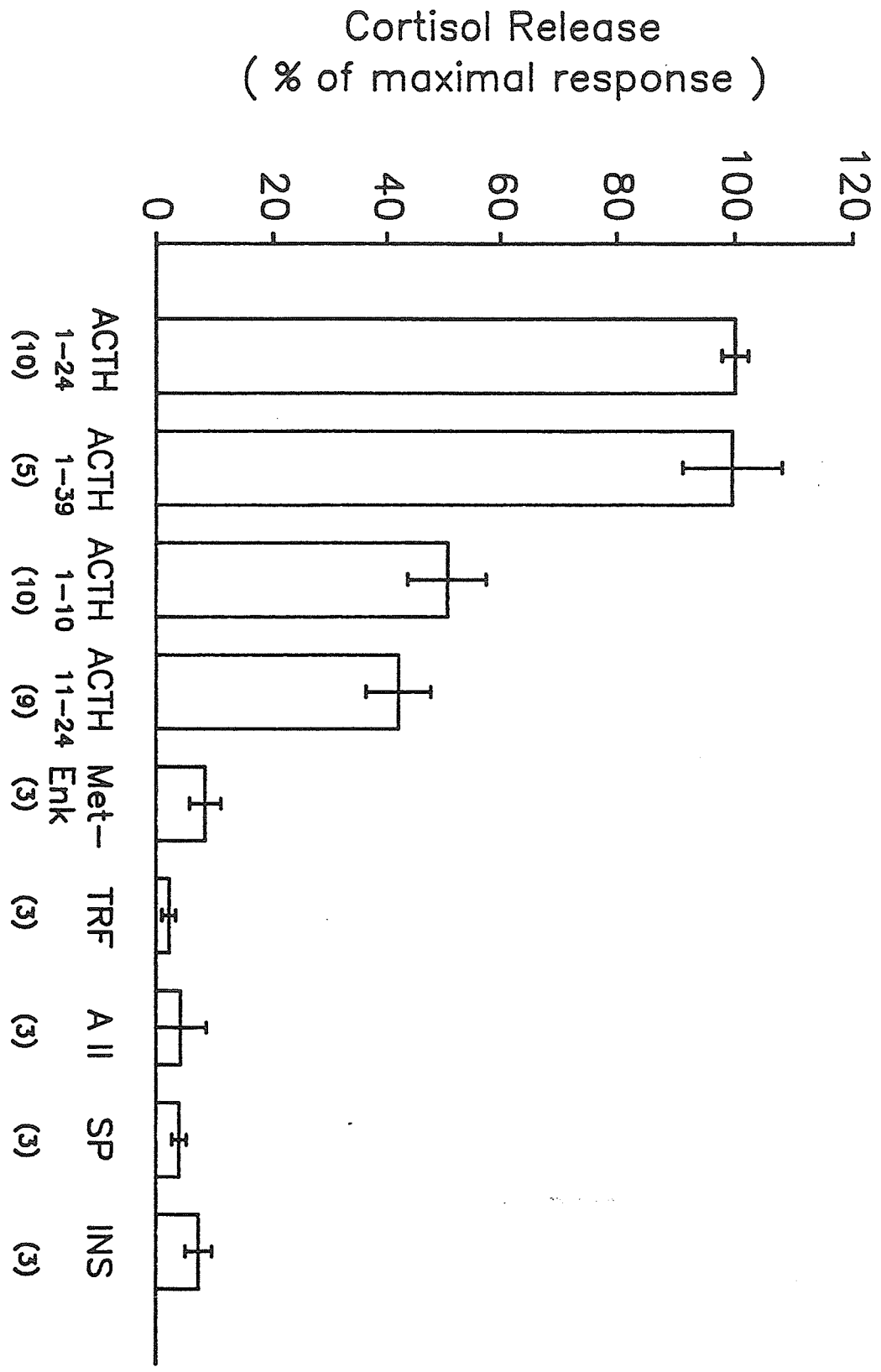

Cortisol Release
(% of maximal response)
0
20
40
60
80
100
120
ACTH 1–24 (10)
ACTH 1–39 (5)
ACTH 1–10 (10)
ACTH 11–24 (9)
Met–Enk (3)
TRF (3)
A II (3)
SP (3)
INS (3)

Fig. 4

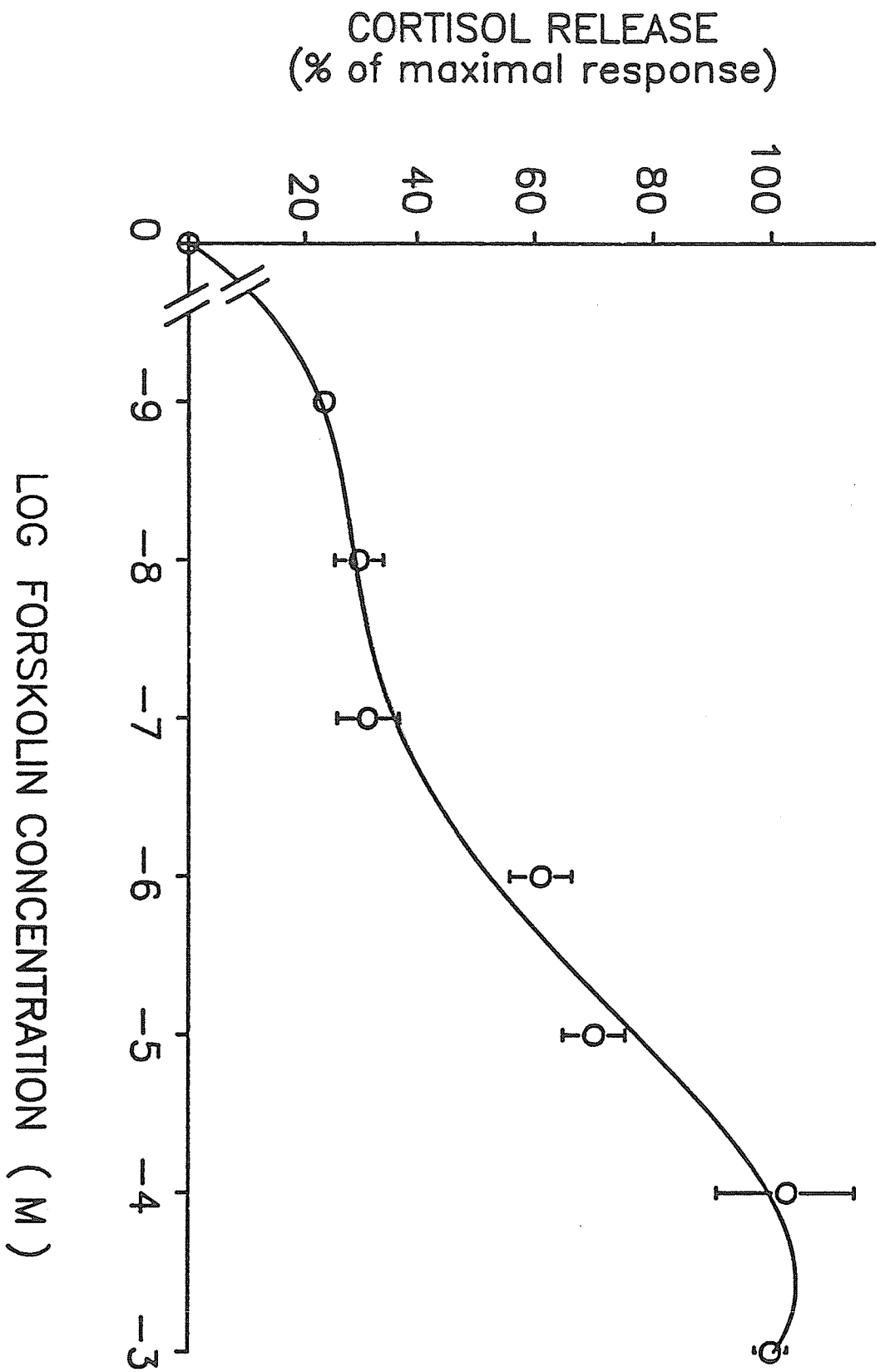

CORTISOL RELEASE
(% of maximal response)
0
20
40
60
80
100
-9
-8
-7
-6
-5
-4
-3
LOG FORSKOLIN CONCENTRATION (M)

Fig. 5

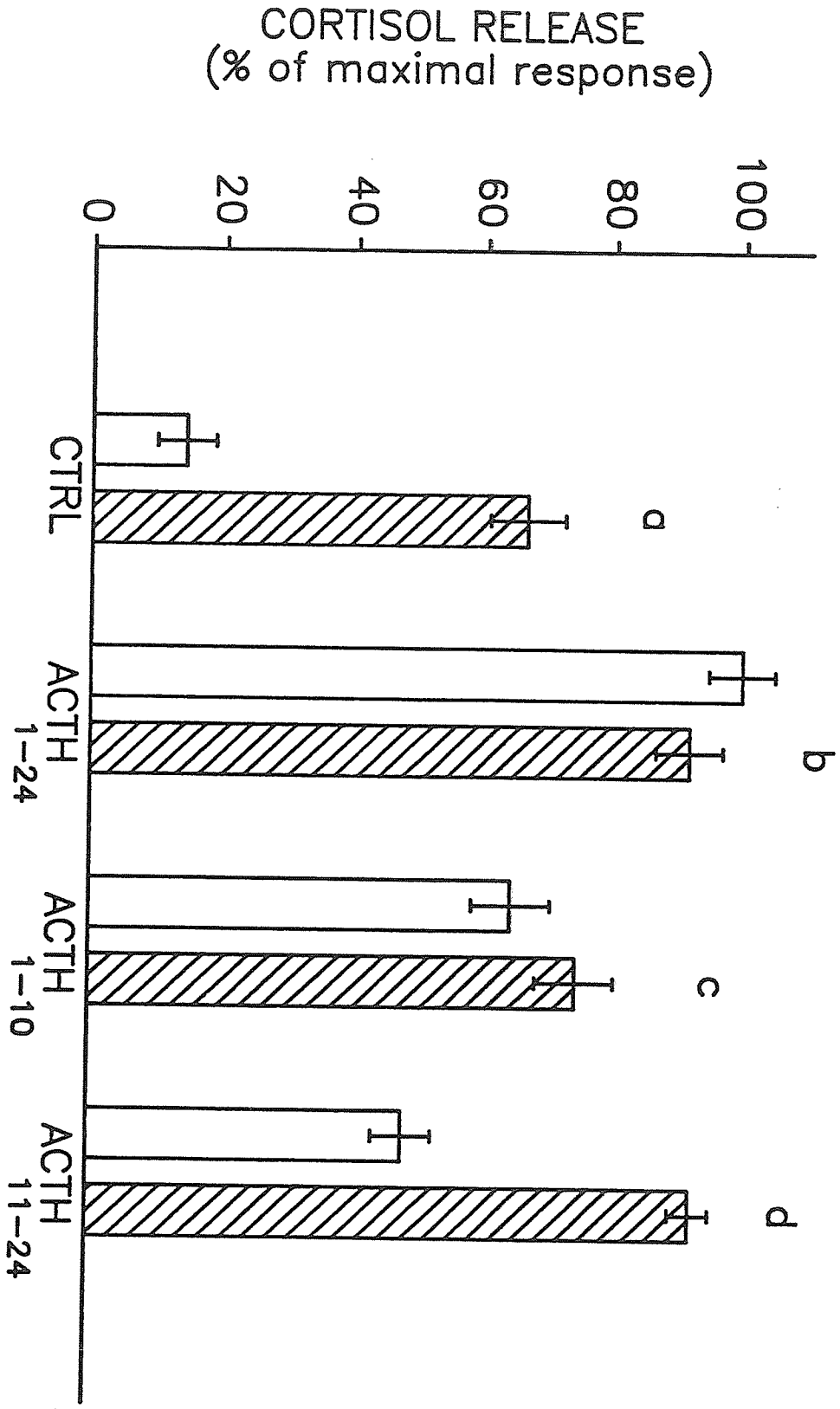
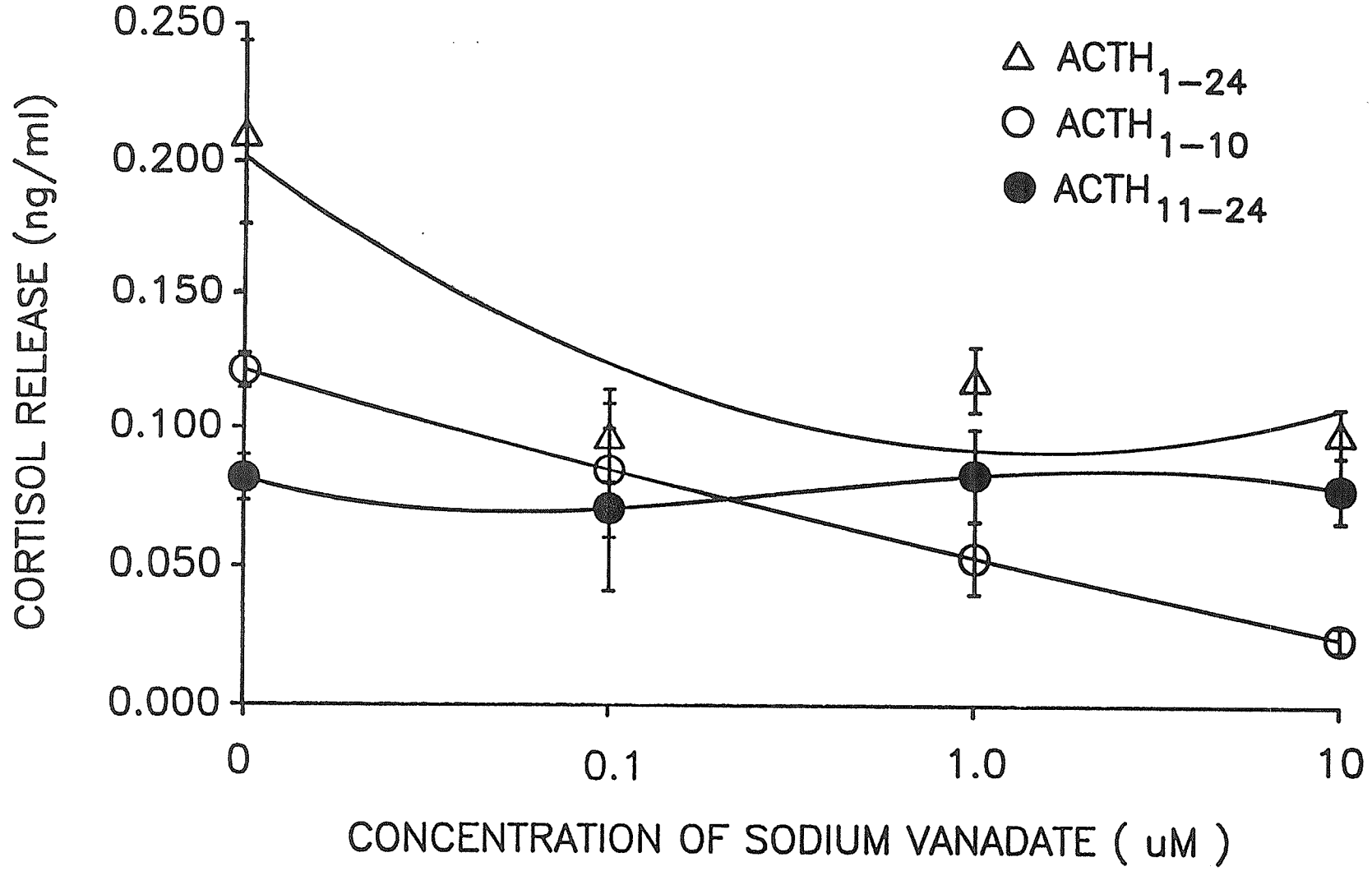

CORTISOL RELEASE
(% of maximal response)
0
20
40
60
80
100
CTRL
a
ACTH 1–24
b
ACTH 1–10
c
ACTH 11–24
d

Fig. 6


0.250
0.200
0.150
0.100
0.050
0.000
CORTISOL RELEASE (ng/ml)
ACTH1-24
ACTH1-10
ACTH11-24
0
0.1
1.0
10
CONCENTRATION OF SODIUM VANADATE (uM)

ACTH, VIP, GRF AND DYNORPHIN COMPETE FOR COMMON RECEPTORS IN BRAIN AND ADRENAL

Zhen-guo Li, Gary Queen and Frank S. LaBella

University of Manitoba, Faculty of Medicine
Department of Pharmacology & Therapeutics
770 Bannatyne Avenue
Winnipeg, Manitoba, Canada R3E 0W3

Corresponding author: Dr. Frank S. LaBella

Abbreviations used: ACTH: adrenocorticotropin; VIP: vasoactive intestinal polypeptide; GRF: growth hormone releasing factor; DYN: dynorphin$_{1-13}$; CRF: corticotropin releasing factor; BSA: bovine serum albumin; PEP T: Peptide T; MET-ENK: methionine-enkephalin; INS: insulin; AII: angiotensin II, SP: substance P; RIA: radioimmunoassay; β-END: β-endorphin; TRH: thyrotropin releasing hormone; α-MSH: α-melanocyte stimulating hormone.

