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Isolation, Tissue Localization and Physiological Action of Corticostatic Peptides

By

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A thesis submitted to the
Faculty of Graduate Studies and Research, McGill University,
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Short Title:

Isolation and physiological action of corticostatic peptides.

This thesis is dedicated to my husband, Qinzhang, in appreciation for his encouragement and assistance and to my sons, Jonathan and Jeffrey, who were born during my Ph.D study

Abstract

Two rabbit and three guinea pig corticostatic (anti-adrenocorticotrophic) peptides were isolated from bone marrow cells and identified. The third guinea pig peptide proved to be a novel 13-member anti-parallel dimer. Removal of the two C-terminal arginines from rabbit corticostatin 1 lowered the biologic activity but removal of the two N-terminal arginines from the guinea pig peptides was without effect. Immunocytochemical localization of rabbit corticostatin 1 in the rabbit indicated that it was localized in immune tissues such as spleen and bone marrow but also in non-immune tissues such as lung, placenta, adrenal, anterior pituitary and various parts of the brain. Rabbit corticostatin 1 was measured in maternal and fetal tissues and in blood at 24, 27 and 30 days of pregnancy in the rabbit and marked changes were noted with increasing gestation. Rabbit corticostatin 1 did not inhibit the action of angiotensin II or Atrial Natriuretic Peptide but it did inhibit α-Melanotrophic Stimulating Hormone binding to specific zona glomerulosa receptors.

Résumé

Deux peptides corticostatiques (anti-adrénocorticotropine) chez le lapin et trois chez le cochon d'Inde ont été isolés et caractérisés à partir de cellules de moëlle osseuse. Le troisième peptide chez le cochon d'Inde s'est avéré être particulier en ce sens qu'il se présente sous forme d'un dimère anti-parallèle de 13 acides aminés. Il est démontré que pour la corticostatine 1 de lapin, l'enlèvement des deux résidus arginine en C-terminal diminuait l'activité biologique alors que l'enlèvement des deux arginines en N-terminal pour les peptides du cochon d'Inde, il n'y avait pas d'effet sur l'activité.

Des études d'immunohistochimie ont démontré que la corticostatine 1 de lapin était présente dans les tissus immunologiques tel que la rate et la moëlle osseuse ainsi que dans les tissus non-immunologiques comme le poumon, le placenta, la surrénale, l'hypophyse antérieure et diverse régions du cerveau. Les niveaux de corticostatine 1 de lapin varient beaucoup dans les tissus et le sang du foetus et de la mère, tel que mesurés à 24, 27 et 30 jours de gestation. La corticostatine 1 de lapin n'inhibe pas l'effet de l'angiotensine 1 ni du peptide natriurétique de l'oreillette, mais compétitionne sur la liaison de l'hormone stimulante de la mélanotropine α aux récepteurs spécifiques de la zona glomerulosa.

Preface

Corticostatins, members of the corticostatin/defensin family of peptides, are a group of cystine-arginine-rich peptides with a wide variety of biological activities. Studies in this thesis were aimed at the comparison of structure-biologic activity of natural and chemically modified corticostatins, the distribution and localization of the most potent corticostatic peptide in rabbit tissues and in cells. In addition we explored the role of corticostatin 1 in pregnancy and in parturition, and its role in the regulation of aldosterone production in adrenal zona glomerulosa cells. All of these studies were performed to gain some insight into the possible physiologic role of the corticostatic peptides.

Most of the studies described in this thesis have been published for publication in the following original articles and abstracts.

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HU, J., Jothy, S. and Solomon, S.

Localization and Measurement of Corticostatin I in non-pregnant and pregnant rabbit tissues during late gestation. *Endocrinology* **132**, 2351-2359 (1993)

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Isolation and Characterization of Corticostatic Peptides From Guinea Pig Bone Marrow. *Biochem. Biophy. Res. Commun.* **180**, 558-565. (1991)

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Isolation and Structure of Corticostatin Peptides From Rabbit Fetal and Adult Lung. *Proc. Natl. Acad. Sci.* **85**,592-596 (1988)

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Hu, J., Jothy, S. and Solomon, S.
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CSI and the Control of Parturition. Program and Abstract of the Endocrine Society 71st Annual Meeting, abstr. 26 (1989).

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ABBREVIATIONS

α-MSH	α-melanocyte stimulating hormone; α-melanotropin
3ß-HSD	3ß-hydroxy-5-ene steroid dehydrogenase
A	alanine
AngioII	angiotensin II
ACTH	adrenocorticotropic hormone
Ala	alanine
ANF	Atrial Natriuretic Factor
Arg	arginine
Asn	asparagine
Asp	aspartic acid
AtT20	a clonal mouse anterior pituitary corticotroph tumor cell line
B	corticosterone
BSA	bovine serum albumin
ß-LPH	ß-lipotropin
C	cysteine
cAMP	cyclic adenosine 3':5' monophosphate
CCCP	M-chloro -phenylhydrazone
cDNA	complementory deoxyribonucleic acid
cGMP	cyclic guanosine 3':5' monophosphate
CHO-K1	epithelial-like lung carcinoma cell line
CLIP	corticotropin-like intermediate lobe peptide
CNS	central nervous system
CRH	corticotropin-releasing hormone
CS	corticostatic peptide
CSI	rabbit corticostatin I
CSII	rabbit corticostatin II
CSIII	rabbit corticostatin III
CSIV	rabbit corticostatin IV
CSV	rabbit corticostatin V
CSVI	rabbit corticostatin VI
Cys	cysteine
D	aspartic acid
DA	dopamine
D-1	dopamine receptor 1
D-2	dopamine receptor 2
DAB	diaminobenzidine
DHA	dehydroepi-androsterone

DHAS	dehydroepi-androsterone sulfate
DHP	dihydropyridines
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease I
DRG	dorsal root ganglion
DTT	dithiothreitol
E	glutamic acid
ED_{50}	fifty percent effective dose
F	phenylalanine
FGF	fibroblast growth factor
G	glycine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GPCS1	guinea pig corticostatin 1
GPCS2	guinea pig corticostatin 2
GPCS3	guinea pig corticostatin 3
GTP	guanosine triphosphate
H	histidine
hACTH	human adrenocorticotropic hormone
hCG	human chorionic gonadotropin
hCS	human chorionic somatomammotropin
HFBA	heptofluorobutyric acid
His	histidine
hPL	human placenta lactogen
HP-1	human defensin peptide 1
HP-2	human defensin peptide 2
HP-3	human defensin peptide 3
HP-4	human corticostatic peptide 4
HPA	hypothalamic-pituitary-adrenal axis
HPLC	high performance liquid chromatography
I	isoleucine
ID_{50}	fifty percent inhibitory dose
IGF-I	insulin-like growth factor I
IGF-II	insulin like growth factor II
IL-1	interleukin 1
IL-1α	interleukin 1α
IL-1ß	interleukin 1ß
IL-2	interleukin 2
IL-6	interleukin 6
Ile	isoleucine
IP3	inositol trisphosphate
K	lysine
Kd	apparent dissociation

	constant
L	leucine
Leu	leucine
Lys	lysine
M	methionine
N	asparagine
NADPH	nicotinamine adenine dinucleotide phosphate (reduced form)
NMDA	N-methyl-D-aspartate
P	proline
PABA	para-aminobenzamidine
PCR	polymerase chain reaction
P_{450c11}	11ß-hydroxylase, 18-hydroxylase and 18-oxidase
P_{450c17}	17α-hydroxylase and 17,20 lyase
P_{450c21}	21-hydroxylase
P_{450scc}	cholesterol side-chain cleavage enzyme
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
Phe	phenylalanine
PI	phosphatidylinositol
PGs	prostaglandins
PGE2	prostaglandin E2
PGE2α	prostaglandin E2α
PLC	phospholipase C
PMN	polymorphonuclear
POMC	proopiomelanocortin
Pro	proline
PTH	phenylthiohydantoin
Q	glutamine
R	arginine
R-1	rat corticostatin/defensin 1
R-2	rat corticostatin/defensin 2
R-3	rat corticostatin/defensin 3
R-4	rat corticostatin/defensin 4
R-5	rat corticostatin/defensin 5
γMSH	γ-melanocyte stimulating hormone; γ-melanotropin
RP-HPLC	reversed-phase high performance liquid chromatography
S	serine
Ser	serine
SK-MES-1	Sloan-Kettering Chinese Hamster Ovarian cell line
T	threonine
TAP	tracheal antimicrobial peptide
TFA	trifluoracetic acid
TF5	thymosin fraction 5
TGF-ß	transforming growth factor ß
TGF-α	fransforming growth

	factor α
TNFα	tumor necrosis factor α
TPA	12-O-tetradecanoyl-phorbol 13-acetate
TRH	thyroid stimulating hormone releasing hormone
Tyr	tyrosine
V	valine
Val	valine
VSCC	voltage-sensitive calcium channels
W	tryptophan
Y	tyrosine