ABSTRACT

It was previously shown in this laboratory that high affinity binding of ^{125}I-ACTH$_{1-24}$ to membranes from rat brain was inhibited by VIP, GRF and DYN, but not by other peptides tested. We now show that these same peptides compete for ^{125}I-ACTH$_{1-24}$ binding in membranes from adrenal cortex. The high affinity sites for ^{125}I-ACTH$_{1-24}$ in the rat brain and bovine adrenal had K_D's (nM) of 0.51 ± 0.41 and 3.9 ± 1.3, respectively; and the K_I's (nM) for VIP of 5.4 ± 4.2 and 1.4 ± 0.51, respectively. The high affinity sites for ^{125}I-VIP in rat brain and bovine adrenal had K_D's (nM) of 2.9 ± 1.7 and 0.5 ± 0.8, respectively; and K_I's (nM) for ACTH of 23.6 ± 14.0 and 22.2 ± 33.0, respectively. Cortisol secretion from isolated bovine adrenal cortical cells was significantly stimulated by 10^{-10} M of either ACTH or VIP, and a maximal response occurred for each at 10^{-8}M. However, maximal cortisol production in response to VIP was only about one-half that by ACTH$_{1-24}$. The combination of both peptides, each at 10^{-10}M, was additive in stimulating cortisol production, whereas each combined at 10^{-8}M caused no greater response than ACTH alone. There was an additive steroidogenic effect of VIP plus ACTH$_{1-10}$ but not VIP plus ACTH$_{11-24}$. In adrenal GRF and DYN competed for binding of labelled ACTH and each enhanced steroidogenesis at 10^{-10}M; maximally effective levels of GRF or DYN stimulated cortisol production to only about half that by ACTH$_{1-24}$. Specific binding of ^{125}I-ACTH$_{11-24}$ in adrenal membranes was inhibited by unlabelled ACTH$_{11-24}$, ACTH$_{1-24}$, VIP, GRF and DYN but not by ACTH$_{1-10}$, PEP T and other peptides, e.g. TRH, α-MSH and β-END; there was no specific binding of ^{125}I-ACTH$_{1-10}$. Previous functional studies and the present binding data, in conjunction with the existence of homologous amino acid sequences, indicate that VIP, GRF and DYN

interact at a subpopulation of ACTH receptors that recognizes a moiety within the 11-24 sequence of the ACTH molecule.

INTRODUCTION

We found that specific binding of ^{125}I-ACTH$_{1-24}$ to brain membranes is inhibited by VIP, GRF and DYN but not by several other peptides tested (Hnatowich et al., in press). Furthermore, we obtained evidence that in the adrenal cortex the ACTH$_{1-10}$ sequence activates a receptor linked to adenylate cyclase and ACTH$_{11-24}$ interacts with a receptor coupled to another second messenger system (Li et al., in press); both receptors mediate steroidogenesis. The present investigation was designed to elucidate the subtype(s) of ACTH receptors that recognize VIP, GRF and DYN. The adrenal was selected for these studies because steroidogenesis represents a functional cellular consequence of receptor activation by ACTH.

MATERIALS AND METHODS

Materials

ACTH$_{1-24}$ and ACTH$_{1-10}$ were generously provided by Organon Inc. (West Orange, NJ); ACTH$_{11-24}$, GRF, CRF, and DYN were purchased from Bachem Inc. (Torrance, CA); VIP (porcine) and [Phe^2,Nle^4]ACTH$_{1-24}$ from IAF Biochemicals (Laval, Quebec); Collagenase/dispase and DNAse from Boehringer Mannheim Corporation (Dorval, Quebec); ^{125}I from Amersham Corporation (Oakville, Ontario); ^{3}H-cortisol from New England Nuclear (Boston, MA); Cortisol antiserum from Endocrine Science (Tarzana, CA); SP from Abbott Laboratories (N. Chicago, Ill.); QUSO G-32 from Philadelphia Quartz (Philadelphia, PA); PEP T from Peninsula Laboratories Inc. (Belmont, CA); BSA, MET-ENK, AII (human), INS, TRH, α-MSH from Sigma Chemical Company (St. Louis, MO); and SurfaSil from Pierce Chemical Co. (Rockford, Ill).

Preparation of brain and adrenal membranes.

The cerebral cortex was dissected from decapitated adult male Sprague-Dawley rats (about 200-300 x g) and meninges carefully removed. The adrenal cortex was dissected from fresh bovine glands obtained from a local slaughterhouse. Adrenal cortex and brain cortex were homogenized in 10% (w/v) sucrose by 5 bursts with a Brinkmann Polytron and centrifuged (600 x g, 15 min) at 0-4°C. The pellet (nuclei and cell debris) was discarded, and the supernatant suspended in 300 mM NaCl and centrifuged at 25,000 x g for 15 min at 0-4°C. The supernatant was discarded and the pellet resuspended in sodium phosphate buffer (300 mM, pH 7.4). Protein concentration of the suspension was determined by the method of Lowry et al. (1951) using BSA as the standard, and the membrane suspensions diluted in buffer to give 1 mg protein/ml.

Iodination of [Phe^2,Nle^4]$ACTH_{1-24}$.

$ACTH_{1-24}$ possesses biological activity equivalent to that of the natural hormone, $ACTH_{1-39}$ (Evans et al., 1966). Iodination of $ACTH_{1-24}$ causes loss of biological activity due to iodination of tyrosine at the 2 position and oxidation of methionine at the 4 position (Hofmann et al., 1986; Lowry et al., 1973). The [Phe^2,Nle^4]$ACTH_{1-24}$ derivative retains biological activity upon iodination of tyrosine at position 23 (Hofmann et al., 1986), and is a practical radiolabel for binding studies.

[Phe^2,Nle^4]$ACTH_{1-24}$ was iodinated following the method of McIlhinney and Schulster (1971). In order to reduce the loss of ACTH through adsorption, all labware was briefly exposured to 10% SurfaSil in N-hexane, rinsed with methanol then water, and air-dried. To a siliconized polypropylene tube, 50 ul of sodium phosphate buffer (0.05 M, pH 7.0),

[Phe^2,Nle_4]$ACTH_{1-24}$ (5-10 ug), lactoperoxidase (4 ug), ^{125}I (2 mCi) and H_2O_2 (0.003%) were added and vortexed frequently at room temperature. An additional 10 ul of H_2O_2 (0.003%) was added at 2 min and 0.5 ml of sodium phosphate buffer at 4 min to stop the iodination reaction. The iodination mixture was transferred to 10 ml of the same buffer containing QUSO G-32 10 mg, BSA 10 mg and mercaptoethanol 50 ul, vortexed for 30 min, then centrifuged at 1700 g for 10 min. The supernatant was aspirated and the pellet washed twice with water to remove free ^{125}I. The ^{125}I labelled ACTH was extracted from QUSO with 0.5 ml of acetone: H_20: acetic acid (40:59:1), stored at 4°C and used within two weeks. The specific activity of monoiodinated [Phe^2,Nle^4]$ACTH_{1-24}$ is approximately 1600 Ci/mmole. Approximately 35% of the added ^{125}I was recovered as labelled peptide.

Daily, 100 ul of the iodination mixture was applied to a uBONDAPAK reverse phase C_{18} HPLC column equilibrated with sodium phosphate buffer (0.1 M, pH 2.1) containing 23% acetonitrile. The sample was eluted with the same buffer at a flow rate of 0.5 ml/min, aliquots of 0.7 ml were collected and radioactivity determined with a LKB Universal Gamma Counter. One or two fractions were collected from the midportion of the major peak (I), which has been assigned to monoiodinated $ACTH_{1-24}$ (Hnatowich et al., in press). The ^{125}I-ACTH was used on the same day or stored at 4°C for use no longer than 24 hours later.

Iodination of VIP.

VIP was iodinated following the method described for ACTH except that the eluting solution applied to HPLC column contained 25% acetonitrile. After HPLC purification three major peaks were obtained, in agreement

with others (Martin et al., 1986). Unlabelled VIP at 0.5 uM inhibited displaceable binding of ^{125}I-VIP from each peak by 67.2%, 62.4% and 60.4%, respectively. The first major peak was collected for routine binding studies in view of the larger yield compared to that of the other two peaks. ^{125}I-VIP was stored at 4°C for up to 7 days and yielded consistent binding properties. The specific activity of ^{125}I-VIP is appoximately 1500 Ci/mmole.

Iodination of $ACTH_{1-10}$ and $ACTH_{11-24}$.

Iodination of $ACTH_{1-10}$ and $ACTH_{11-24}$ with ^{125}I and the removal of free ^{125}I were done as described above, but the iodinated mixtures were not purified further.

Radioligand binding assay

Eppendorf centrifuge tubes contained ^{125}I peptide in 200 ul, 50 ul membrane suspension (1 mg/ml), 10 ul unlabeled peptide dissolved and diluted to designed concentration with 0.5% BSA (w/v), and sodium phosphate buffer (300 mM, pH 7.4) to 500 ul. All labware was siliconized. The final BSA concentration in incubate is 0.01% (w/v). After incubation at 0°C for 1 hr, the incubates were centrifuged (8000 x g, 15 min at 0-4^{o}C). The supernatants were aspirated and the pellet surfaces and assay tube walls gently washed twice with ice-cold 0.9% saline. The tips of the centrifuge tubes were cut off and ^{125}I determined in a gamma-counter.

Isolation of adrenal cortical cells

Fresh bovine adrenal glands were obtained from the local

slaughterhouse and kept in 10% sucrose at room temperature. After stripping off the fat, the cortical portions of the adrenal were dissected and sliced in McIlwain Tissue Chopper. The slices were incubated with Krebs bicarbonate buffer (pH 7.4) containing BSA 0.5% (w/v), collagenase (2 mg/ml), and DNAse (25 ug/ml) on a gyratory shaker for 30 min at 37°C under an atmosphere of 95% O_2:5% CO_2. The tissue was dispersed by repeated pipetting through plastic pipettes of decreasing diameters. The undigested fragments were allowed to settle and the digested fragments decanted through nylon mesh (160 μm pore). The cells were collected by centrifugation at 100 x g for 5 min, and the pellets washed twice and put in an atmosphere of O_2:CO_2 (95:5). The undigested fragments were again exposed to the same procedure and the dispersed cells mixed and washed with Krebs/BSA. Viability of free cells was determined with trypan blue (Tennant, 1964). Cell number was determined with a hemocytometer.

Measurement of steroid release from adrenal cells

The incubate contained adrenal cell suspension in 0.99 ml Krebs/BSA (1-6 X 10^6 cells) and peptides were added in 0.01 ml. After incubation at 37° for 1 hr, incubates were centrifuged (1000 x g, 10 min) and aliquots of the supernatant analyzed for steroids by radioimmunoassay. Briefly, 0.05 ml of supernatant was mixed with 0.20 ml of ^{3}H-cortisol/antiserum mixture (1,200,000 dpm of ^{3}H-cortisol, 0.30 ml of 2.5% human gamma globulin, 0.24 ml of 10% BSA, 0.10 ml of anti-cortisol antiserum, and 12 ml of borate buffer, 0.05 M, pH 8.0) and incubated for 1.5 hours at 37°C. Bound ^{3}H-cortisol was precipitated with addition of 0.25 ml of saturated $(NH_4)_2SO_4$. Unbound ^{3}H-cortisol was counted with a

Scintillation β-counter and cortisol calculated by weighted linear regression.

RESULTS

Specific binding of ^{125}I-ACTH to membranes from both brain and adrenal was inhibited by either unlabeled ACTH or unlabeled VIP in a dose-dependent manner (Fig. 1). Binding data from multiple experiments were pooled and analyzed with the computer program LIGAND (Munson and Rodbard, 1980). For ACTH in brain a two-site model with a high affinity, low capacity site and a low affinity, high capacity site was statistically a better "fit" of data than a one-site model ($p < 0.05$). In the adrenal a one-component binding model was statistically a better "fit" of the data than a two-component model.

Similarly, specific binding of ^{125}I-VIP to membrane preparations from brain and adrenal was inhibited by both unlabelled VIP and ACTH in a dose-dependent fashion (Fig. 1). A two-site model for VIP binding provided a significantly improved "fit" compared to a one-site model ($p < 0.05$) in both tissues. The binding parameters are summarized in Table 1.

Unlabelled VIP, ACTH and several other peptides were tested at 1 uM against ^{125}I-VIP binding in brain. As shown in Figure 2, both VIP and ACTH displaced ^{125}I-VIP binding, whereas INS and AII were inactive and MET-ENK, SP and CRF insignificantly increased binding of ^{125}I-VIP ($P > 0.05$).

ACTH and VIP each enhanced steroidogenesis in a dose-dependent manner (Fig. 3). For either peptide significant stimulation of cortisol occurred at 10^{-10} M and a maximal response at 10^{-8} M. Maximal cortisol production induced by VIP was about 40% of that by ACTH.

To determine if VIP stimulates cortical cells via the same receptor as ACTH, submaximal or maximally effective concentrations of VIP and ACTH were combined. As shown in Table 2, 10^{-10} M of VIP plus 10^{-10} M of ACTH significantly increased cortisol production over either alone, whereas the combination of peptides each at 10^{-8} M concentration led to no greater effect than ACTH alone at that concentration.

As we have shown, $ACTH_{1-10}$ or $ACTH_{11-24}$ each stimulates steroidogenesis maximally at a concentration of 10 uM (Li, Park and LaBella, in press). Maximal steroidogenesis by VIP (1 uM) was significantly enhanced by the addition of 10 uM $ACTH_{1-10}$ but not by 10 uM of $ACTH_{11-24}$ (Fig. 4).

GRF and DYN also stimulated steroidogenesis in a dose-dependent fashion (Fig. 5), although, again, maximal cortisol release by either peptide was about half that induced by $ACTH_{1-24}$. Cortisol release from adrenal cells was stimulated by as low as 10^{-10} M of each peptide.

As shown in Figure 6, GRF and DYN at 1 uM significantly inhibited ^{125}I-$ACTH_{1-24}$ binding to adrenal membranes, whereas PEP T, β-END, α-MSH and TRH were ineffective.

Figure 7 shows that the specific binding of ^{125}I-$ACTH_{11-24}$ in adrenal membranes was inhibited, at 1 uM, by unlabelled $ACTH_{11-24}$, $ACTH_{1-24}$, VIP, GRF and DYN, by 63, 69, 40, 50, and 83%, respectively. $ACTH_{1-10}$ and PEP T were inactive. Other inactive peptides tested in single experiment, included TRF, α-MSH and β-END.

DISCUSSION

In this study VIP was a potent competitor of ^{125}I-ACTH binding, and ACTH a potent competitor of ^{125}I-VIP binding, both in brain and adrenal.

Of many peptides tested in an ^{125}I-VIP binding assay on brain membranes, ACTH was a potent competitor, several peptides were inactive and three peptides actually increased ^{125}I-VIP binding slightly; the mechanism of the enhanced affinity is unknown. But the use of membrane preparations and an incubation temperature of 0°C, rules out metabolic mechanisms. We found a large variability in the estimates of parameters of ACTH binding data, probably due in large part to the extraordinary propensity of ACTH to adhere to surfaces. Although all labware was siliconized with SurfaSil to minimize adsorption, it is not possible to abolish it completely.

The high affinity binding of ^{125}I-ACTH$_{1-24}$ to membranes from rat brain is potently inhibited by VIP, GRF and DYN (Hnatowich et al., unpublished). We now show that VIP, GRF and DYN also compete for high affinity binding of ^{125}I-ACTH$_{1-24}$ in membranes from adrenal cortex. These three peptides show amino acid sequence homologies with ACTH (Fig. 8). VIP is reported to stimulate steroidogenesis in cultured adrenal tumour cells (Kowal et al., 1977; Morera et al., 1979), ovarian granulosa cells (Davoren and Hsueh, 1985), testicular cells (Kasson et al., 1986) and in rat (Nussdorfer and Mazzocchi, 1987; Cunningham and Holzwarth, 1988) and amphibian (Leboulenger et al., 1984) adrenal glands. GRF is reported to interact with a VIP receptor in rat and human intestinal epithelial cell membranes (Laburthe et al., 1983; Laburthe et al., 1986), guinea pig pancreatic acini (Pandol et al., 1984), rat pancreatic plasma membranes (Waelbroeck et al., 1985), and, like VIP, to stimulate adenylate cyclase activity in these preparations. Opioid peptides influence corticoid synthesis by isolated rat adrenocortical cells; e.g. β-endorphin stimulated corticosterone production but the combination of maximally

steroidogenic concentrations of ACTH and β-endorphin gave no further increase (Guaza et al., 1986; Shanker and Sharma, 1979). Szalay and Stark (1981) reported that β-endorphin elicited a u-shaped dose-response pattern in isolated zona fasciculata cells: low concentrations decreased and high concentrations increased steroid production. $Dynorphin_{1-17}$ enhanced steroidogenesis by a low concentrations of ACTH but did not affect the response to a maximally effective dose of ACTH (Guaza et al., 1986). These observations suggest that VIP, DYN and GRF and ACTH compete for at least one population of receptors in the adrenal cell and elicit steroidogenesis, but maximal hydrocortisone secretion mediated through that species of receptor accommodating VIP, DYN and GRF is only about one-half that evoked by $ACTH_{1-24}$.

In a previous study (Li et al., in press) we found that the 1-10 or 11-24 amino acid fragments of ACTH elicited steroidogenesis, each by a separate receptor system, and that the 1-10 but not the 11-24-activated receptor was linked to adenylate cyclase. Individually, each of the fragments evoked a steroid response that was only one half that of $ACTH_{1-24}$, but the two fragments together elicited a response approximately equal to that to the whole molecule. VIP enhanced steroidogenesis by $ACTH_{1-10}$, but not that by $ACTH_{11-24}$. Binding of $^{125}I\text{-}ACTH_{11-24}$ was inhibited by $ACTH_{11-24}$, and by VIP, GRF and DYN, but not by $ACTH_{1-10}$ and PEP T. The latter peptide was tested because its amino acid sequence is contained within the sequence of VIP and was postulated to interact with VIP receptors (Ruff et al., 1987). The amino acid homologies among the peptides appear to comprise the 11-24 region of ACTH. We failed to demonstrate displaceable binding of $^{125}I\text{-}ACTH_{1-10}$, probably because iodination of ^{2}Tyr in the peptide yields a biologically inactive peptide,

presumably because of the failure of the receptor to recognize the iodinated species, as was demonstrated for ^{125}I-(^{2}Tyr)$ACTH_{1-24}$ (Lowry et al., 1973).

Kowal et al. (1977) reported that VIP stimulated steroidogenesis in cultured murine adrenal tumor cells (with less potency but the same efficacy as $ACTH_{1-24}$). Our results indicate that VIP stimulated corticoid production in adrenocortical cells with the same potency but with less efficacy than does $ACTH_{1-24}$. The apparent inconsistencies may be explained by differences in cell types or in steroid assay methods. Kowal et al. measured 20α-dihydroprogesterone (20-OHA) and 11β-hydroxy,20α-dihydroprogesterone in the supernatant from cultured murine tumor cells using a fluorometric method (Kowal and Fiedler, 1968). We measured cortisol levels in supernatant from bovine adrenocortical cells by radioimmunoassay. In our hands, also, the fluorometric method showed VIP to stimulate steroid production with the same efficacy and potency as ACTH (data not shown). The reason(s) for the lower steroidogenic activity of VIP, as indicated by the more specific radioimmunoassay, may reflect: (1) lower intrinsic activity of VIP, (2) multiple intracellular pathways involved in steroid biosynthesis that VIP and ACTH affect differently, or (3) activation by VIP of fewer receptors that mediate steroid production. The data accumulated in this paper seem to favor the last notion that VIP (also, DYN and GRF) interacts with only a certain subtype of ACTH receptor that recognizes $ACTH_{11-24}$.

ACKNOWLEDGEMENTS

Supported by grants from the Medical Research Council of Canada and the Health Sciences Centre Research Foundation (Winnipeg). Z. Li was supported by Studentships from the Manitoba Health Research Council and the MRCC. F.S. LaBella is a Career Investigator of the MRCC.

REFERENCES

Cunningham L.A. and Holzwarth M.A. (1988) Vasoactive intestinal peptide stimulates adrenal aldosterone and corticosterone secretion. Endocrinol. 122, 2090-2093.

Davoren J.B. and Hsueh A.J.W. (1985) Vasoactive intestinal peptide: a novel stimulator of steroidogenesis by cultured rat granulosa cells. Biol. Reprod. 33, 37-52.

Evans H.M., Sparks L.L. and Dixon J.S. (1966) The physiology and chemistry of adrenocorticotrophin, in The Pituitary Gland (Harris G.W. and Donovan B.T., eds), pp. 317-373, v. 1, Butterworths, London.

Guaza C., Zubiaur M. and Borrell J. (1986) Corticosteroidogenesis modulation by β-endorphin and dynorphin$_{1-17}$ in isolated rat adrenocortical cells. Peptides 7, 237-240.

Hnatowich M.R., Queen G., Stein D. and LaBella, F.S. Specific binding/sequestration of ^{125}I-[Phe^2,Nle^4] ACTH$_{1-24}$ by rat brain slices. Canad. J. Physiol. Pharmacol., in press.

Hofmann K.K., Romovacek H., Stehle C.J. and Finn F.M. (1986) Radioactive probes for adrenocorticotrophin hormone receptors. Biochemistry 25, 1339-1346, 1986.

Kasson B.G., Lim P. and Hsueh A.J.W. (1986) Vasoactive intestinal peptide stimulates androgen biosynthesis by cultured neonatal testicular cells. Mol. Cell Endocrinol. 48, 21-29.

Kowal J. and Fiedler R. (1968) Adrenal cells in tissue culture. 1. Assay of steroid products; steroidogenic responses to peptide hormones. Arch. Biochem. Biophys. 128, 406-421, 1968.

Kowal J., Horst J., Pensky J. and Alfonzo M. (1977) A comparison of effects of ACTH, vasoactive intestinal peptide, and cholera toxin on adrenal cAMP and steroid synthesis. Ann. N.Y. Acad. Sci. 247, 314-328.

Laburthe M., Amiranoff B., Boige N., Rouyer-Fessard C., Tatemoto K. and Moroder L. (1983) Interaction of GRF with VIP receptors and stimulation of adenylate cyclase in rat and human intestinal epithelial membranes. Comparison with PHI and secretin. FEBS Lett. 159, 89-92.

Laburthe M., Courineau A. and Rouyer-Fessard C. (1986) Study of species specificity in growth hormone-releasing factor (GRF) interaction with vasoactive intestinal peptide (VIP) receptors using GRF and intestinal VIP receptors from rat and human: evidence that Ac-Tyr^{1}h GRF is a competitive VIP antagonist in the rat. Mol. Pharmacol. 29, 23-27, 1986.

Leboulenger F., Perroteau I., Netchitaile P., Lihrmann I., Leroux P., Delarue C., Coy D.H. and Vaudry H. (1984) Action of vasoactive intestinal peptide (VIP) on amphibian adrenocorticcal function in vitro. Peptides 4, 299-303.

Li Z.G., Park D., Queen, G. and LaBella F.S. $ACTH_{1-10}$ and $ACTH_{11-24}$ promote adrenal steroidogenesis by different mechanisms. Endocrinology, in press.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951) Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Lowry P.J., McMartin C. and Peters J. (1973) Properties of a simplified bioassay for adrenocorticotrophic activity using the steroidogenic response of isolated adreanl cells. J. Endocr. 59, 43-55.

Martin J.L., Rose K., Hughes G.H. and Magistretti P.J. (1986) [mono[^{125}I]iodo-Tyr^{10},Met0^{17}]-vasoactive intestinal polypeptide. J. Biol. Chem. 261, 5320-5327.

Morera A.M., Cathiard A.M., Laburthe M. and Saez J.M. (1979) Interaction of vasoactive peptide (VIP) with a mouse adrenal cell line (y-1): specific binding and biological effects. Biochem. Biophys. Res. Commun. 90, 78-85.

Munson P.J. and Rodbard D. (1980) LIGAND: A versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107, 220-239.

McIlhenny R.A.J. and Schulster D. (1971) The preparation of biologically active ^{125}I-labelled adrenocorticotropic hormone by a simple enzymic radioiodination procedure utilizing lactoperoxidase. Endocrinol. 94, 1259-1264.

Nussdorfer G.G. and Mazzocchi G. (1987) Vasoactive intestinal peptide stimulates aldosterone secretion by rat adrenal gland in vivo. J. Steroid Biochem. 26, 203-205.

Pandol S.J., Seifert H., Thomas M.W., Rivier J. and Vale W. (1984) Growth hormone-releasing factor stimulates pancreatic enyzme secretion. Science 225, 326-328.

Ruff M.R., Martin B.M., Ginns E.I., Farrar W.L. and Pert C.B. (1987) CD4 receptor binding peptides that block HIV infectivity cause human monocyte chemotaxis. Relationship to vasoactive intestinal peptide. FEBS Lett. 211, 17-22.

Shanker G. and Sharma P.K. (1979) β-endorphin stimulates corticosterone synthesis in isolated rat adrenal cells. Biochem. Biophys. Res. Commun. 86, 1-5.

Szalay K.S. and Stark E. (1981) Effect of beta-endorphin on the steroid production of isolated zona glomerulosa and zona fasciculata cells. Life Sci. 29, 1355-1361.

Tennant J.R. (1964) Evaluation of the trypan blue technique for determination of cell viability. Transplantation 2: 685-694.

Waelbroeck M., Robberecht P., Coy D.M., Camus J.C., Neef P.D. and Christophe J. (1985) Interaction of growth hormone-releasing factor and 14 GRF analogs with VIP receptors of rat pancreas. Discovery of (N-Ac-Tyr^1,D-Phe^2) GRF (1-29)-NH_2 as a vasoactive intestinal peptide antagonist. Endocrinol. 116: 2643-2649.

Table 1. Binding Parameters for ACTH and VIP in Membranes from Brain and Adrenal Cortex.

		^{125}I-VIP			^{125}I-ACTH		
		K_D (nM)	K_I (nM)	Bmax (fmole/mg)	K_D (nM)	K_I (nM)	Bmax (fmole/mg)
BRAIN	VIP	2.9 ± 1.7 167 ± 112		121 ± 81 6,450 ± 4,390		5.4 ± 4.2 6,000 ± 9,800	
	ACTH		23.6 ± 14.0 5,240 ± 10,600		0.51 ± 0.41 87 ± 40		13.5 ± 9.3 2,780 ± 1,340
ADRENAL	VIP	0.5 ± 0.8 14.3 ± 6.6		257 ± 367 12,200 ± 4,750		1.4 ± 0.51	
	ACTH		22.2 ± 33.0 not resolved		3.9 ± 1.3		2,820 ± 929

K_D, K_I and B_{max} were determined using the computer program LIGAND (see Materials and Methods). Data are expressed as the Mean ± SEM from the same experiments shown in Figure. 1.

TABLE 2. Steroidogenic effect of VIP and ACTH from adrenal cell suspensions.

		Cortisol release (ng/10^6 cells/hour mean ± SE)
Control		0.15 ± 0.01
VIP	0.1 nM	0.32 ± 0.02
	100 nM	0.59 ± 0.10
ACTH	0.1 nM	0.77 ± 0.25
	100 nM	1.37 ± 0.03
VIP + ACTH	0.1 nM + 0.1 nM	1.22 ± 0.11**
VIP + ACTH	100 nM + 100 nM	1.47 ± 0.07(a)

** $p < 0.01$ vs ACTH 0.1 nM
$p < 0.01$ vs VIP 0.1 nM

(a) NS vs ACTH 100 nM (Tukey's multiple comparison)

LEGENDS

Fig. 1. Displacement of ^{125}I-ACTH or ^{125}I-VIP by unlabelled ACTH and VIP from brain and adrenal membranes. Panels A and B represent the displacement of ^{125}I-VIP and ^{125}I-ACTH, respectively, from brain membranes. Panels C and D represent their displacement from adrenal membranes. Experimental data were pooled and analyzed using the computer program LIGAND (see Materials and Methods) to generate displacement curves. Each ligand-derived displacement curve is based on data pooled from multiple experiments (n). $ACTH_{1-24}$ (solid line) n=9,4,3,2, in panels A,B,C,D, respectively. VIP (dashed line), n=10,2,2,2 in panels A,B,C,D, respectively. Each point represents the Mean ± SEM from experiments performed in 5 replicates. In experiments where a particular dose was used only once (5 replicates), no standard error bars are shown.

Fig. 2. Inhibition of I^{125}-VIP binding to rat brain membranes by peptides at 1 uM concentration. Total ^{125}I-VIP binding in the absence of unlabelled peptides is taken as 100%. Each bar represents percentage of the total binding. Mean ± SEM of two experiments, each with 4 replicates.

(a) vs CTRL, $p < 0.01$;

(b) vs CTRL, $p < 0.01$;

(c) vs CTRL, NS;

(d) vs CTRL, NS;

(e) vs CTRL, NS;

(a) vs (b), NS (Tukey's multiple test)

Fig. 3. Steroidogenic activity of $ACTH_{1-24}$ and VIP. Adrenal cortical cell suspensions were incubated in the presence of increasing concentrations of ACTH and VIP. Each point represents the Mean ± SEM of three experiments performed in triplicate. ○ ACTH, ● VIP,

Fig. 4. Effect of addition of VIP upon $ACTH_{1-10}$ and $ACTH_{11-24}$-induced steroidogenesis. VIP (1 uM), ACTH fragments (10 uM), CTRL, no peptides.

(a) vs VIP, $p < 0.01$;

(a) vs $ACTH_{1-10}$, $p < 0.01$;

(b) vs VIP, NS;

(b) vs $ACTH_{11-24}$, NS; (Tukey's multiple test).

Fig. 5. Steroidogenic effect of peptides. Data are expressed as the percentage of the maximal steroidogenic activity elicited by $ACTH_{1-24}$. Each point represents mean ± SEM of three experiments performed in triplicate. ○ ACTH, △ DYN, □ GRF, ● VIP.

Fig. 6. Inhibition of ^{125}I-$ACTH_{1-24}$ binding to adrenal membranes by 1 uM VIP, DYN, GRF, TRH, β-END, α-MSH and PEP T. The specific binding was obtained by substracting the binding in the presence of 1 uM $ACTH_{1-24}$ from the total binding. Each bar represents the Mean ± SEM for three experiments of five replicates each. ** $p < 0.01$ vs CTRL (Dunnett's test).

Fig. 7. Competition for ^{125}I-$ACTH_{11-24}$ binding to adrenal membranes by $ACTH_{11-24}$, $ACTH_{1-10}$, $ACTH_{1-24}$, GRF, DYN, VIP and PEP T, all at 1 uM. The specific binding is the difference between total binding and binding in the presence of 5 uM of $ACTH_{11-24}$. Each

bar represents the Mean ± SEM of three experiments, each with five replicates. ** $p < 0.01$ compared to CTRL (Dunnett's test).

Fig. 8. Amino acid sequence homologies among $ACTH_{11-24}$, VIP, GRF and DYN. The enclosed are identical amino acid residues, while the amino acid residues within the same classification (basic, neutral, acidic, etc.) are underlined.