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CHAPTER 1: INTRODUCTION

1.1 General Introduction

It is generally accepted that the nervous system and the endocrine system, which have an intimate link at the level of the hypothalamus and the pituitary, regulate all specialized tissues to function in an integrated fashion as components of intact organisms. In the past 30 years, evidence has been accumulated to indicate that most specialized tissues such as the heart or immune system can also secrete some factors into the circulation to regulate distal organs or even the nervous system as well as the endocrine system. Therefore the boundary between the endocrine system and other systems has been diminished in recent years.

The adrenal gland is one of most important glands in the endocrine system. The secretion of its major products, glucocorticoids and mineralocorticoid, is controlled mainly by pituitary ACTH, and the renin-angiotensin system respectively. Recently it has been shown that a number of non-endocrine factors such as ANF (1-4), TGFβ (5), TNFα (6, 7) and corticostatic peptides (8) are capable of modulating the steroid synthesis in the adrenal gland.

About five years ago members of our laboratory reported in the Proceedings of the National Academy of Science USA the

isolation of a family of anti-ACTH peptides, named corticostatins, from nonendocrine tissues, and fetal and adult rabbit lung. In this thesis, I will systematically describe this family of peptides that I have been studying. Due to the varied nature of these studies, it is impossible to review all aspects of endocrinology, immunology and neurology as they relate to the project. The main purpose of this introduction is three fold: to provide a basic introduction of those topics that are most closely related to the present studies; to establish the relevance of these studies to current medical research and to review the current state of knowledge in this field.

1.2. Physiologic Action of Adrenal Steroids

The adrenal glands are complex polyfunctional organs whose secretions are required for maintenance of life. Without them, deranged electrolyte or carbohydrate metabolism leads to circulatory collapse or hypoglycemic coma and death. The hormones, produced by the adrenal cortex, are steroids which have broad physiological effects mainly on carbohydrate and lipid metabolism, on immunological funtion and inflammatory processes, on musculoskeletal and connective tissues, on fluid and electrolyte homeostasis and others such as neuropsychiatric and behavioral effects, gastrointestinal effects, development effects and dissociation of biological effects.

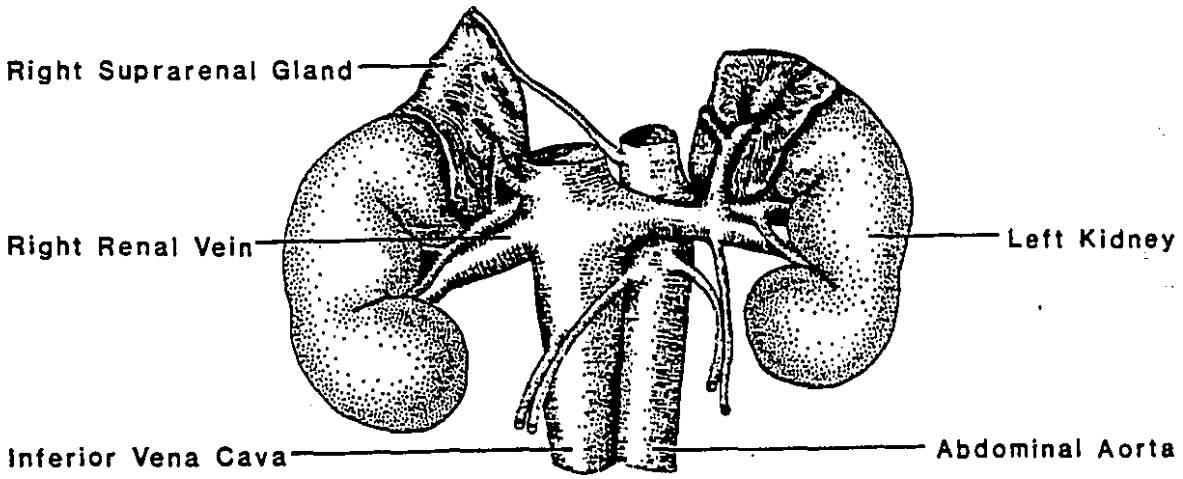
1.2.1. Anatomy and Histology of the adrenal gland

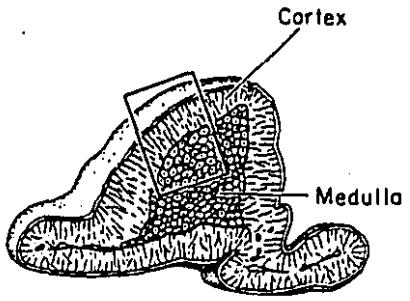
CHAPTER 1. INTRODUCTION

The "discovery" of the adrenal glands has been attributed to an anatomist of the sixteenth century, Bartholomeo Eustachius (9). The adrenal glands were first called suprarenal glands because of their location adjacent to the upper surface of each kidney, as illustrated in Fig.1-1. Each adrenal consists of two functionally distinct endocrine glands within a single capsule. In the embryonic development of humans and most mammals, the cortex derives from mesenchymal cells attached to the coelomic cavity lining adjacent to the urogenital ridge. It is then invaded by neuroectodermal cells that will form the medulla. It becomes quite vascular and increases rapidly in size, to become larger than the kidney at midgestation (10). By the second trimester, the adrenal cortex can be divided into two distinct zones, the thin outer "definitive" zone that will form the adult cortex and the inner "fetal" zone which makes up most of the adrenal mass even at birth but degenerates rapidly after birth.

In the adult, the adrenal cortex constitutes 80-85% of the adrenal gland and consists of three layers. The histology of the adrenal cortex was an early indication of its diverse functions. The division of the mammalian adrenal cortex into three areas or zones was first recorded by Arnold in 1866 (11). He named the three zones: zona glomerulosa, zona fasciculata, and zona reticularis. Arnold based his nomenclature on the arrangement of the cortical cells as well as the connective tissue fibres and blood vessels within the cortex. These differences are clearly

Figure 1-1. Location and structure of the adrenal glands. (Adapted from Hadley ME,In: Endocrinology, Prentice-Hall, Inc., Englewood Cliffs, 1984)





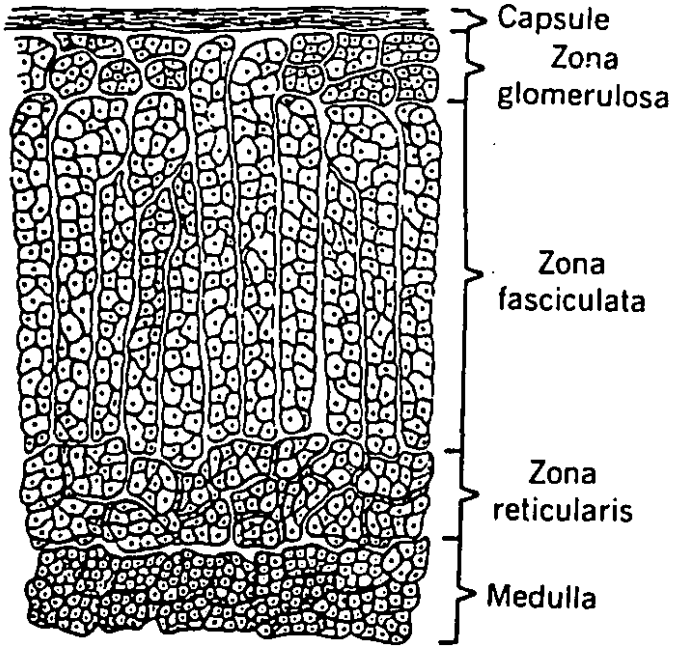
visible in histological sections of the adrenal cortex. The histological differences between the zones of the adrenal cortex and chromaffin-producing cell of the adrenal medulla are illustrated in Fig. 1-2.

The outer zone, the zona glomerulosa, immediately adjacent to the connective tissue capsule, consists of small, many-sided epithelial cells arranged in rounded groups or curved columns. The glomerulosa cell cytoplasm contains numerous microtubules and elongated mitochondria, as well as an abundance of granular endoplasmic reticulum, a characteristic feature of cells that synthesize steroids. The principal steroid made by zona glomerulosa is the mineralocorticoid, aldosterone.

Internal to the zona glomerulosa is the broader and better defined zona fasciculata. The cells of this zone are arranged in straight columns two cells thick, and have numerous droplets containing large amounts of phospholipids, fats, fatty acids, and cholesterol. These substances are embedded in a complex arrangement of granular endoplasmic reticulum. The mitochondria are spherical and the Golgi complex is extensive. The innermost zone of cortex, the zona reticularis, consists of branching and joining columns of rounded cells whose cytoplasm contains much smooth endoplasmic reticulum, numerous lysosomes, and some pigment bodies. The fasciculata-reticularis areas of the adrenal cortex synthesize cortisol and several other glucocorticoids.

In humans and many other mammals the chromaffin tissue is

Figure 1-2. Zones of the adrenal cortex. (Adapted from Yates et al. In: Medical Physiology, 14th ed. Edited by Mountcastle. Mosby, St. Louis, 1980.)



surrounded by steroidogenic tissue and the vascular relationships are such that the secretory products of the cortex perfuse the medulla through a portal network. As noted, adrenal chromaffin epinephrine synthesis is dependent on steroid hormonal support (12). There is evidence that chromaffin tissue has the capacity to carry out many enzymatic reactions characteristic of the cortex and is therefore capable of converting incomplete steroid intermediates passing through the medulla into active glucocorticoids. The close anatomical coupling and functional interrelationship between the cortex and medulla suggest that these two tissues, although of diverse origin, may constitute an integrated functional unit, at least in higher vertebrates. This relationship may be of particular adaptive importance under conditions of stress when both adrenaline and glucocorticoids are in particular demand (13).

1.2.2. The physiological effects of adrenal steroids

Although the "discovery" of the adrenal took place as early as the sixteen century, little was known about its function until Thomas Addison described, in 1855, the disease now known as Addison's disease. Since 1930 when Hartman et al first discovered that lipid extracts of adrenal cortex had high potency for maintaining normal health and growth of adrenalectomized cats (14-16), knowledge of steroid structure and synthesis of such potent synthetic steroids such as prednisone, prednisolone, dexamethasone, and triamcinolone have increased rapidly. The

availability of highly specific techniques for testing adrenocortical function has greatly enhanced and simplified the evaluation of adrenal function. The importance of hormones of the adrenal cortex for survival becomes very clear.

Diverse effects of adrenal cortex steroids can be classified, somewhat arbitrarily, into two general categories based on their ability to protect against these two causes of death. Glucocorticoid effects are those concerned with intermediary metabolism, inflammation, immunity, wound healing, and muscle and myocardial integrity. Cortisol and, to a lesser extent, corticosterone are the physiologically important glucocorticoids. Mineralocorticoid effects are those concerned with salt, water, and other mineral metabolism. Aldosterone is the physiologically important mineralocorticoid, although some deoxycorticosterone, another potent mineralocorticoid, is also produced by the normal adrenal gland. The adrenal cortex also produces androgens which have biological effects similar to those of the male gonadal hormones.

Maintaining of carbohydrate reserves is the hallmark of glucocorticoid activity. Glucocorticoids promote the conversion of protein to carbohydrate (gluconeogenesis) and the storage of carbohydrate as glycogen. The diminished urinary nitrogen, plasma glucose, and liver glycogen characteristic of adrenalectomized animals can be restored to normal by administration of adrenal steroids. Another remarkable effect of glucocorticoids is their

anti-inflammatory effect. However, we are still far from understanding the physiological importance of glucocorticoids in the respose to tissue injury and the manner in which they temper the inflammatory response. High concentration of glucocorticoids suppress cellular immunity by blocking lymphokine production and thus decrease normal proliferation of B-lymphocytes, and they may also kill T-lymphocytes. It has been suggested that the physiological role of the suppressive effects of glucocorticoids on humoral and cellular immunity might be to prevent development of autoimmunity that would otherwise follow from the release of fragments of injured cells. The major action of aldosterone is on the excretion of electrolytes by kidney. Aldosterone enhances the reabsorption of sodium and the excretion of potassium, ammonium, and magnesium. Aldosterone also increases the ratio of sodium to potassium concentrations in sweat and salivary secretion. Since perspiration can be an important avenue for sodium loss, the actions of aldosterone on sweat glands is physiologically significant. Because the maintenance of glucose and electrolyte homeostasis are absolutely essential to survival, it would seem that the adrenal cortex is very important to life in this regard.

1.2.3. Adrenal steroid biosynthesis

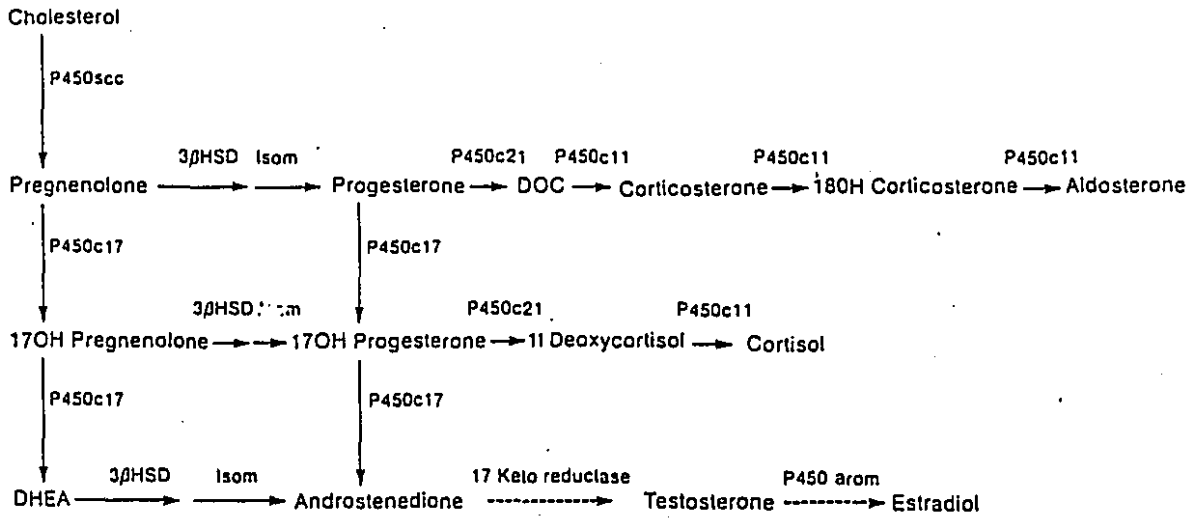
Most aspects of adrenocortical hormone biosynthesis, including the intracellular location and structure of these

enzymes and the genes that encode them, have now been elucidated (17), and these will be summarized as follows.