Fig. 1

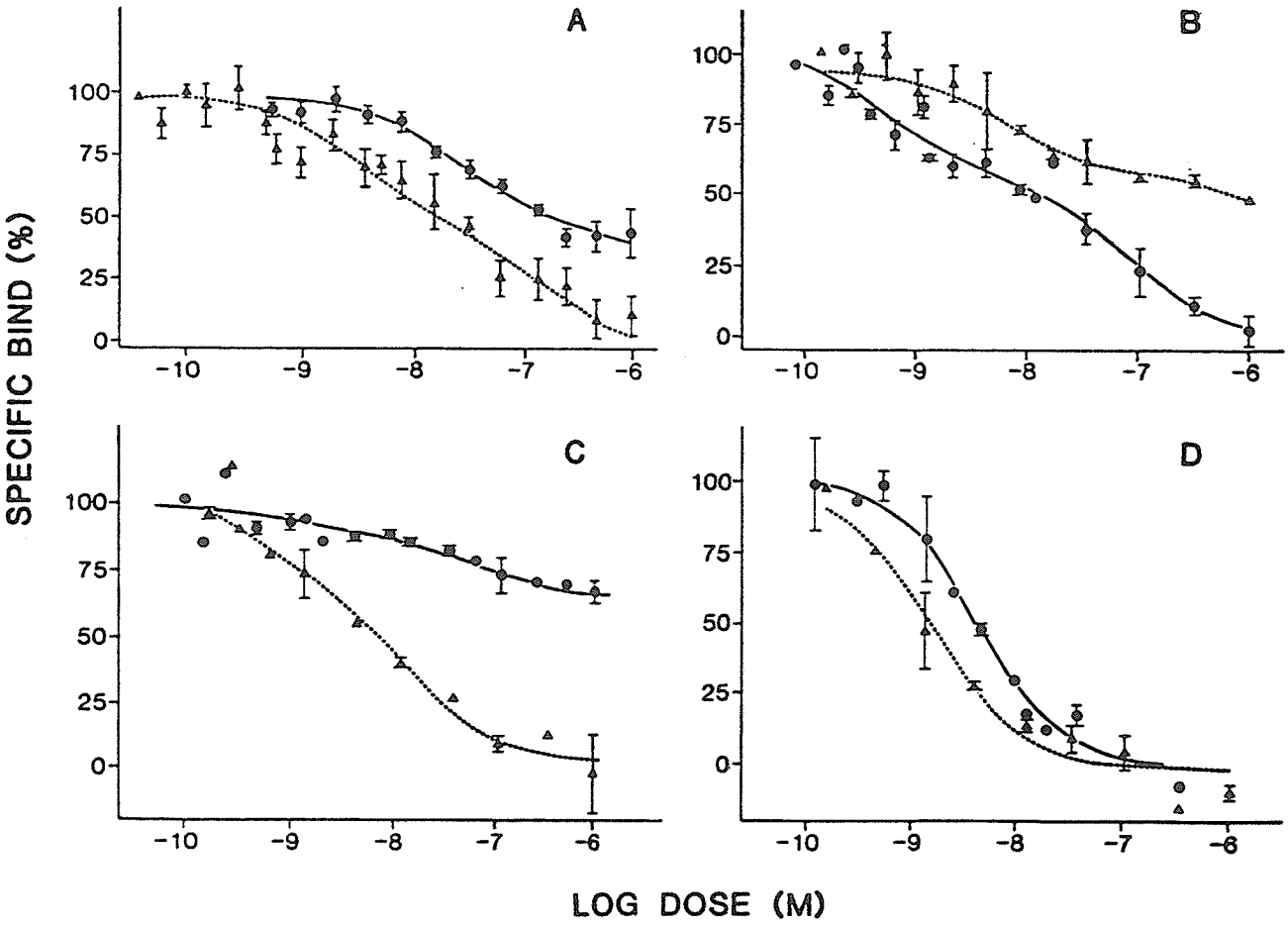


Fig. 2

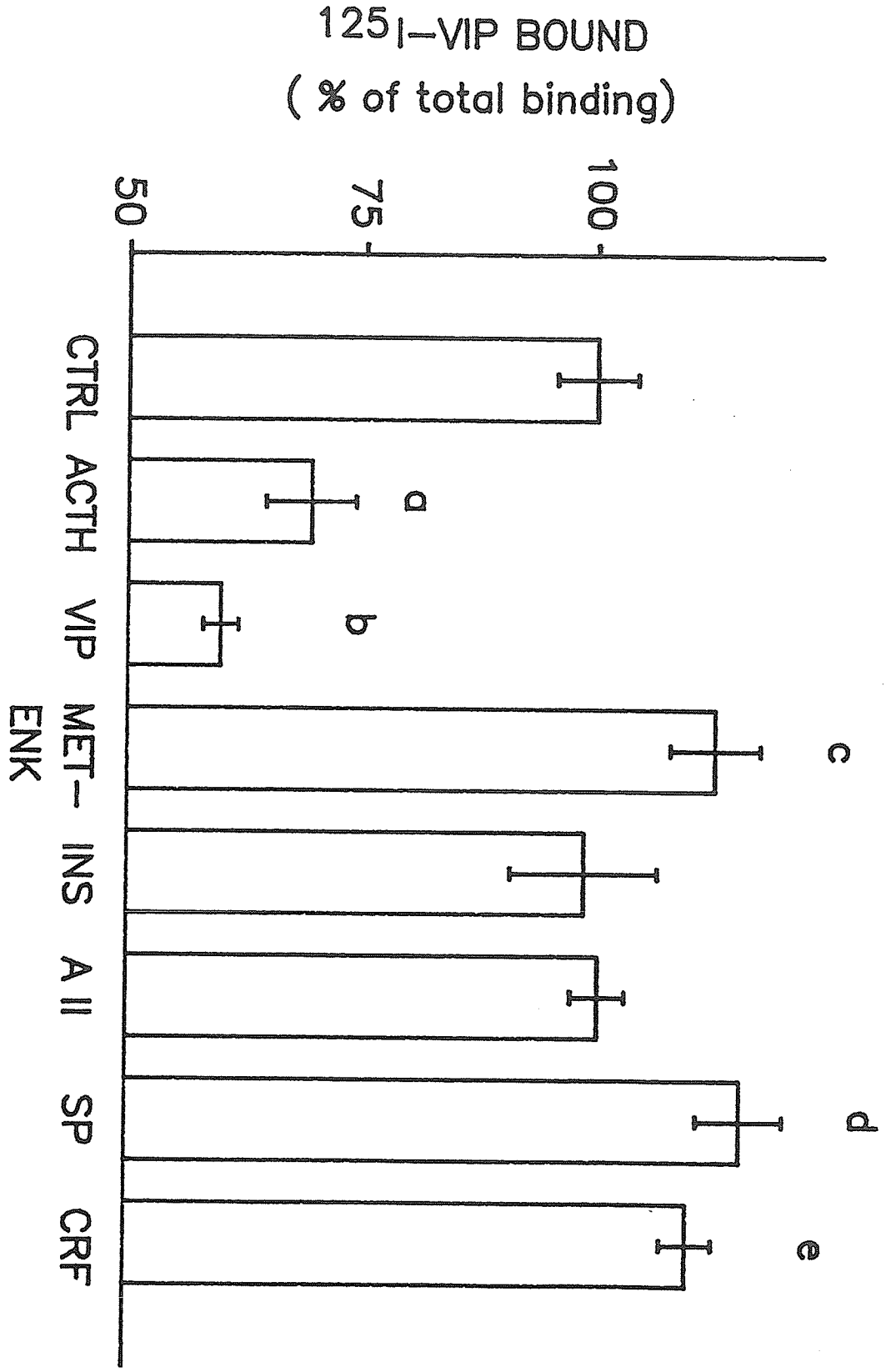

125I–VIP BOUND
(% of total binding)
50
75
100
CTRL
ACTH
VIP
MET–ENK
INS
A II
SP
CRF
a
b
c
d
e

Fig. 3

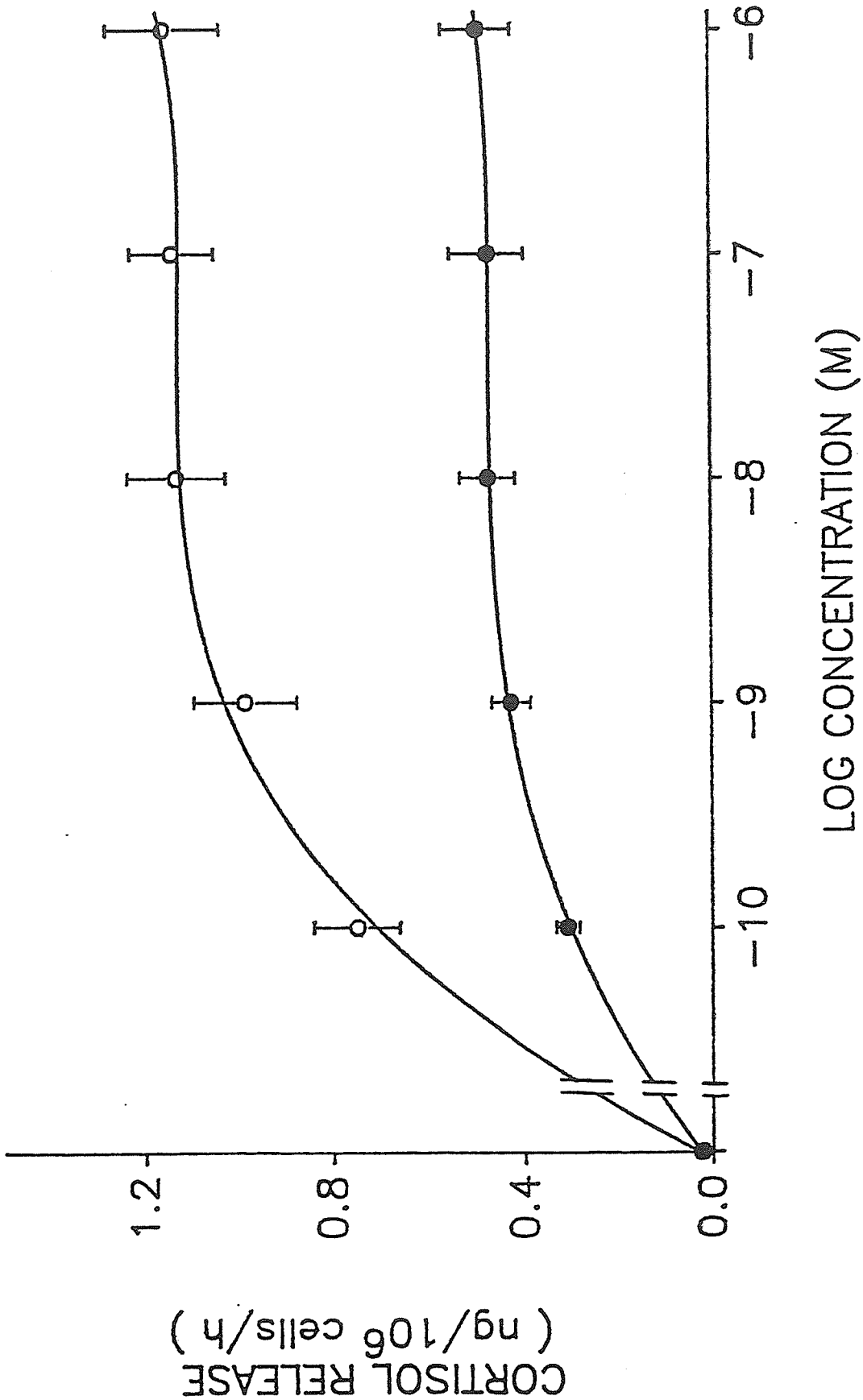

CORTISOL RELEASE
(ng/10⁶ cells/h)
0.0
0.4
0.8
1.2
-10
-9
-8
-7
-6
LOG CONCENTRATION (M)

Fig. 4

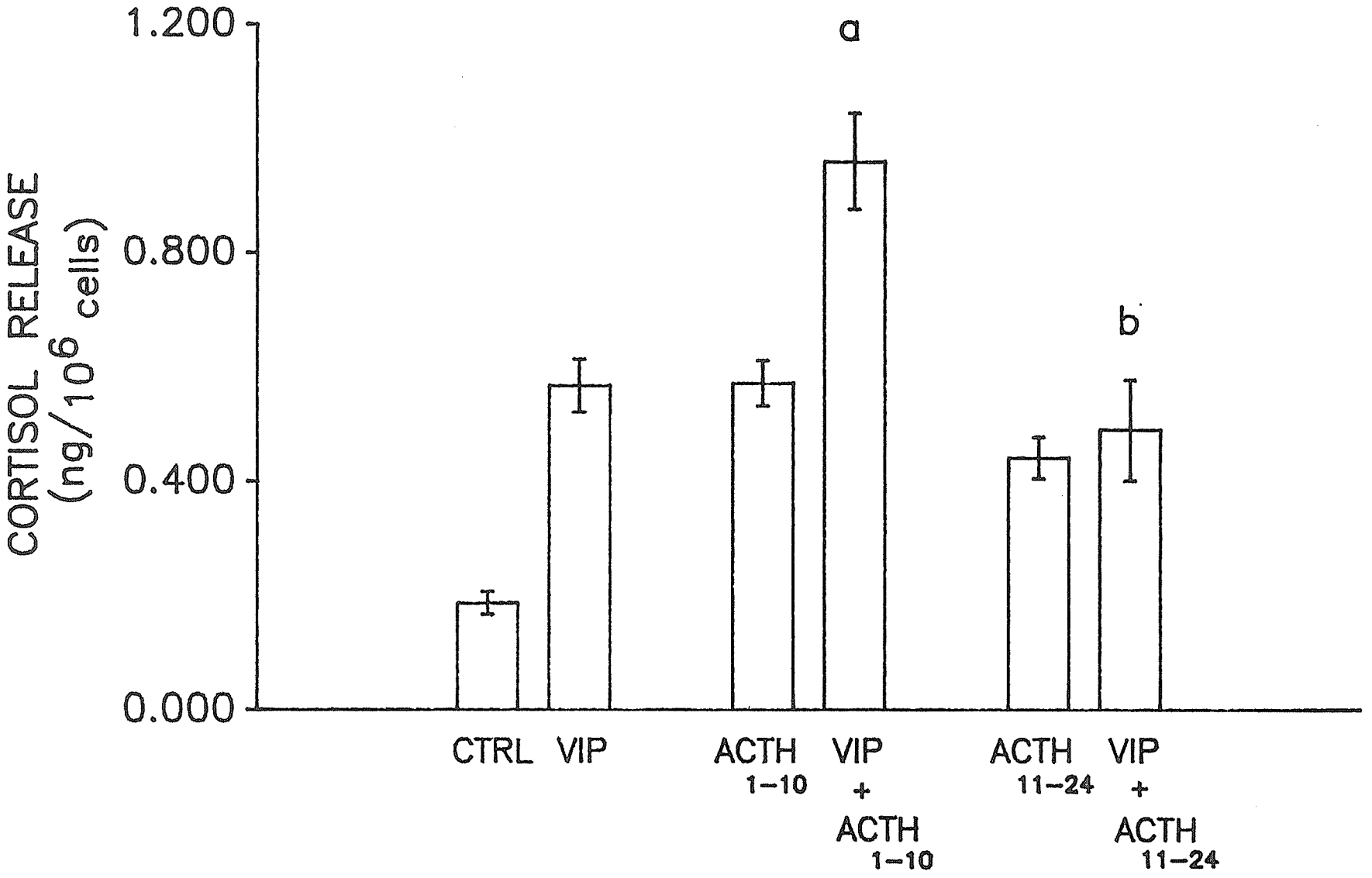

1.200
0.800
0.400
0.000
CORTISOL RELEASE
(ng/10^6 cells)
a
b
CTRL
VIP
ACTH 1-10
VIP + ACTH 1-10
ACTH 11-24
VIP + ACTH 11-24