All the adrenocortical hormones are steroid compounds. They are synthesized in the cell via cholesterol formation from acetate. In addition , cholesterol is extracted from circulating blood directly into the steroidogenic cell. The common biosynthetic pathway from cholesterol is via pregnenolone, the stem precursor for the three major groups of adrenal steroids: glucocorticoids, mineralocorticoids, and androgens. The principal steps in the formation of these important steroid products are indicated in Fig. 1-3. Essentially all these steps occur in two of the organelles of the cell, the mitochondria and the endoplasmic reticulum, some steps occurring in one of these organelles and some in the other. Each step is catalyzed by a specific enzyme system. A change in even a single enzyme in the schema can cause vastly different types of hormones to be formed, such as especially large quantities of masculinizing or, very rarely, feminizing sex hormones or other steroid compounds not normally present in the blood but that have either mineralocorticoid or glucocorticoid actions or a combination of both.

Most steroidogenic enzymes are members of the cytochrome P450 group of oxidases (18,19). Cytochrome P450 is a generic term for a large number of oxidative enzymes, all of which consist of about 500 amino acids and contain a single heme group.

Figure 1-3. Principal pathways of human adrenal steroid hormone synthesis. (Adapted from Miller W, Moleculor Biology of steroid hormone synthesis. Endocrine Reviews Vol.9, No.1 1988)



They are so called P450 (pigment 450) because all exhibit a characteristic shift in the absorbent peak from 420 to 450 nm upon reduction with carbon monoxide. Most cytochrome P450 enzymes are found in the endoplasmic reticulum of the liver, where they metabolize an endless and innumerable array of endogenous and exogenous toxins, drugs, xenobiotics, and environment pollutants (19,20,21). There are four distinct P450 enzymes which are involved in adrenal steroidogenesis (Fig.1-3). P450scc, found in adrenal mitochondria, is the cholesterol side-chain cleavage enzyme mediating the series of reactions formerly termed 20,22-desmolase. P450c11, also found in mitochondria, mediates 11-hydroxylase, 18-hydroxylase and 18-methyloxidase activity. P450c17, found in the endoplasmic reticulum, mediates both 17α-hydroxylase and 17,20-lyase activity, while P450c21 mediates the 21-hydroxylation of both glucocorticoids and mineralocorticoid.

The conversion of cholesterol to pregnenolone represents a rate-limiting step in the biosynthesis of steroids in many tissues as well as in the adrenal cortex. The 20α- and 22-hydroxylations and cleavage of the cholesterol side chain between carbon atoms 20 and 22 to yield pregnenolone are mediated via a single species of P450scc located in the inner mitochondrial membrane (22). Each of the latter reactions requires a pair of electrons and molecular oxygen. P450scc functions as the terminal oxidase in a mitochondrial electron transport system. Electrons

from NADPH are accepted by an adrenodoxin reductase flavoprotein, also associated with the inner mitochondrial membrane. Adrenodoxin reductase transfers the electrons to an iron sulfur protein, adrenodoxin, located in solution in the mitochondrial matrix, which then denotes them to cytochrome P450scc. A single gene for P450scc has been cloned and sequenced from the bovine (23) and human (24,25) adrenal, and it lies on human chromosome 15 (17). Adrenodoxin reductase has also been cloned in the human and exists as a single gene on the long arm of chromosome 17 (26).

During the biosynthesis of the corticosteroids, the newly synthesized pregnenolone is returned to the cytosolic compartment, where it is converted to progesterone by dehydrogenation of the 3-hydroxyl group of pregnenolone and isomerization of the double bond at C-5. These reactions occur as a result of the sequential action of the enzyme 3ß-hydroxysteroid dehydrogenase and two or three different isomerases. A single form of the dehydrogenase is thought to exist in all steroidogenic tissues (27) but various substrate-specific isomerase isoenzymes have been characterized (28). These enzymes have been found in both mitochondrial and microsomal fractions, but the enzymatic processes responsible are poorly understood.

In the adrenal cortex, the pregnenolone is also converted by a second form of cytochrome P450c17 to 17α-hydroxypregnenolone. In humans and cattle 17-hydroxysteroids are produced by the zona

fasciculata and zona reticularis. However some mammalian adrenal lacks a 17α-hydroxylase, and therefore synthesizes corticosterone, a glucocorticoid which does not contain a 17α-hydroxyl group. For example, corticosterone is the principal steroid of the rat adrenal, except for the zona glomerulosa which also produces aldosterone (29). Sequential actions by the enzymes 18-hydroxylase and 18-hydroxysteroid dehydrogenase convert corticosterone to aldosterone. The latter enzyme is found only in the glomerulosa.

The progesterone or 17α-hydroxyprogesterone then undergoes hydroxylation by a third cytochrome P450c21, which leads to the production of deoxycorticosterone (DOC) and 11-deoxycortisol, respectively. 21-hydroxylation is mediated by P450c21 found in smooth endoplasmic reticulum. 21-Hydroxylation was the first steroidogenic activity ascribed to any cytochrome P450 (30). P450c21 employs the same flavoprotein intermediate, P450 reductase, used by P450c17 to transport electrons from NADPH. Isolation of P450c21 from bovine adrenals showed a single species of protein that could 21-hydroxylate both progesterone and 17OHP (31), but it was not until the cDNAs and genes were cloned that it was proven that there is only a single functional 21-hydroxylase gene in man (32).

DOC and 11-deoxycortisol then leave the endoplasmic reticulum and enter the mitochondria for the final step in glucocorticoid production, which is catalyzed by P450c11. P450c11

is also localized in the inner mitochondrial membrane (33,34) and utilizes reducing equivalents provided by adrenodoxin and adrenodoxin reductase, as described for P450scc (35-37). Thus DOC is converted into corticosterone (B), which is the major glucocorticoid in rodents, while 11-deoxycortisol is converted into cortisol (F), the primary glucocorticoid in humans. These reactions take place in the fasciculata-reticularis cells of the adrenal cortex.

A single bovine P450c11 protein has 11-hydroxylase, 18-hydroxylase, and 18-oxidase activities, but this series of enzymatic steps may be mediated by more than one enzyme in rats. Enzymatic assays of purified rat mitochondrial proteins have suggested that one enzyme found in all zones of the adrenal cortex has both 11- and 18-hydroxylase activities, whereas another enzyme, found exclusively in the zone glomerulosa, catalyzes 18-hydroxylation and 18-oxidation of corticosterone (38). Another study also confirmed that rats synthesize two similar, but distinct, P450c11 mRNAs (39). One, P450c11A, is found both in the zona glomerulosa and fasciculata/reticularis, whereas the second, P450c11B, is found only in the zona glomerulosa. When rats receive a low-salt diet, P450c11A mRNA decreases and P450c11B mRNA increases. In situ hybridization studies show that only the P450c11 found in the zona glomerulosa is regulated by salt treatment in vivo, whereas glucocorticoid treatment in vivo regulates P450c11 in all zones (39). These

experimental results suggest that the regulation of glucocorticoid and aldosterone production in the rat adrenal occurs by different mechanisms.

Two important facts should be stressed regarding the substrates, enzymes, and zones involved in adrenal steroidogenesis. First, although the same enzymes are present throughout all zones of the adrenal cortex, these enzymes produce different steroids at different zones depending on the substrate and the coenzyme; for example, the enzyme 11-hydroxylase in the zona glomerulosa converts DOC to corticosterone, whereas in the zona fasciculata the same enzyme converts 11-deoxycortisol to cortisol. Second, although the three zones function as independent units, the substances formed in one zone serve as substrates for synthesis of the products in an adjacent zone; for example, pregnenolone and progesterone synthesized by zona glomerulosa are not only essential for the synthesis of mineralocorticoid by that zone but are also utilized by the zona fasciculata as substrates for the synthesis of glucocorticoid precursors.

1.3. Modulators of adrenal steroid synthesis

So far there is no solid evidence to support the existence of a "ready-to-secrete" pool for steroid hormone as for most of the protein hormones. In general it is believed that steroid secretion takes place by passive diffusion after it is

synthesized in the adrenal gland. Since addition of most of the metabolic intermediates, but not the precursor cholesterol, to whole adrenal or dispersed adrenal cells in the adrenal bioassay system can cause an immediate increase of the definitive steroid output, the conversion of cholesterol to pregnenolone seems to be the rate-limiting step regulated by hormonally controlled mechanisms. A number of factors such as ACTH, α-MSH, angiotensin II, cAMP, or some toxins (forskolin, cholera toxin) will stimulate steroidogenesis, while other factors such as corticostatic peptides, somatostatin, TGFß, ANF and interleukins can inhibit basal or stimulated steroidogenesis.

1.3.1. Mechanism of hormone action

In general, the mechanism of action of a hormone is a description of the chain of events, on the molecular level, that results eventually in the observed physiologic change. Hormones are transported to all the cells of the body through the circulatory system. The specificity of the hormone for the cells of its target tissues depends upon the fit of the hormone to its receptor. The formation of the hormone-receptor complex initiates other cellular and subcellular events. Although a number of factors have been documented as the modulators of adrenal steroidogenesis, only part of their mechanisms involved in the steroidogenesis has been elucidated so far.

1.3.1.1. *cAMP and its role in adrenal steroidogenesis*

It has become increasingly evident that cAMP has marked

effects on steroid hormone synthesis. The function of cAMP, as proposed by Sutherland et al.(40,41) is to act as a "second messenger" by accepting the information offered by the hormone-receptor complex and transmitting it to its molecular destination within the cell. This is achieved by the activation of protein kinase enzymes in the cell. Most of the cAMP-activated protein kinases are characterized by the presence of two different subunits which bind cAMP resulting in the dissociation of this regulatory subunit from a protein kinase unit that has either been inhibited or inactivated. The new active part of the protein kinase, the catalytic subunit, is phosphorylated in the presence of ATP and then proceeds to initiate the intracellular activities that are characteristic of the hormone.

Like ACTH, which will be discussed in section 1.3.2.1., cAMP stimulates steroidogenesis and increases P450scc and P450c11 proteins and their mRNA in bovine adrenal cells (7, 42-44). In cultured human fetal adrenal cells, cAMP increases P450scc and P450c17 at physiological concentrations (45). The accumulation of mRNAs for adrenodoxin and adrenal steroidogenic P450s apparently occurs via cAMP-mediated stimulation of gene transcription in primary cultures of bovine adrenocortical cells(46,47). The rat P450scc gene was also regulated by cAMP in vitro (48). Additional studies were performed to define the mechanism by which cAMP increased steroid synthetic function. Cycloheximide, an inhibitor of protein synthesis, inhibited the stimulation of the mRNAs for

multiple enzymes by cAMP (46,49). It suggested that this might be a general cellular strategy for regulating steroidogenesis. The cAMP responsiveness of bovine P450scc gene expression is mediated by sequences different from canonical consensus regulatory elements (50). Whether or not there are sequences conferring cAMP responsiveness which are common both to P450scc and the other steroidogenic P450 genes remains to be established. However, the human gene for P450scc has been found to be repressed by TPA, a phorbol ester, plus A23187, a calcium ionophore, by mechanisms and sequences independent of those that mediate induction by cAMP (51).

The dominant role played by cAMP-dependent protein kinase in the kinase-deficient mouse Y1 adrenal tumor cells was demonstrated by experiments showing the regulation of cAMP-regulated gene expression by protein kinase C requires basal cAMP-dependent protein kinase activity. Steroidogenesis in these kinase-deficient cells was no longer stimulated by cAMP analogues, and the expression of cAMP-regulated gene (ornithine decarboxylase) could no longer be induced (52). The mitochondria, the microsomes and the cytosol have been described as possible sites of cAMP-dependent phosphorylation. Dada et al (53) have shown that cytosol is the site of phosphorylation of the cAMP-dependent protein kinase in adrenal steroidogenesis, but not the microsomes.

The participation of adenylate cyclase and cAMP in the

mechanism of hormone action has been verified by numerous in vivo and in vitro experiments. Cyclic guanosine monophosphate (cGMP), synthesized from the substrate guanosine triphosphate (GTP) (54) is locally abundant in many tissues, but its actual intracellular concentration is about a tenth that of cAMP. In addition, unlike cAMP, cGMP has not been shown to mimic hormone effects.

1.3.1.2. *Calcium, Calcium Channels and their roles in adrenal steroid synthesis*

Calcium clearly plays a pivotal role as an intracellular second messenger. Ca^{++} has a potent effect on many vital physiological functions, including cell division, blood clotting, and muscle contraction. In many endocrine control systems, calcium is necessary for hormone secretion and it plays an important role in the hormonal regulation of metabolic pathways. When the concentration of free cytoplasmic Ca^{++} rises to the micromolar range, many important cellular events are initiated. Although cells contain a great deal of Ca^{++}, most of it is not free. Thus, Ca^{++} can be bound by a variety of proteins and can be sequestered by organelles such as the mitochondria and endoplasmic reticulum(55,56). Furthermore, several pumps and exchange systems remove free Ca^{++} from the cytoplasm if its concentration begins to rise (57). All these factors maintain the resting Ca^{++} at very low level- less than 10^{-7}M. Because the Ca^{++} concentration gradient is about four orders of magnitude across

the cell membrane, it is much greater than that for Na^+ or K^+.

A transient increase in Ca^{++}, which acts as an important intracellular signal, can be initiated in two major ways. (i) Calcium can be released from intracellular stores associated with the endoplasmic reticulum (58-60). Several important stimuli (such as hormones) act upon cell surface receptors and stimulate the breakdown of the phospholipid phosphatidylinositol bisphosphate (60). This generates diacylglycerol and inositol triphosphate (IP_3) Intracellular receptors for IP_3 exist that, when activated, lead to the release of Ca^{++} into the cytoplasm (58,59). Although the precise mechanism of this effect is unclear, it is the topic of intensive investigation. (ii) The Ca^{++} may also rise due to an increase in the Ca^{++} permeability of the plasma membrane, which is normally virtually impermeable to Ca^{++}.This is achieved by the opening of channels through which Ca^{++} can pass into the cytoplasm down its electrochemical gradient. Some cells have Ca^{++} channels that can be opened by the action of an agonist on a receptor (61). For example, Ca^{++} can pass through the channels opened by nicotinic cholinergic agonists (62) or by the excitatory amino acid N-methyl-D-aspartate (NMDA) (63). However, several cells have channels that open and close in response to changes in membrane potential. These voltage-sensitive calcium channels (VSCC) (64,65) are found in some types of eggs and endocrine cells, a variety of types of muscle cells, neurons, and a variety of other cell types.

CHAPTER 1. INTRODUCTION

Nowycky et al. and several other groups recently advanced our understanding significantly by demonstrating that chick dorsal root ganglion (DRG) neurons in culture possessed three distinct types of VSCC (66-69). The first type of VSCC, designated T, gives rise to a small transient Ca^{++} current elicited by small depolarizing steps from negative holding potentials. With stronger depolarization a second component of the current is observed that only slightly inactivates over a time course of several hundred milliseconds. The second type of VSCC (N), of intermediate size, was responsible for the second inactivating phase of the Ca^{++} current observed at strong depolarization. The third VSCC (L), which was also activated by strong depolarization, was responsible for the noninactivating component of the current. The pharmacological properties of these three types of VSCC proved most interesting. L channels were modulated by dihydropyridines (DHP) agonists and antagonists but T and N channels were not. L and N channels were potently blocked by Cd^{++}, whereas T channels were much less sensitive. Finally, in chick DRG cells a 27-amino acid toxin from the venom of the marine snail Conus geographicus, called ornega conotoxin blocked both N and L channels, but not T channels (70).