Fig. 5

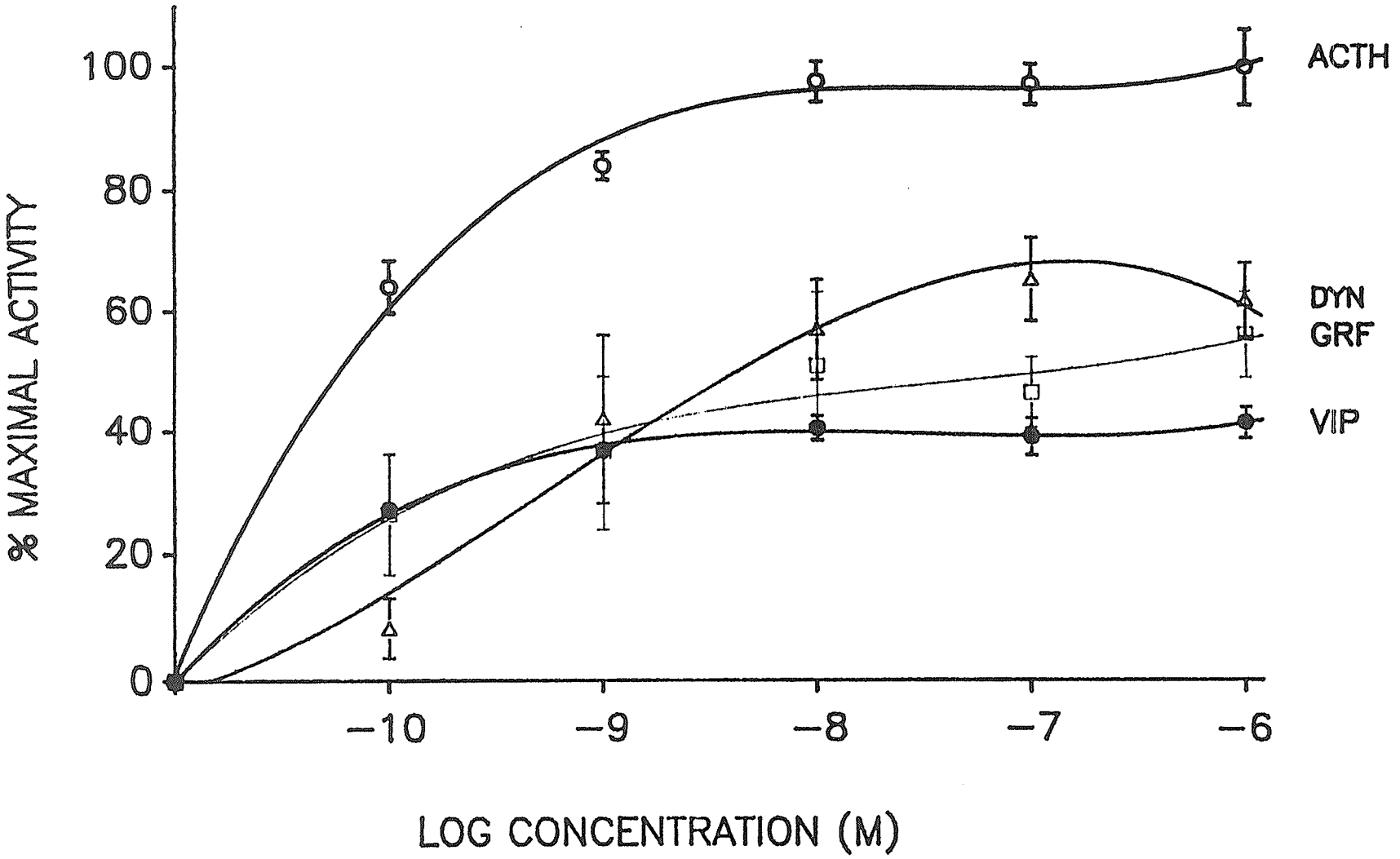

% MAXIMAL ACTIVITY
100
80
60
40
20
0
−10
−9
−8
−7
−6
LOG CONCENTRATION (M)
ACTH
DYN
GRF
VIP

Fig. 6

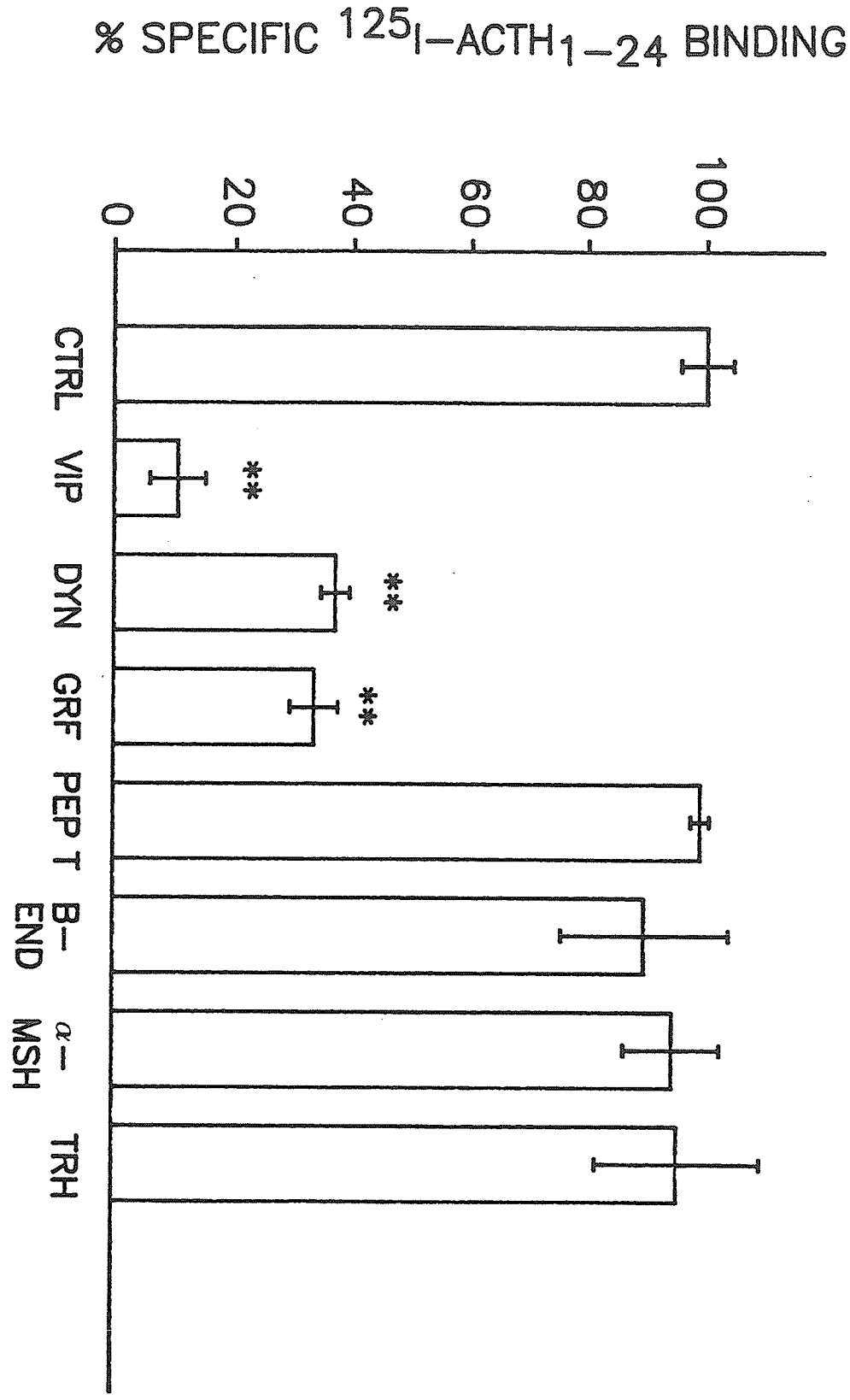

% SPECIFIC 125I–ACTH1–24 BINDING
0
20
40
60
80
100
CTRL
VIP
DYN
GRF
PEP T
B–END
α–MSH
TRH
**
**
**

Fig. 7

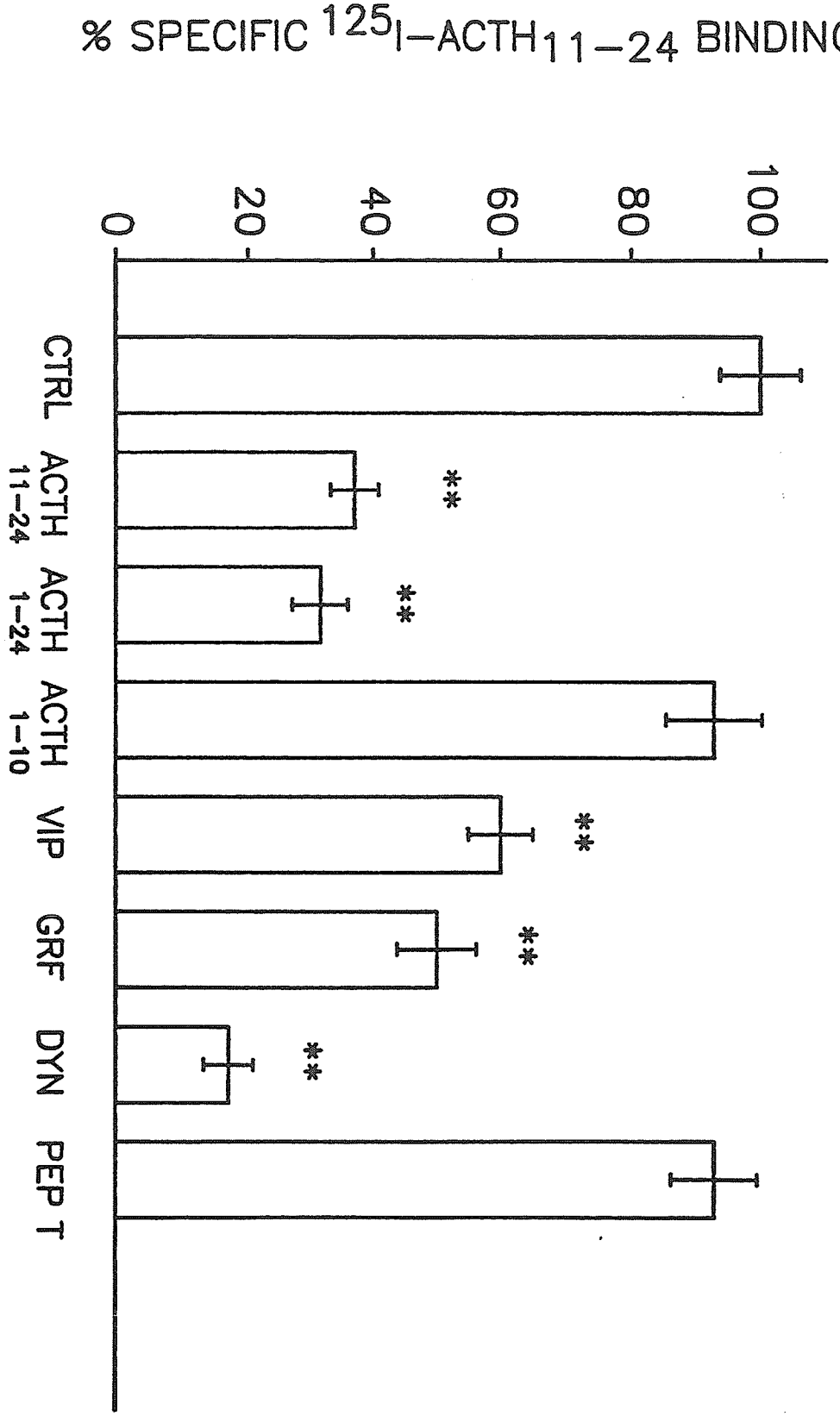
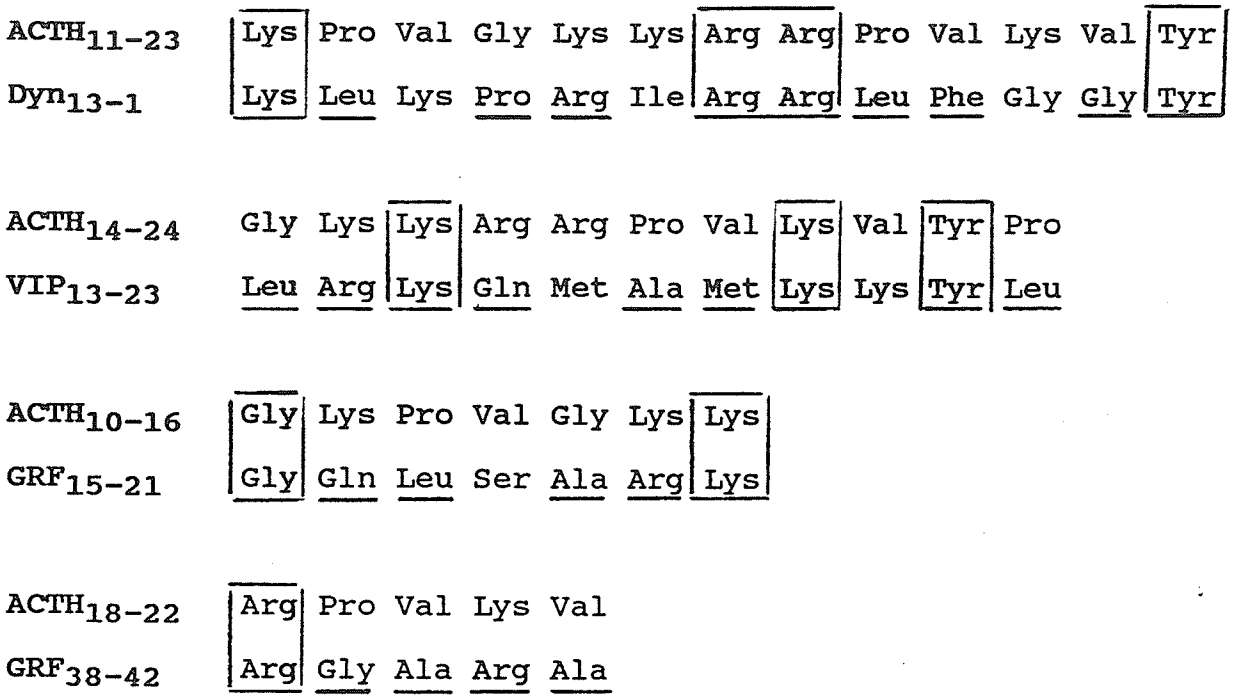

% SPECIFIC ^{125}I–ACTH$_{11-24}$ BINDING
0
20
40
60
80
100
CTRL
ACTH 11–24
ACTH 1–24
ACTH 1–10
VIP
GRF
DYN
PEP T
**
**
**
**
**

Fig. 8


ACTH11-23 Lys Pro Val Gly Lys Lys Arg Arg Pro Val Lys Val Tyr
Dyn13-1 Lys Leu Lys Pro Arg Ile Arg Arg Leu Phe Gly Gly Tyr
ACTH14-24 Gly Lys Lys Arg Arg Pro Val Lys Val Tyr Pro
VIP13-23 Leu Arg Lys Gln Met Ala Met Lys Lys Tyr Leu
ACTH10-16 Gly Lys Pro Val Gly Lys Lys
GRF15-21 Gly Gln Leu Ser Ala Arg Lys
ACTH18-22 Arg Pro Val Lys Val
GRF38-42 Arg Gly Ala Arg Ala

ACTH/VIP RECEPTORS MEDIATE CYCLIC AMP GENERATION IN BRAIN AND ADRENAL. DIFFERENTIAL EFFECTS OF $ACTH_{1-10}$ AND $ACTH_{11-24}$.

Zhen-guo Li and Frank S. LaBella

Department of Pharmacology and Therapeutics
University of Manitoba, Faculty of Medicine
770 Bannatyne Avenue
Winnipeg, Manitoba, Canada R3E 0W3

Abbreviations

ACTH: adrenocorticotropin, ATP: adenosine 5'-triphosphate, BSA: bovine serum albumin, cAMP: adenosine 3',5'-cyclic monophosphate, DTT: dithiothreitol, GTP: guanosine 5'-triphosphate, VIP: vasoactive intestinal peptide.

Abstract

Our previous studies indicated that ACTH and VIP share a common species of receptor in both brain and adrenal. In this study ACTH and VIP are shown to stimulate cAMP production in brain in a dose-dependent manner. In adrenal both $ACTH_{1-24}$ (1 uM) and $ACTH_{1-10}$ (10 uM) maximally induce cAMP formation, whereas $ACTH_{11-24}$ is inactive. These results now provide direct evidence for our earlier conclusion that promotion of adrenal steroidogenesis by $ACTH_{1-10}$ is mediated through a receptor coupled to adenylate cyclase and by $ACTH_{11-24}$ through a receptor linked to another second messenger system.

Introduction

There is substantial evidence for a role of cAMP in the process whereby ACTH stimulates steroidogenesis (1-4). The interaction of ACTH with membrane receptors gives rise to activation of adenylate cyclase, and the consequent increase in intracellular cAMP level activates steroidogenesis acutely, and in the long-term induces steroidogenic enzymes through the action of cAMP-dependent protein kinase (5). It has been recognized generally that not all ACTH receptors are linked to adenylate cyclase. Rasmussen and Barrett (6) proposed that there are at least two types of ACTH receptors on adrenal cortical cells, one linked to cAMP and the other to calcium influx.

Our previous work indicated indirectly that there are at least two types of ACTH receptors, one recognizing the sequence $ACTH_{1-10}$ and coupled to cAMP formation, the other accommodating $ACTH_{11-24}$ (7). In the adrenal, VIP and certain other steroidogenic peptides appear to act through the $ACTH_{11-24}$ receptor. In this study we present direct data on cAMP formation in membrane preparations from brain and adrenal cortex upon exposure to $ACTH_{1-24}$, $ACTH_{1-10}$, $ACTH_{11-24}$ and VIP that provide additional support for the existence of two types of ACTH receptors.

Experimental

Materials

ATP, cAMP, GTP, DTT, neutral alumina (type WN-3), creatine phosphokinase, and imidazole were obtained from Sigma Chemical Company (St. Louis, MO); α-^{32}p-ATP from ICN Radiochemicals (Irvine, CA), ^{3}H-cAMP from Amersham Corporation (Oakville, Ont); creatine phosphate from Boehringer Mannheim Corporation (Dorval, Quebec); Dowex AG 50W-X4 from

Bio-Rad Laboratories (Richmond, Calif.); plastic columns (175 x 7 mm internal diameter) with funnels from Kontes Scientific (Vineland, NJ); VIP and $ACTH_{11-24}$ from Bachem Inc. (Torrance, CA); and $ACTH_{1-24}$ and $ACTH_{1-10}$ were generously provided by Organon (W. Orange, NJ).

Isolation of cAMP.

Isolation and measurement of ^{32}P-cAMP was performed by the sequential chromoatographic method of Solomon (8), with some modification. Dowex AG50W-X4 (100-400 mesh) was washed with deionized water until the effluent was colour-free. A 1:1 slurry (Dowex:water) (2.5 ml) was added to the glass wool-stoppered columns to form the first column. Neutral alumina (WN-3) (0.55 g) was added to each column, washed with 10 ml of imidazole buffer (1.0 M, pH 7.3) to form the second column, followed by 20 ml of imidazole buffer (0.1 M, pH 7.3). The columns were placed on a special rack constructed to fit into each other in pairs, with the columns in the upper and lower racks vertically aligned. 'Stop solution' (1 ml) containing 10,000 cpm α-^{32}p-ATP and 10,000 cpm ^{3}H-cAMP was added to each column and allowed to drain completely into the vials containing 4 ml of scintillation fluid. Then, consecutive 0.5 ml water effluents from each column were collected into vials containing 4 ml of scintillation fluid for a total of 14 fractions. To all the vials which received 0.5 ml effluent, 0.5 ml H_2O was added and counted for ^{3}H and ^{32}P. The counts in ^{3}H and ^{32}P channels were plotted against the elution volume (ml). 'Stop solution' (1 ml) containing 10,000 cpm ^{3}H-cAMP was loaded into Dowex columns. Effluent allowed to drain completely and washed with 2 ml of water. The rack containing the Dowex columns was placed on top of a rack containing the alumina columns. Each Dowex column was eluted with 3.5 ml of water and the effluent was allowed to

pass through both columns into scintillation vials containing 14 ml of scintillation fluid. The upper rack containing Dowex columns was removed and alumina columns were eluted with consecutive 10 x 0.5 ml aliquots of 0.1 M imidazole buffer (pH 7.3) into vials containing 4 ml of scintillation fluid. 0.5 ml of imidazole was added to all the vials and counted in ^{3}H and ^{32}P channels. The counts were plotted against the elution volume (ml) and the elution profiles determined.

Measurement of ^{32}P-cAMP formation from ^{32}P-ATP

On the day of experiment, 2-6 x 10^{6} cpm α-^{32}P-ATP was added to 1 ml of reaction mixture containing creatine phosphate 25 mM, creatine phosphokinase (250 u), Tris acetate buffer (ph 7.6, 125 mM), magnesium acetate (25 mM), ATP (2.5 mM), cAMP (0.25 mM), DTT (5.0 mM), BSA (0.5 mg) and GTP (0.1 uM). Into plastic centrifuge tube (1.5 ml) held in an ice bath, 10 ul of reaction mixture, 10 ul peptides and 10 ul theophyline (4 mM) were added. The reaction was initiated by adding 20 ul of fresh adrenal or brain membrane preparation (15 ug protein/assay tube), mixed well and incubated at 30°C for 15-30 min. The tubes were placed in an ice bath and 1.0 ml of 'stop solution' containing 0.2 mg of ATP and 0.1 mg of cAMP and 50 ul of ^{3}H-cAMP (10,000 cpm) was added to each assay tube. The tubes were centrifuged at 8000 g for 15 minutes. Aliquots of 0.9 ml supernatants were collected and applied to sequential chromatography on Dowex columns and alumina columns. The elutes from alumina columns were counted in both ^{3}H and ^{32}P channels and the gross amount of cAMP is calculated as follows:

$$cAMP_{gross} = \frac{H}{S} \times \frac{(S'-B'-F) \times 1,000}{Rsp \times E}$$

H and H' represent the cpm for ^{3}H-cAMP standard in ^{3}H and ^{32}P

channel, respectively; P and P' for the ^{32}P-ATP standard, S and S' for the sample and B and B' for the background in the ^{3}H and ^{32}P channels respectively. E is membrane protein (ug/per assay tube). F, the correction for ^{3}H counts in the ^{32}P-channel is $\frac{H'-B'}{H \times S}$

Rsp, the specific radioactivity of α-^{32}P-ATP is $\frac{P' \times 100}{A}$ (cpm/p mole),

where A represents ATP (pmole/assay tube). The blank value (Z) is calculated similarly, and the net cAMP formation $cAMP_{net} = cAMP_{gross} - Z$.