Ca^{++} is well known to exert a second massage role in rat adrenal glomerulosa cells in which voltage-dependent Ca^{++} channels have been described (71-75). There are two types of Ca^{++} channels, T and L, which were identified in bovine adrenal glomerulosa

cells (72,73). In cultured glomerulosa cells, there are three different components of the Ca^{++} current, T, L and N (76). In the dispersed bovine adrenal glomerulosa cell system, T-type Ca^{++} channel has been found to have an important role in mediating stimulus-secretion coupling in response to high K^+ or Angio II (74,75). Recently, Yanagibashi et al. reported Voltage-dependent Ca^{++} channels are involved in the regulation of the intracellular events of steroid synthesis in bovine but not in rat fasciculata cells (77).

The importance of Ca^{++} in the stimulation of steroidogenesis in the adrenal cortex by ACTH was first pointed out by Birmingham et al.(78). Subsequently, several groups have shown that extracellular calcium is required for ACTH-induced steroidogenesis in isolated adrenocortical cells derived from several species (79-82). There have been numerous attempts to elucidate the precise role of the ion in the action of ACTH. It has been suggested by Yanagishi et al.(83) that there are two types of ACTH-receptors on adrenocortical cells, e.g., one is the high affinity receptor which is concerned with an increased Ca-influx regulated steroidogenesis, another is the low affinity receptor which is coupled to adenylate cyclase to generate cAMP. Lefkowitz et al. (84) investigated the binding of ^{125}I-labelled ACTH preparations to a subcellular fraction derived from a mouse adrenal tumor and proposed their results as evidence for the existence of two classes of ACTH receptors with vastly differing

affinity and capacities. However, the existence of the very high affinity sites were not confirmed, and other investigators could detect only the low affinity sites in adrenal membrane preparations. Based on these results, Lefkowitz et al. concluded that calcium is not required for the interaction of ACTH with the adrenal receptor(s) (85). In contrast Cheitlin et al.(86) utilized specific photoaffinity labelling of ACTH receptors (87,88) to distinguish between the role of calcium in the binding of the hormone to its receptor and the role of the ion in post-binding events. They found the concentration of extracellular Ca^{++} required for restoring steroidogenesis was 10-fold lower than the concentration of Ca^{++} needed for optimal binding of ACTH to its receptor, suggesting that the primary role of extracellular Ca^{++} in the action of ACTH is to facilitate the association of the hormone with its receptor.

Potassium ion is also involved in steroidogenesis, especially in zona glomerulosa cells. These effects of potassium will be discussed together with the angiotensin system in section 1.3.2.2..

1.3.2. Control of steroid synthesis

The biological roles of adrenal glucocorticoids and mineralocorticoid differ considerably. It is not unexpected, therefore, that the control of synthesis and secretion of these two classes of steroid hormones is also different. Adrenal glucocorticoid synthesis and secretion is controlled by pituitary

adrenocorticotropin (ACTH), whereas aldosterone secretion is controlled primarily by the renin-angiotensin system.

1.3.2.1. ***The role of CRF and ACTH in glucocorticoid synthesis***

Glucocorticoid production by the cells of the zona fasciculata is regulated by pituitary ACTH. The release of ACTH is in turn regulated by a hypothalamic corticotrophin releasing hormone (CRH) (89). Unilateral adrenalectomy is followed by contralateral adrenal hypertrophy and hyperplasia. After bilateral adrenalectomy or in primary adrenal insufficiency, there is a striking increase in plasma ACTH levels (90-93). These elevated levels of ACTH can be returned to normal by administration of glucocorticoids (94,95), suggesting a negative feedback regulation of ACTH secretion by the steroids. Glucocorticoid negative feedback may be mediated at the level of pituitary, hypothalamus, or even higher brain centres.

ACTH regulates glucocorticoid production through binding to specific plasma membrane receptors in the zona fasciculata cells of the adrenal cortex (84,96). ACTH binds to receptors that couple to heterotrimeric guanine nucleotide-binding proteins (G proteins) (97) that activate adenylate cyclase (98-101) leading to an increase of cAMP-dependent protein kinase activity (102). As mentioned before, calcium is required for ACTH activation of adenylate cyclase but not for binding of the peptide to adrenal cells (103).

In the past few years a number of G-protein coupled

receptors have been characterized and found to belong to a large gene family. ACTH, and possiblly α-MSH as well, binds to receptors that couple to G-proteins that activate adenylate cyclase and were therefore expected to have sequence similarity with other members of this large gene family (104). A DNA fragment that encodes part of the α-MSH receptor was isolated by the use of the polymerase chain reaction (PCR) (105) amplification of cDNA from a human melanoma that contained a large number of α-MSH binding sites (106). Very recently, Mountjoy et al.(107) cloned a family of genes that encode the melanocortin receptors which include murine and human α-MSH receptors and a human ACTH receptor. The coding regions of the murine and human α-MSH receptors and human ACTH receptor were sequenced, and the predicted amino acid sequences were aligned with three representative G protein-coupled receptors: the cannabinoid receptor (108,109), the bombesin receptor(110), and the ß-adrenergic receptor (111). The human α-MSH receptor amino acid sequence was 76% identical and collinear with the murine α-MSH receptor cDNA sequence, whereas the human ACTH receptor was 39% identical and nearly collinear with the human α-MSH receptor. A comparison of the α-MSH and ACTH receptors amino acid sequences with the cannabinoid (-32% identity), ß2-adrenergic (-30% identity), and bombesin (-25% identity) receptors define the melanocortin receptors as a subfamily of the major G-protein-coupled receptor gene family. The α-MSH receptor mRNA expressed

in several human melanoma samples and two primary human melanocyte samples. In situ hybridization of a fragment of the human ACTH receptor to sections of rhesus adrenal gland localized the expression of this receptor exclusively to the cortex; no hybridization to the medulla or capsule was apparent.

ACTH, mainly through a cAMP mediated mechanism, has two effects on the adrenal glands; acute phase action within minutes and a prolonged trophic action. Acute stimulation by ACTH involves transformation of an inactive precursor protein to an active labile one by phosphorylation (112) and concomitantly activates an inactive phosphatase possibly for the rapid termination of the steroidogenic response. This still-to-be defined labile protein regulates the conversion of cholesterol to pregnenolone, the rate-limiting step catalyzed by P450scc (113), through several ways (114,115), by an unknown mechanism, including 1) to increase the availability of free cholesterol by stimulating cholesterol esterase and inhibiting cholesterol ester synthetase (116-118); facilitating transport of cholesterol into mitochondria (119); 3) promoting the binding of cholesterol to P450scc (120); and 4) stimulates the release of newly synthesized pregnenolone (121). The details of ACTH mediated stimulation of these early steps have been reviewed elsewhere (122-125).

In contrast, ACTH has a prolonged trophic action on the adrenal cell. These effects include increased synthesis of most of the enzymes of the steroidogenic pathway and more general

actions on adrenocortical cell protein, RNA and DNA synthesis, and cell growth (126-128). The long-term chronic effects of ACTH are mediated directly at the level of the steroidogenic enzymes. ACTH is required to maintain normal amounts of P450scc in mitochondria, while supraphysiological concentrations of ACTH increase the synthesis of P450scc protein, and the complete absence of ACTH virtually abolishes production of P450scc mRNA (129). ACTH stimulates steroidogenesis and increases the rate of synthesis of all steroidogenic cytochrome P-450 enzymes, including P-450scc (120), P-457c21(131), P-450c17 (132), and P-450c11 (133), plus the electron transport protein adrenodoxin (134) and adrenodoxin reductase (128) in bovine adrenal cells. The accumulation of mRNA of these enzymes by ACTH is due to increased rates of gene transcription rather than to changes in the rates of mRNA turnover (128,135). Somewhat less is known about the regulation of other steroidogenic enzymes. Pharmacological doses of ACTH greatly increase bovine adrenal P450c21 protein, but P450c21 mRNA assayed by cell-free translation increased only slightly, and 21-hydroxylase activity remained unchanged (130). The significance of this study remains unclear, because ACTH and cAMP increase P450scc and P450c11 proteins and their mRNAs in bovine adrenal cells (42). ACTH also stimulates the synthesis of other proteins required for steroidogenesis, such as the LDL receptor (124), which is required for uptake of circulating cholesterol; adrenodoxin

(132), which is needed for transfer of reducing equivalents; and sterol carrier protein 2 (136,137), which is required for transport of cholesterol from intracellular lipid stores to mitochondria. Furthermore, ACTH has been implicated in the maintenance of adrenocortical steroidogenic capacity (138) as well as in promoting ACTH receptor expression in adrenal cells (139).