Preparation of membranes

Fresh bovine adrenal glands were obtained from a local slaughterhouse and kept in 10% sucrose at room temperature. After removing fat, the adrenals were bissected and cortical portions collected. Male Sprague-Dawley rats (weighing 150-200 g) were decapitated and brains removed rapidly and stripped of meninges. Tissues were minced with scissors and homogenized using 5 strokes of a glass-teflon homogenizer. The homogenate was centrifuged for 15 min at 1000 g (4°C), and the supernatant centrifuged for 30 min at 20,000 x g (4°C). The resulting pellet was resuspended in Tris-acetate buffer (0.01 M, pH 7.4) containing EDTA (0.8 mM), theophylline (4 mM). Protein concentration was determined by the method of Lowry et al. (9) and protein content in each assay tube was approximately 15 ug.

Results

A typical elution profile of the Dowex column is shown at Figure 1. Over 99.9% of ATP added was eluted in the first 1.5 ml volume, and cAMP after the 2.5 ml elution volume. ^{32}P-ATP was separated from the ^{32}P-cAMP

by washing the column with 2 ml, then by 4 ml of water to elute the ^{32}P-cAMP into the alumina column.

Figure 2 shows the typical elution profile of an alumina column. cAMP was not eluted until 1.0 ml elution volume of imidazole buffer was applied. ^{32}P-cAMP was purified by washing the column with 0.5 ml of imidazole (to eliminate residual ^{32}P-ATP) followed by 3.5 ml of the same buffer into the collecting vials. With this elution profile, more than 80% of cAMP was recovered.

VIP at 10^{-10}M induces detectable cAMP production from brain membranes, and at 10^{-6}M is maximally effective (Fig. 3). The dose of VIP required to stimulate maximal cAMP production by 50% (EC_{50}) is 7 x 10^{-10} M. Similarly, ACTH stimulates adenylate cyclase activity from brain membrane in a dose-dependent manner (Figure 4). Detectable cAMP formation was stimulated by ACTH at 10^{-10} M, and maximal cAMP generation at 10^{-7}M. The EC_{50} is about 1.2 x 10^{-9}M. At 10^{-6}M ACTH seems to be less effective.

In adrenal cortical membranes, ACTH (1 uM) induces cAMP formation in a time-related manner (Fig. 5). In the presence of the phosphodiesterase inhibitor theophyline (4 mM), a linear relationship between cAMP formation and incubation time up to 60 min was found. Both $ACTH_{1-10}$ and $ACTH_{1-24}$ enhance cAMP formation (148% and 238% of basal level, respectively), whereas $ACTH_{11-24}$ was ineffective (Fig. 6).

Discussion

ACTH augments cAMP levels in nervous tissue, including synaptosomal plasma membranes from rat brain (10), cultured murine brain cells (11) and cultured mouse cortical neurons (12). Binding of VIP to its receptor

sites is coupled to activation of an adenylate cyclase (13), e.g. in plasma membrane preparations from rat fat cells (14,15), heart (16) and lung (17); in isolated rat and chicken adipose cells (18,19), guinea pig pancreatic acinar cells (20), epithelial cells of rat ventral prostate (21) and horizontal cells of the teleost retina (22); and in cultured bovine choroid plexus epithelial cells (23). In the central nervous system, VIP stimulates cAMP formation in guinea pig brain (24) and various regions of rat brain (25-29). Our results confirm that VIP and ACTH stimulate adenylate cyclase activity in rat brain. From the study on time-course of cAMP generation to ACTH, cAMP generation increases over 0-60 min. The incubation times in all the experiments were 15-30 min which is within the linear portion of the curve. These observations also indicate that the two column separation method for cAMP is sensitive and reliable.

Since the classic report of Haynes and Berthet (1) implicating cAMP in adrenal steroidogenesis, many studies have related the action of ACTH to the generation of cAMP (2-4). However, it is generally recognized that there is not a consistent association between ACTH induced steroidogenesis and cAMP formation (30-34). For example, low concentrations of ACTH evoke steroidogenic response without evoking detectable changes in cAMP levels. To explain this discrepancy, several hypotheses have been proposed, e.g. the 'receptor-reserve model' by Ramachandran (35) and the 'compartment guidance concept' by Schulster and Schwyzer (36). Evidence has since accumulated to indicate two types of ACTH receptors on adrenocortical cells, only one of which is coupled with cAMP formation.

Early binding studies demonstrated two classes of binding sites for

ACTH in rabbit adrenal glands (37), mouse adrenocortical tumour cell membranes (38) and isolated rat adrenocortical cells (39). McIlhinney and Schulster (39) correlated their binding data to previous measurements of cAMP production in response to increasing concentration of ACTH (31). The K_D value of the low affinity site (1×10^{-8}M) was consistent with an EC_{50} value of ACTH action on cAMP formation (1×10^{-8}M). They suggested that this low affinity high capacity binding site was coupled to the cAMP system. Later, Moyle et al. (40) reported that an O-nitrophenyl sulfenyl derivative of ACTH, NPS-ACTH, appeared to inhibit, in a competitive fashion, the effect of ACTH on cAMP production, but not steroid production, indicating that the effects of ACTH on cAMP formation and steroidogenesis involved an interaction of the hormone with two types of receptors on the adrenal cells. Yanagibashi (41) identified two distinct ACTH receptors in rat adrenocortical cells and correlated binding data to steroidogenesis, calcium influx and cAMP synthesis induced by $ACTH_{1-24}$. From double reciprocal plots analysis, the apparent dissociation constant (K_D) of ACTH effect on steroidogenesis, 2.6×10^{-10}M in the presence of Ca^{++}, was in good agreement with the K_D value for the high affinity ACTH receptor, while the apparent dissociation constant (K_D) of the ACTH effect on cAMP formation, 5.4×10^{-9}M, was very close to K_D value of the low-affinity receptor, thus indicating that the high affinity ACTH receptor was linked to steroidogenesis and the low affinity receptor to cAMP formation. Bristow et al. (42) found that the action of either $ACTH_{1-39}$ or $ACTH_{5-25}$ (a full agonist of $ACTH_{1-39}$), was inhibited by $ACTH_{6-24}$ to different extents, indicating that the steroidogenic action of these two agonists might be mediated by two different receptor types. Recently, Kojima et al, (43) provided evidence to support the dual

receptor theory and proposed that both types of ACTH receptors on adrenal cortical plasma membrane elicit steroidogenesis. ACTH binds to one type of receptor to activate adenylate cyclase and binds to the other type of receptor to promote calcium influx. Increases in both cAMP formation and calcium influx act synergistically to induce a cellular steroidogenic response.

Our present report agrees with Kojima et al. (43) and indicated that of two classes of ACTH receptors mediating steroidogenesis, one recognizes the $ACTH_{1-10}$ fragment and is linked to cAMP system, whereas the other one recognizes $ACTH_{11-24}$ fragment and is coupled with another second messenger system (7). In this study we show directly that $ACTH_{1-10}$ and $ACTH_{1-24}$ elicit cAMP formation, whereas $ACTH_{11-24}$ is ineffective. Differential effects of $ACTH_{1-10}$ and $ACTH_{11-24}$ on cAMP production directly confirms conclusions drawn in our previous study and support the concept of two types of ACTH receptors.

References

1. Haynes RC and Berthet L. Studies on the mechanism of action of adrenocorticotropic hormone. J. Biol. Chem. 225: 115-124, 1957.

2. Grahame-Smith DG, Butcher RW, Ney KL and Sutherland EW. Adenosine 3',5'-monophosphate as the intracellular mediator of action of adrenocorticotropic hormone on the adrenal cortex. J. Biol. Chem. 242: 5535-5541, 1967.

3. Schulster D, Tait SAS, Tait JF and Mrotek J. Production of steroids by in vitro superfusion of endocrine tissue. III. Corticosterone output from rat adrenals stimulated by adrenocorticotropin or cyclic 3',5'-adenosine monosphosphate and the inhibitory effect of cycloheximide. Endocrinol. 86: 487-502, 1970.

4. Taunton OD, Roth J and Pastan I. Studies on the adrenocorticotropic hormone-activated adenyl cyclase of a functional adrenal cortex. J. Biol. Chem. 244: 247-253, 1969.

5. Ramachandran J, Isubokawa M, and Gohil K. Corticotropin receptors. Ann. N.Y. Acad. Sci. 512: 415-425, 1987.

6. Rasmussen H and Barrett P. Temporal and spatial events in the calcium messenger system. In: Ehrlich YH et al. Eds. Molecular Mechanisms of Neuronal Responses. Advances in Experimental Medicine and Biology. Plenum Press, New York, 221: 19-31, 1987.

7. Li ZG, Park D and LaBella FS. $ACTH_{1-10}$ and $ACTH_{11-24}$ promote adrenal steroidogenesis by different mechanisms. Submitted.

8. Salomon Y. Adenylate cyclase assay. In: Brooker G et al. Eds. Advances in Cyclic Nucleotide Research. Raven Press, New York, 10: 35-55, 1979.

9. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275, 1951.

10. Wiegant VM, Dunn AJ, Schotman P and Gispen WH. ACTH-like neurotropic peptides: possible regulators of rat brain cyclic AMP. Brain Res. 168: 565-585, 1979.

11. Van Calker D, Loffler F and Hamprecht B. Corticotropin peptides and melanotropins elevate the level of adenosine 3',5'-cyclic monophosphate in cultured murine brain cells. J. Neurochem. 40: 418-427, 1983.

12. Weiss S, Sebben M and Bockaert J. Corticotropin-peptide regulation of intracellular cyclic AMP production in cortical neurons in primary culture. J. Neurochem. 45: 869-874, 1985.

13. Said SI. Vasoactive intestinal peptide. J. Endocrinol. Invest. 9:191-200, 1986.

14. Desbuquois B, Laudat MH and Laudat P. Vasoactive intestinal polypeptide and glucagon. Stimulation of adenylate cyclase activity via distinct receptors in liver and fat cell membranes. Biochem. Biophys. Res. Commun. 53: 1187-1194, 1973.

15. Bataille D, Freychet P and Rosselin G. Interaction of glucagon, gut glucagon, vasoactive intestinal polypeptide and secretin with liver and fat cell plasma membranes: Binding to specific sites and stimulation of adenylate cyclase. Endocrinol. 95: 713-721, 1974.

16. Christophe J, Waelbroeck M, Chatelain P and Robberecht P. Heart receptors for VIP, PHI and secretin are able to activate adenylate cyclase and to mediate inotropic and chronotropic effects. Species variations and physiopathology. Peptides. 5: 341-353, 1984.

17. Robberecht P, Chatelain P, Neef PD, Camus JC, Waelbroeck M and Christophe J. Presence of vasoactive intestinal peptide receptors coupled to adenylate cyclase in rat lung membranes. Biochim. Biophys. Acta. 678: 76-82, 1981.

18. Frandsen EK and Moody AJ. Lipolytic action of a newly isolated vasoactive intestinal polypeptide. Horm. Metab. Res. 5: 196-199, 1974.

19. Kitabgi P, Rosselin G and Bataille D. Interaction of glucagon and related peptides with chicken adipose tissue. Horm. Metab. Res. 8: 266-270, 1976.

20. Robberecht P, Conlon TP and Gardner JD. Interaction of porcine vasoactive intestinal peptide with dispersed pancreatic acinar cells from the guinea pig. Structural requirements for effects of VIP and secretin on cellular adenosine 3',5'-monophosphate. J. Biol. Chem. 251: 4635-4639, 1976.

21. Carmena MJ and Prieto JC. Cyclic AMP-stimulating effect of vasoactive intestinal peptide in isolated epithelial cells of rat ventral prostate. Biochim. Biophys. Acta. 763: 414-418, 1983.

22. Watling K and Dowling JE. Effect of vasoactive intestinal peptide and other peptides on cyclic AMP accumulation in intact pieces and isolated horizontal cells of the teleost retina. J. Neurochem. 41: 1205-1213, 1983.

23. Crook R and Prusiner S. Vasoactive intestinal peptide stimulates cyclic AMP metabolism in choroid plexus epithelial cells. Brain Res. 384: 138-144, 1986.

24. Deschodt-Lanckman M, Robberecht P and Christophe J. Characterization of VIP-sensitive adenylate cyclase in guinea pig brain. FEBS Lett. 83: 76-80, 1977.

25. Quik M, Iversen LL and Bloom SR. Effect of vasoactive intestinal peptide (VIP) and other peptides on cAMP accumulation in rat brain. Biochem. Pharmacol. 27: 2209-2213, 1978.

26. Borghi C, Nicosia S, Glachetti A and Said SI. Vasoactive intestinal polypeptide (VIP) stimulated adenylate cyclase in selective areas of rat brain. Life Sci. 24: 65-70, 1979.

27. Kerwin RW, Pay S, Shoola KD and Pycock CJ. VIP-sensitive adenylate cyclase in rat brain: Regional distribution and localization on hypothalamic neurons. J. Pharmac. 32: 561-566, 1980.

28. Schorderet M, Sovilla JY and Magistretti PI. Vasoactive intestinal peptide and glucagon-induced formation of cyclic AMP in intact retina in vitro. Eur. J. Pharmacol. 71: 131-134, 1981.

29. Etgen AM and Browning ET. Activation of cyclic adenosine 3',5'-monophosphate accumulation in rat hippocampal slices: Action of vasoactive intestinal peptide (VIP). J. Neurosci. 3: 2487-2493, 1983.

30. Beall RJ and Sayer G. Isolated adrenal cells: Steroidogenesis and cyclic AMP stimulation in response to ACTH. Arch. Biochem. Biophys. 148: 70-76, 1972.

31. Mackie C, Richardson MC and Schulster D. Kinetics and dose-response characteristics of adenosine 3',5'-monophosphate production by isolated rat adrenal cells stimulated with adrenocorticotrophic hormone. FEBS Lett. 23: 345-348, 1972.

32. Bowyer F and Kitabchi AE. Dual role of calcium in steroidogenesis in the isolated adrenal cell of the rat. Biochem. Biophys. Res. Commun. 57: 100-105, 1974.

33. Sharma RK, Ahmed NK and Shanker G. Metabolic regulation of steroidogenesis in isolated adrenal cells of the rat. Eur. J. Biochem. 70: 427-433, 1976.

34. Honn KV and Chavin W. In vitro temporal cAMP and cortisol responses to ACTH by the normal human adrenal gland. Acta Endocr. 85: 823-831, 1977.

35. Ramachandran J. Corticotropin receptors, cyclic AMP and steroidogenesis. Endocr. Res. 10: 347-363, 1984-1985.

36. Schulster D and Schwyzer. ACTH receptors. In: Schulster D and Levitzki A. eds. Cellular Receptors for Hormones and Neurotransmitters. John Wiley & Sons, London and New York, 197-217, 1980.

37. Wolfsen AR, McIntyre MB and Odell WD. Adrenocorticotropin measurement by competitive binding receptor assay. J. Clin. Endocr. 34: 684-689, 1972.

38. Lefkowitz RJ, Roth J and Pastan I. ACTH-receptor interaction in the adrenal: a model for the initial step in the action of hormones that stimulates adenyl cyclase. Ann. N.Y. Acad. Sci. 185: 195-209, 1971.

39. McIlhinney RAJ and Schulster D. Studies on the binding of ^{125}I-labelled corticotropin to isolated rat adrenocortical cells. J. Endocr. 64: 175-184, 1975.

40. Moyle WR, Kong YC and Ramachandran J. Steroidogenesis and cyclic adenosine 3',5'-monophosphate accumulation in rat adrenal cells: divergent effects of adrenocorticotropin and its 0-nitrophenyl sulfenyl derivative. J. Biol. Chem. 248: 2409-2417, 1973.

41. Yanagibashi K, Kamiya N, Lin G and Matsuha M. Studies on adrenocorticotropic hormone receptor using isolated rat adrenocortical cells. Endocrinol. Jap. 25: 545-551, 1978.

42. Bristow AF, Gleed C, Fauchere JL and Schwyzer R. Effects of ACTH (corticotropin) analogues on steroidogenesis and cyclic AMP in rat adrenocortical cells. Biochem. J. 186: 599-603, 1980.

43. Kojima I, Kojima K and Rasmussen H. Role of calcium and cAMP in the action of adrenocorticotropin on aldosterone secretion. J. Biol. Chem. 260: 4248-4256, 1985.

Legends

Fig. 1. Elution profile of Dowex column. 1 ml 'stop solution' containing 10,000 cpm $-^{32}$P-ATP and 10,000 cpm ^{3}H-cAMP was applied to the column. Consecutive 0.5 ml volumes of water from the column was collected and counted (see Materials and Methods). Each point represents the radioactivity in 0.5 ml of effluent in a typical experiment.

Fig. 2. Elution profile of Alumina column. 1 ml 'stop solution' containing 10,000 cpm ^{3}H-cAMP was applied to alumina column and eluted as described under the Materials and Methods. Each point represents the radioactivity in 0.5 ml of effluent in one typical separation.