In humans there is a diurnal rhythm of ACTH and cortisol secretion and 17-hydroxycorticoid secretion (140,141). The regularity of this rhythm appears to be a function of the sleep-wake habits of the individual. In those individuals who sleep largely at the same hours each day have a sharp increase in ACTH-cortisol secretion which occurs during the third to fifth hours of sleep and becomes maximal about an hour after awakening. Minimal levels of these hormones are reached a few hours before and after resumption of sleep. In humans this rhythm of activity has a cycle length of about 24 hours and cannot be synchronized with environmental lighting regimes. In a free-running environment with an absence of clues to the true local time the rhythm persists but is slightly and consistently longer or shorter than 24 hours (141).

Voutilainen and Miller (142) have demonstrated that mRNAs for IGF-II and the cytochrome P-450 cholesterol side-chain cleavage (P450scc) enzyme which catalyzes the utilization of cholesterol for steroidogenesis in human fetal adrenal cell

cultures were both increased by ACTH. Interestingly, cAMP also increased the mRNAs for IGF-II and P450scc. They suggest that pituitary hormones that stimulate steroidogenesis via cAMP also stimulate cell-specific accumulation of IGF-II mRNA by the same cAMP mechanism. In contrast to these findings, McAllister and Hornsby (143) have demonstrated that stimulation of cAMP-independent protein kinase C by the phorbol ester TPA was associated with increased growth of human fetal adrenal definitive zone cells in culture. TPA, however, also modified the pattern of enzymes in the fetal adrenal important to the production of androgens and cortisol (144). Thus, factors such as IGF-I, which have been demonstrated to modulate protein kinase C (145), may not only play an important role in modulating growth but may also participate in regulating the pattern of steroidogenesis elicited by the human fetal adrenal gland. In a recent study, Naaman et al.(146) have demonstrated that IGF-I enhanced growth and differentiation of ovine fetal adrenal cells in vitro either in the absence or presence of IGF-I and ACTH did not enhance their cAMP response to ACTH. The exact mechanism of action of IGF-I on the adrenal ACTH-sensitive adenylate cyclase system is not known, but these results suggest that these effects are exerted at sites close to the ACTH receptor, since forskolin-induced cAMP output was not modified by IGF-I pretreatment. They also demonstrated that IGF-I increased the output of corticosteroids of ovine fetal adrenal cells stimulated by cAMP,

suggesting an effect of the growth factor at sites beyond cAMP (146).

1.3.2.2. ***The renin-angiotensin system and the control of aldosterone synthesis***

The synthesis and secretion of aldosterone by adrenal glomerulosa cells is under the control of three major stimulatory extracellular messengers: angiotensin II, extracellular potassium, and to a lesser extent ACTH (147,148). These factors have complex regulating interaction (147,149). In certain species PTH (150), vasopressin (151,152), and acetylcholine (153) also enhance the aldosterone secretory response.

Angio II, an octapeptide, is derived from the decapeptide angiotensin I through the action of a converting enzyme (154,155). Angiotensin I is derived from a precursor protein, appropriately designated as angiotensinogen, which originates in the liver. The conversion of angiotensinogen to angiotensin I results from the enzymatic action of an enzyme, renin, which is synthesized in the juxtaglomerular apparatus of the kidney. The level of circulating renin is the rate-limiting factor in this process (149). The secretion of renin is controlled mainly by renal arteriolar blood pressure, the sodium concentration of tubular fluid sensed by the macula densa, and renal sympathetic nerve activity (156). The action of Angio II on adrenal glomerulosa cells is terminated by angiotensinases which split the octapeptide into smaller inactive fragments (154,155).

Extrarenal sources of renin, isorenins, have been described (brain, uterus, adrenal gland) but their significance is undefined. Angiotensin I converting enzyme has been localized to a variety of vascular beds. The presence of this enzyme in these vessels may be indicative of a functional role of angiotensin in the local control of blood flow. These extrarenal sites of angiotensin production comprise the so-called tissue angiotensinase system (154,155).

The steroidogenic action of Angio II in the adrenal glomerulosa is initiated by the binding of the hormone to its membrane receptor, which may be coupled to one or more G-proteins (147). Using Scatchard analysis, two classes of Angio II receptors have been described in bovine, canine, and human glomerulosa cells (157,158): a high-affinity site possibly linked to PI-specific PLC and a low affinity site linked to adenylate cyclase. Using photoaffinity labelling with an Angio II analog (159) has resulted in the identification of an Angio II receptor, which in the bovine adrenal possesses a molecular weight of 58 Kd. This receptor exhibits remarkable heterogeneity, differing not only among species (the rat adrenal receptor is 79 K) but also among Angio II-target tissues within species (160). This heterogeneity may be due in part to differences in glycosylation, as enzymatic deglycosylation converts the rat and bovine adrenal Angio II receptor into a 34 Kd and 35 Kd protein, respectively. Very recently, cDNA encoding type-1 Angio II receptor has been

isolated from bovine and rat (161-163). The receptor encodes a protein of 359 amino acid residues with a trans-membrane topology similar to that of other G protein-coupled receptors. A comparison of the rat and bovine sequences shows 27 differences of which eight are conservative changes (164). Northern blot analysis revealed that the messenger RNA for this receptor is expressed in bovine adrenal medulla, cortex and kidney (162).

The adrenal glomerulosa cell is an exquisite sensor of extracellular K^+, such that small elevations of plasma K^+ within the physiological range induce significant increases in aldosterone secretion (165). Accordingly, as K^+ is raised above 3.5 mM, there is a sharp rise in the rate of aldosterone secretion, reaching a peak value (4- to 8-fold increase) between 8 and 12 mM K^+ (166-168). However, if extracellular K^+ is raised further, the rate of aldosterone secretion does not continue to increase, but declines sharply to a nadir at 18-20 mM (166-168).

K^+ not only is an agonist itself but also profoundly affects the response of the glomerulosa cell to Angio II. At low concentration (<2.0 mM) of K^+, Angio II has little or no effect on sustained aldosterone secretion, even though the hormone activates PI turnover (167). Between 2.5 and 7.5 mM K^+, Angio II enhances aldosterone production with its maximal stimulatory effects occurring between 4 and 6 mM K^+ (166). At these K^+ concentration Angio II also enhances Ca^{++} influx, but at low extracellular K^+, Angio II has no significant effect on Ca^{++}

influx. Therefore, a direct correlation exists between the ability of Angio II to increase Ca^{++} influx and its ability to induce a sustained increase in the aldosterone secretion rate. This effect of K^+ on Angio II-elicited increase in Ca^{++} influx and aldosterone secretion is thought to be the result of depolarizing the plasma membrane which activates voltage-sensitive Ca^{++} channels (169,170). The mechanism of the effect of K^+ on aldosterone production in guinea pig adrenal cortex was examined by Pushkarev et al.(171). At high K^+ (8mM), aldosterone output and content was elevated, and there was a significant increase in the phosphorylation of intracellular proteins and of protein kinase C activity. Cyclic AMP levels showed a less significant increase. At low K^+ concentrations, addition of EDTA led to a significant accumulation of cAMP (171). These results suggest the participation of cAMP and protein kinase C in the regulation of aldosterone biosynthesis by K^+.