Fig. 3. Dose-response curve for VIP-stimulated cAMP formation from brain membrane preparations. Incubation conditions and elution profiles were determined as described above (see Materials and Methods). Data are expressed as the Mean ± SEM of two experiments pooled in duplicates.

Fig. 4. Dose response curve for $ACTH_{1-24}$-stimulated cAMP generation from brain membrane preparations. Data are expressed as the Mean ± SEM of pooled two experiments performed in duplicates.

Fig. 5. Time course of ACTH-induced cAMP formation from adrenal membranes. Adrenal membranes (15 ug/tube) were incubated with theophyline (4 mM), ACTH (1 uM) for 0-60 minutes. Each point represents the mean value of duplicates from one experiment.

Fig. 6. cAMP generation by $ACTH_{1-10}$, $ACTH_{11-24}$ and $ACTH_{1-24}$. Adrenal cortical membrane was incubated in the presence of $ACTH_{1-24}$ (1 uM) or $ACTH_{1-10}$ (10 uM) or $ACTH_{11-24}$ (10 uM). Each bar represents the mean ± SEM of two experiments, each in duplicate.

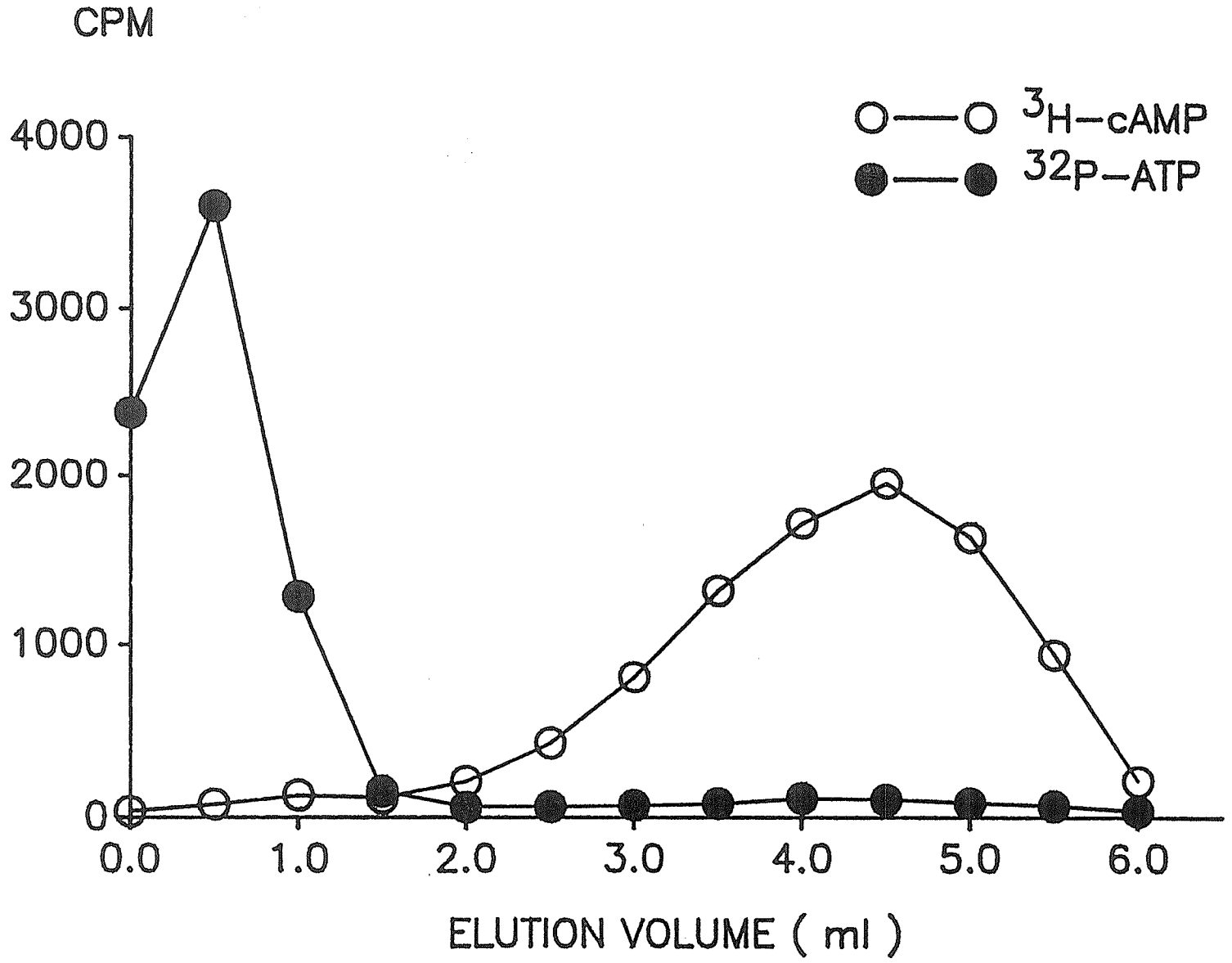

CPM
4000
3000
2000
1000
0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
ELUTION VOLUME (ml)
O—O 3H–cAMP
●—● 32P–ATP

Fig. 1

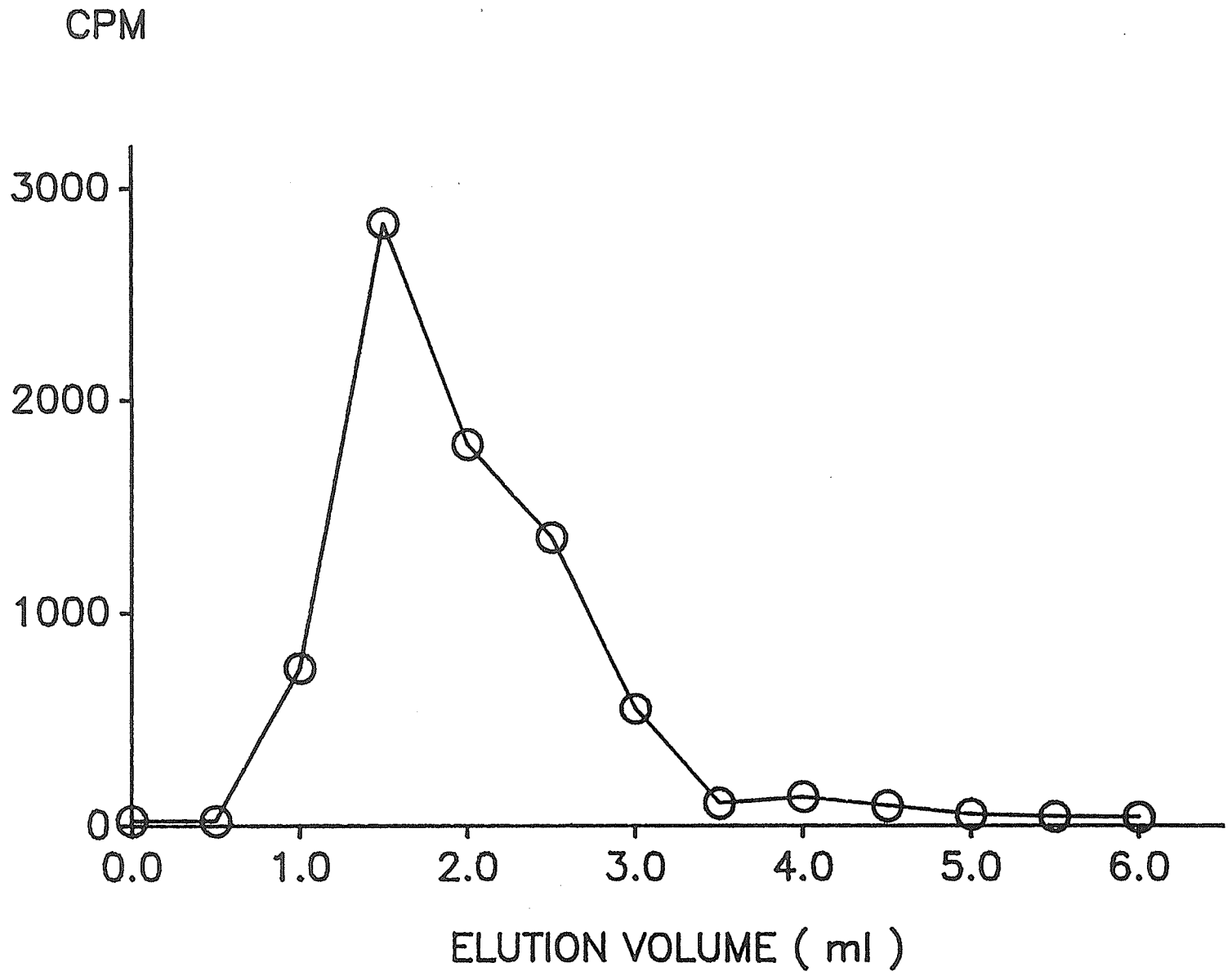


Fig. 2

Fig. 3

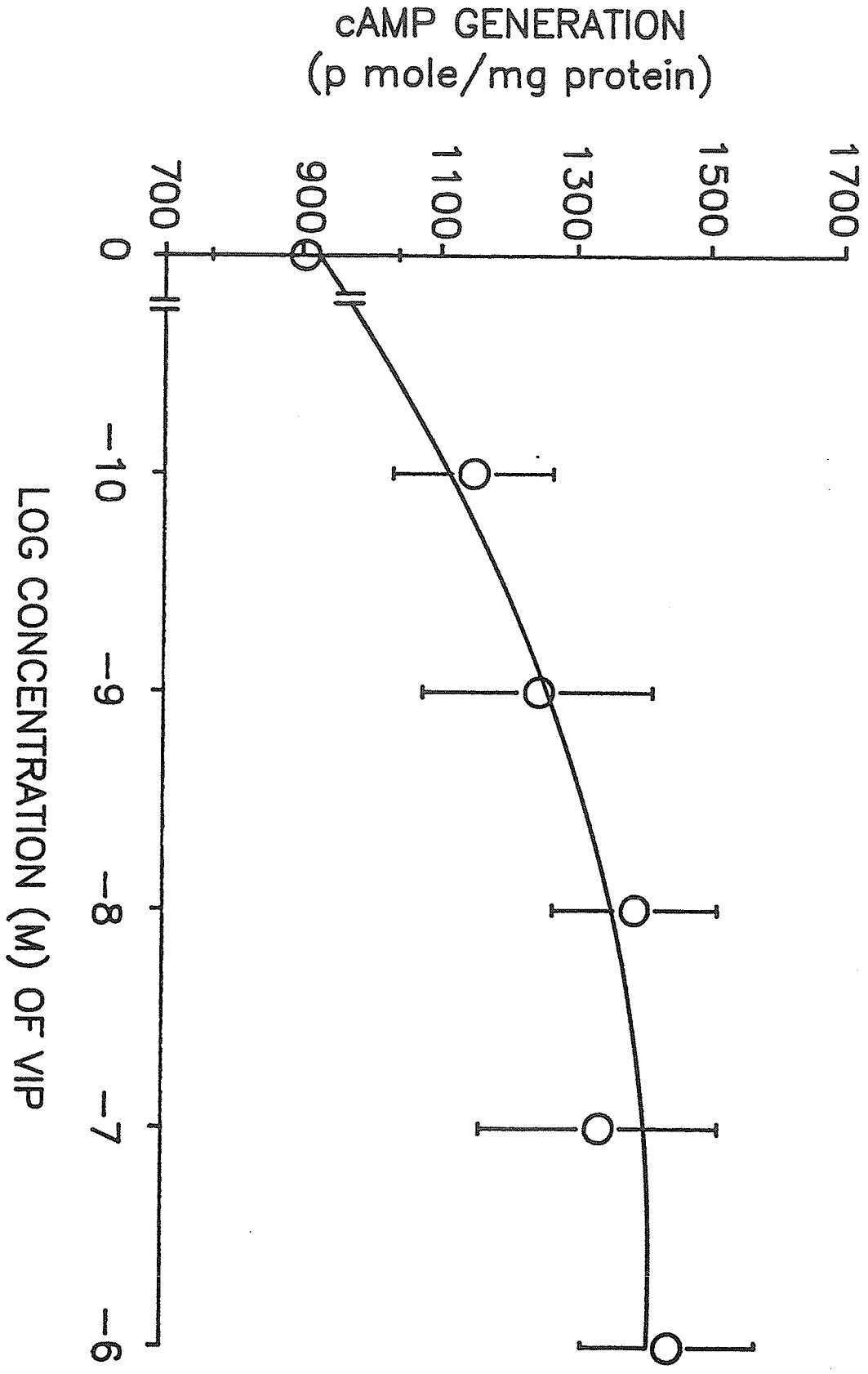

cAMP GENERATION
(p mole/mg protein)
700
900
1100
1300
1500
1700
0
-10
-9
-8
-7
-6
LOG CONCENTRATION (M) OF VIP

Fig. 4

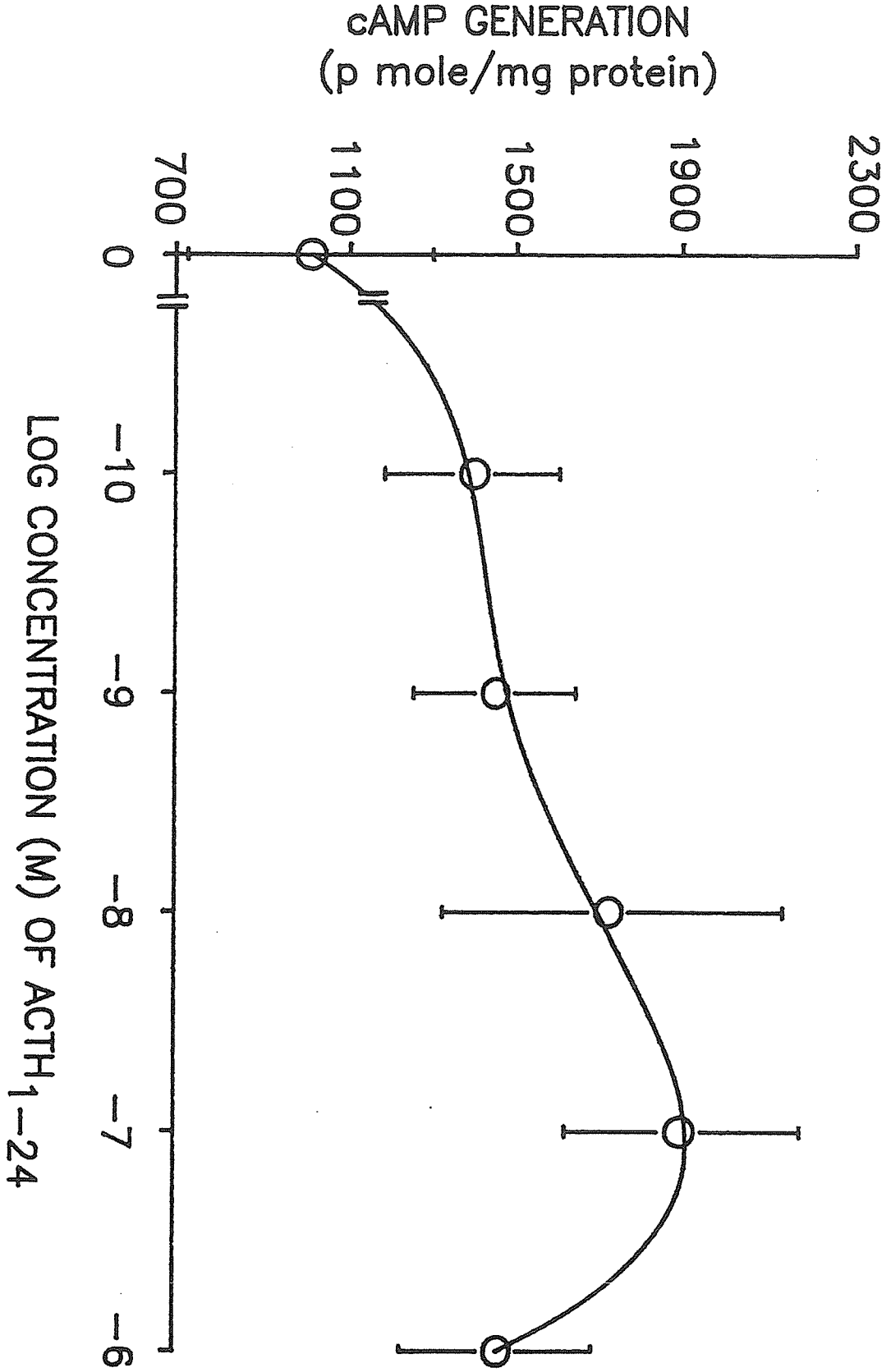

cAMP GENERATION
(p mole/mg protein)
700
1100
1500
1900
2300
0
-10
-9
-8
-7
-6
LOG CONCENTRATION (M) OF ACTH1-24

Fig. 5

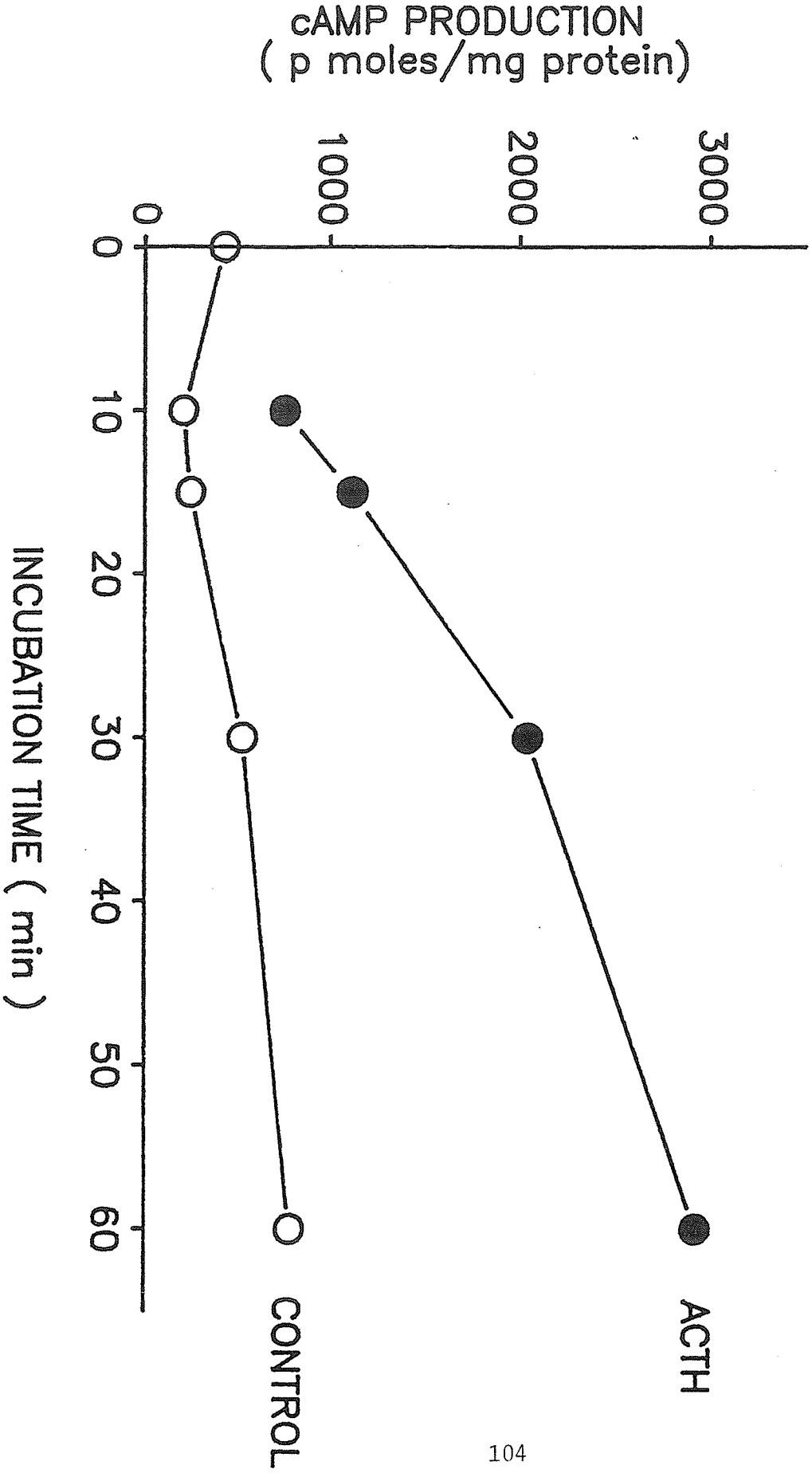
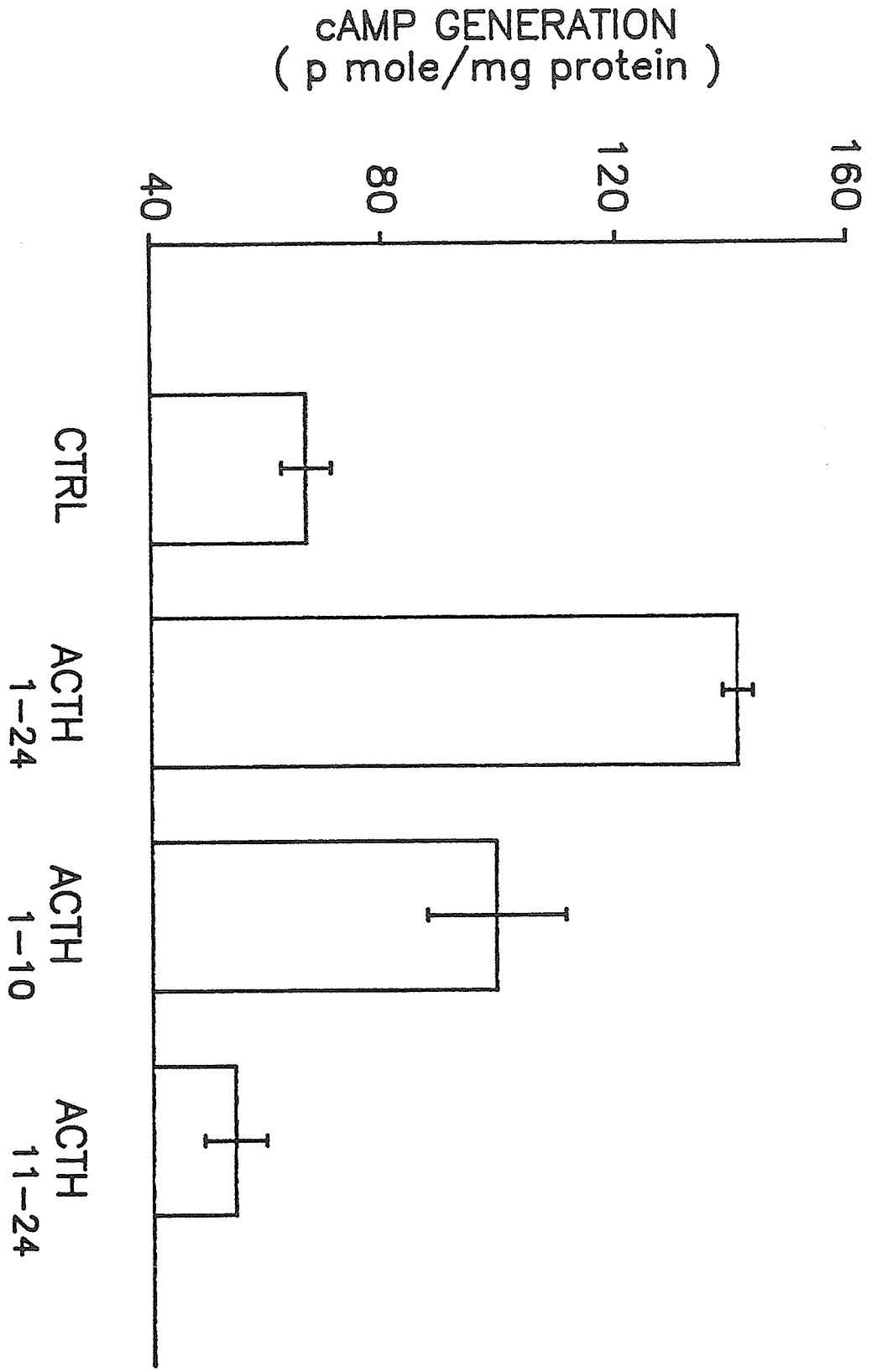

cAMP PRODUCTION
(p moles/mg protein)
0
1000
2000
3000
0
10
20
30
40
50
60
INCUBATION TIME (min)
CONTROL
ACTH

Fig. 6


cAMP GENERATION
(p mole/mg protein)
40
80
120
160
CTRL
ACTH 1–24
ACTH 1–10
ACTH 11–24

General Discussion and Overall Conclusion

Previous studies in this laboratory showed that high affinity binding of ^{125}I-ACTH$_{1-24}$ to membranes from rat brain is potently inhibited by the peptides, VIP, GRF and DYN. We now provide evidence that all four peptides also compete for the high affinity binding of ^{125}I-ACTH$_{1-24}$ to membranes from bovine adrenal cortex, and of ^{125}I-VIP to membranes from rat brain and bovine adrenal cortex. In addition, VIP, GRF and DYN stimulate cortisol production in isolated bovine adrenocortical cells, suggesting that ACTH, VIP, GRF and DYN interact with a common species of receptor that mediates steroidogenesis.

There has been no report describing the interaction of VIP, (GRF,DYN) with the ACTH receptor. Kowal et al. (1977) reported that VIP stimulated steroid production in murine tumor cell cultures. The maximal steroid secretion in response to VIP was almost identical to that by ACTH, but with much less potency. Our results show that VIP elicits cortisol production in isolated bovine adrenal cortex cells with the same potency, but with less efficacy than does ACTH$_{1-24}$. The apparent inconsistency may be caused by differences in cell types and/or in assay method for steroids. Kowal et al. measured steroids represented by 20α-dihydroprogesterone (20-OHA) and 11β-hydroxy, 20α-dihydroprogesterone from cultured murine adrenal tumor cells using a fluorometric method. We measured cortisol levels from dispersed bovine adrenocortical cells using radioimmunoassay. With the fluorometric method we have observed that VIP elicited steroid secretion with the same efficacy and potency as does ACTH. The reason for "overestimation" of steroid levels by fluorometric method is unclear.

The lower steroidogenic activity of VIP (GRF and DYN) may reflect

that these peptides 1) have lower intrinsic activity, 2) affect differently the multiple intracellular pathways involved in steroidogenesis from ACTH, or 3) occupy fewer receptors mediating steroid production. The finding that there are two classes of ACTH receptors mediating steroidogenesis in adrenal favors the last notion. Schwyzer (1980); Rasmussen et al. (1986) proposed the dual receptor model of ACTH action in steroidogenesis. However, the relation between the two receptors and ACTH structure has not been clear. We demonstrated that $ACTH_{1-10}$ and $ACTH_{11-24}$ each stimulates cortisol release in isolated and cultured bovine adrenocortical cells to about one-half the extent by $ACTH_{1-24}$. The combination of $ACTH_{1-10}$ and $ACTH_{11-24}$ elicits cortisol secretion to the maximal level promoted by $ACTH_{1-24}$. The maximal cortisol secretion by $ACTH_{11-24}$ is enhanced by forskolin, whereas the maximal cortisol production by $ACTH_{1-10}$ or $ACTH_{1-24}$ is not promoted by forskolin. On the other hand, vanadium in a dose-dependent manner inhibits cortisol release elicited by $ACTH_{1-10}$ and $ACTH_{1-24}$, but not by $ACTH_{11-24}$. cAMP production was induced in bovine adrenocortical membrane preparation by $ACTH_{1-10}$ and $ACTH_{1-24}$, but not by $ACTH_{11-24}$. These observations suggest that there are two classes of ACTH receptor sites mediating ACTH-induced steroid secretion, one recognizing $ACTH_{1-10}$ and coupled with the cAMP messenger system, and the other recognizing $ACTH_{11-24}$ and linked to a non-cAMP messenger pathway.

With respect to the steroidogenic activity of $ACTH_{1-10}$ and $ACTH_{11-24}$, there is controversy in the literature. In an early study, Schwyzer et al. (1971) reported that $ACTH_{1-10}$ stimulated the maximal steroid secretion equivalent to that by $ACTH_{1-24}$, but with less potency. However in a recent study by Hinson and Birmingham, $ACTH_{1-10}$ was reported to

elicit only a fraction of the ACTH response and with a less potency as well. Our results are in agreement with Hinson and Birmingham. As regarding $ACTH_{11-24}$, many have reported that $ACTH_{11-24}$ was inactive in steroidogenic activity, except for Goverd and Smals (1984), who reported that $ACTH_{11-24}$ steroidogenic potency was comparable to that of $ACTH_{1-10}$. This latter finding is consistent with ours. Goverde and Smalls (1984) also reported that $ACTH_{11-24}$ promoted steroid secretion with an equal efficacy to $ACTH_{1-39}$, whereas we found that $ACTH_{11-24}$ only induced a maximal steroid synthesis corresponding to about one-half that by $ACTH_{1-24}$.

Specific binding of ^{125}I-$ACTH_{11-24}$ to adrenal membranes is inhibited by VIP, GRF and DYN, but not by $ACTH_{1-10}$ and other peptides tested. Maximal cortisol production in response to VIP, GRF and DYN is only about one-half that by $ACTH_{1-24}$. An additive steroidogenic effect of VIP plus $ACTH_{1-10}$ but not plus $ACTH_{11-24}$ has been shown. These binding and functional studies, in conjunction with the existence of structural homology among VIP, GRF, DYN and $ACTH_{11-24}$, indicate that a common ACTH receptor site, probably the $ACTH_{11-24}$ recognition site accommodates VIP, GRF, DYN and mediates their steroidogenic actions.

Schwyzer (1980) proposed that one class of adrenal ACTH receptor, receptor A, elicited steroidogenesis through cAMP pathway, whereas a second species of receptor, receptor B, stimulated steroidogenesis through another mechanism(s). He also stated that the N-terminal region of ACTH molecule contained all the message sequences responsible for activating both receptor A and B. In our study we agree with the dual receptor concept. However the message sequences within ACTH molecule for activating receptor A and B appear not be restricted to the N-terminal

region as proposed by Schwyzer. In contrast, the two message sequence appear to be situated more distant one from the other. The message sequence for activating receptor A seems to be situated in the 1-10 region, whereas that for receptor B seems to be located in the 11-24 region. Opmeer et al. (1978) reported that $ACTH_{11-24}$ induced lipolysis in rat adipocytes. Greven et al. (1977) reported that $ACTH_{11-24}$ contained a message sequence for activity in the central nervous system. In this context, it is reasonable that $ACTH_{11-24}$ contains a message sequence for activating receptor A and steroidogenesis.

The work in this study extends the previous demonstration of high affinity specific binding of ^{125}I-ACTH in rat brain. It constitutes binding and functional studies towards further characterizing ACTH receptors in brain and adrenal, demonstrating the existence of two classes of ACTH receptors, identifying specific regions within the ACTH molecule for activating each type of receptor, and elucidating the 'second messenger system' activated by occupancy of these receptors. We feel we have contributed to understanding the mechanism of action of ACTH in steroidogenesis and provided an example of how several structurally related neuropeptides may interact at one common recognition site to elicit a common physiological response.

References

Goverde HJM and Smals AGH. The anomalous effect of some ACTH-fragments missing the amino acid sequence 1-10 on the corticosteroidogenesis in purified isolated rat adrenal cells. FEBS Lett 173: 23-26, 1984.

Greven H and De Wied D. Influence of peptides structurally related to ACTH and MSH on active avoidance behaviour in rats. Front. Hormone Res. 4: 140-152, Karger, Basel, 1977.

Hinson J and Birmingham MK. ACTH and adrenal aerobic glycolysis II: Effects of aminoterminal peptide fragments on lactic acid and steroid production by mouse adrenocortical cells. J. Endocrinol. 115: 71-76, 1987.

Kowal J, Horst J, Pensky J and Alfonzo M. A comparison of effects of ACTH, vasoactive intestinal peptide, and cholera toxin on adrenal cAMP and steroid synthesis. Ann. N.Y. Acad. Sci. 247: 314-328, 1977.

Opmeer FA, Van Ree JM and De Wied, D. ACTH-induced lipolysis in rat adipocytes: structure-activity relationships. Naunyn-Schmiedeberg's Arch. Pharmacol. 302: 31-36, 1978.

Rasmussen H, Kojima I, Apfeldorf W and Barrett P. Cellular mechanism of hormone action in the kidney: Messenger function of calcium and cyclic AMP. Kidney International 29: 90-97, 1986.

Schwyzer R, Schiller P, Seelig S and Sayers G. Isolated adrenal cells: Log dose response curves for steroidogenesis induced by $ACTH_{1-24}$, $ACTH_{1-10}$, $ACTH_{4-10}$ and $ACTH_{5-10}$. FEBS Lett. 19: 229-231, 1971.

Schwyzer R. Organization and transduction of peptide information. Trends Pharmacol. Sci. 1: 327-331, 1980.

THE UNIVERSITY OF MANITOBA

FACULTY OF MEDICINE
Department of Pharmacology and Therapeutics

770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3

(204) 788-6553

October 23, 1990

Dr. Zhen-guo Li
McMaster University
Department of Pediatrics
HSC 3H31
1200 Main St. W.
Hamilton, Ontario
L8N 3Z5

Dear Dr. Li:

You have my permission to use, as you see fit, the material in your Ph.D. Thesis including those sections which bear my name as co-author.

Sincerely yours,

Frank S. LaBella, Ph.D.
Professor

FSL/cp

THE UNIVERSITY OF MANITOBA

FACULTY OF MEDICINE
Department of Pharmacology and Therapeutics

770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3

(204) 788-6553
FAX: (204) 783-6915

November 9, 1992

Dr. Zhen-guo Li
McMaster University
Department of Pediatrics
HSC 3H31
1200 Main St. W.
Hamilton, Ontario
L8N 3Z5

Dear Dr. Li:

You have my permission to use, as you see fit, the material in your Ph.D. Thesis including those sections which bear my name as co-author.

Sincerely yours,

Gary Queen

GQ/cp

NOV 17 1992

Don Park

November 2, 1992

Dr. Zhen-guo Li
McMaster University
Department of Pediatrics
HSC 3H31
1200 Main St. W.
Hamilton, Ontario
L8N 3Z5

Dear Dr. Li:

You have my permission to use, as you see fit, the material in you Ph.D. Thesis including those sections which bear my name as co-author.

Sincerely yours,

Donald Park