ACTH can also increase aldosterone secretion by binding to specific glomerulosa cell-surface receptors, activating adenylate cyclase as it does in increasing glucocorticoid production by fasciculata cells (section 1.3.2.1.). But unlike glucocorticoid synthesis, ACTH only plays a minor role in aldosterone secretion. This conclusion is based on two sets of observations: 1) aldosterone secretion usually remains normal after depletion of ACTH by hypophysectomy; 2) ACTH acutely increases aldosterone secretion in humans, but in contrast to the effect of angiotensin

II, this effect lasts less than 24h despite continued ACTH administration (172). ACTH has been found not only to have down regulation effects on ACTH receptors on the glomerulosa cells (173) but also inhibit Angio II-stimulated inositol phosphate accumulation in rat adrenal glomerulosa cells (174). In bovine zona glomerulosa cells, Yoshida et al. found ACTH has a modulatory effect on cAMP via the angio II receptor and, therefore, can induce the inhibition of angio II on aldosterone production (175).

1.3.3. Other activators

1.3.3.1. α-MSH

α-MSH is derived from the same precursor called proopiomelanocortin (POMC) as ACTH (a detailed discussion on POMC derived peptides will be given in section 1.5. of this chapter). α-MSH, α-melanocyte stimulating hormone, as its name implies regulates integumental pigmentation mainly in nonmammalian vertebrates for rapid physiological color changes. The biological significance in mammals for α-MSH is still not very obvious but recent studies mainly in the rat have found that α-MSH has profound effects on zona glomerulosa function (176). Studies also show that acute adminstration causes stimulation of aldosterone secretion both in vivo and vitro (176,177), while more prolonged adminstration causes an increase in the growth and steroidogenic capacity of the rat adrenal zona glomerulosa (178). The adrenal sensitivity to α-MSH varies with the physiological status of the

animal, with a significant increase in sensitivity following dietary sodium depletion (179,180). It has been shown that α-MSH, but not ACTH, can selectively restore aldosterone secretion in hypophysectomized rats (181). These data strongly suggest a role for α-MSH in the control of aldosterone secretion in the rat. As mentioned in section 1.3.2.1 the α-MSH receptor has been cloned and characterized. It belongs to the G-protein coupled receptor gene family.

1.3.3.2. *Other hormonal activators*

There are several other factors that also stimulate steroid hormone secretion from the adrenal cortex such as serotonin, substance P, IGF-I and insulin. Serotonin is a recognized stimulator of aldosterone secretion in vivo and in vitro (182-185). This effect of serotonin is believed to be mediated by type 2 serotonin receptors, since it is blocked by the selective antagonist ketanserin (186,187). A recent study of its mechanism of action in the adrenal glomerulosa shows that at low concentrations (ED_{50}), serotonin potentiates the stimulatory effect of Angio II on aldosterone secretion(188). This result suggests that under some physiological conditions, serotonin may play a role in regulating adrenal sensitivity to Angio II. Very recently, Lefebvre et al (189) reported that serotonin induced a stimulation of cortisol secretion from human adrenocortical tissue which is mediated through activation of a serotonin 4

receptor subtype.

Cortisol secretion also can be induced by substance P (SP) in bovine adrenocortical cells (190). Adrenaline has been found to stimulate cholesterol side-chain cleavage cytochrome P450 mRNA accumulation in bovine adrenocortical cells (191). This stimulatory action on cortisol formation requires ß-adrenergic receptors and is due to a cAMP-mediated increases in the accumulation of mRNA encoding P450scc. IGF-I and insulin enhance the steroidogenic response to Angio II. These effects have been shown on Gi proteins and Angio II-induced phosphoinositide breakdown in cultured bovine adrenal cells (192).

1.3.3.3. ***Interleukin 1***

One of the hallmarks of glucocorticoid action is its capacity to regulate immune function (193,194). There is increasing evidence which suggests that the immune system can, in turn, activate the adrenocortical axis and provide a shortcut by which immune recognition of an infectious challenge rapidly activates the stress response. During times of antigenic challenge to the immune system, glucocorticoid secretion is provoked by lymphokines and monokines (195). The mechanisms involved in the regulation of steroidogenesis for most of cytokines are still not clear. Among them, Interleukin-1 (IL-1) seems to have the most profound effects on the steroidogenesis. One thing to keep in mind is that all the cytokines, especially IL-1, are multi-functional factors (196) and they play a very

important role in the cytokine cascade in the immune system.

IL-1, a protein produced predominantly by stimulated macrophages and monocytes, exerts several biological actions. The immunological effects of IL-1 are the control of differentiation and activation of lymphocytes and the stimulation of lymphokine production (197-200). IL-1 also acts as an endogenous pyrogen, stimulates hepatocytes to elaborate acute-phase proteins, augments granulocyte superoxide production, and alters fibroblast growth and collagenase and prostaglandin production (201-207).

The genes for human and murine IL-1 have been cloned (208,209), and two distinct IL-1 subtypes, IL-1α and IL-1ß showing only 26% sequence homology, have been identified in humans by cDNA sequencing (208). However, both molecules bind to the same receptor and mediate similar actions (210-214). An unusual structural feature of these molecules is the lack of the conventional leader sequence that allows the proteolytic cleavage of the protein and passage into the extracellular space. It is unclear how the IL-1 is translocated within the cells without the hydrophobic sequence.

Recent studies proposed that IL-1 is an important stimulator of the HPA axis. First, IL-1 has been found to activate the HPA axis at the level of the brain, stimulating the release of the hormone CRF from the hypothalamus (215,216). Infusion of IL-1 induced a significant secretion of CRF into the circulation from rat hypothalamus, whereas immunoneutralization of CRF blocked the

stimulatory effect of IL-1 on glucocorticoid secretion (215). After blockade of fast axonal transport in hypothalamic neurons by colchicine, IL-1 administration decreased the CRF immunostaining in the median eminence indicating an enhanced release of CRF in response to IL-1 (216). The IL-1-induced increase in ACTH is highly specific in that blood levels of oxytocin (215), MSH, GH, and vasopressin (216) are not affected. In view of the presence of IL-1 binding sites in the hypothalamus (217), it is possible that IL-1 may induce CRF secretion by direct action in the hypothalamus on CRF neurones.

IL-1 has been reported to stimulate ACTH secretion by AtT-20 cells, a mouse pituitary tumor line (218). Based on recombinant human IL-1ß stimulated the secretion of ACTH in a monolayer culture of rat pituitary cells, Bernton et al. suggested the possibility that IL-1 may not only act indirectly on pituitary secretion through hypothalamic CRF (215,216)but also has a direct effect on the secretion of ACTH by pituitary (219).

The effects of interleukines on adrenal steriodogenesis and their mode of action were studied by Tominaga et al.using cultured rat adrenal cells (220). Their results indicate that 1) IL-1α and IL-2 directly stimulate glucocorticoid synthesis in a dose- and time-dependent manner; 2) a half-maximal effective concentration of ACTH acts synergistically with IL-1α and IL-2 in stimulating glucocorticoidogenesis; 3) the stimulatory process initially requires PGs, followed by the activation of the

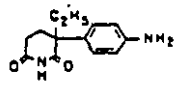
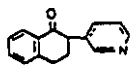
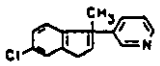
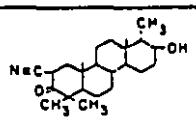
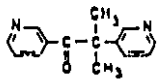
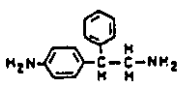
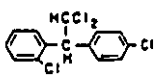
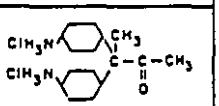
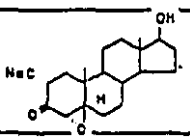
adenylate cyclase system; 4) although the profiles of steroidogenic action of IL-1α and IL-2 are quite similar, they may exert their effects through different mechanisms in their early steps of PGE2 production; and 5) the low effective concentrations of both cytokines suggest possible physiological or pathophysiological roles of circulating cytokines in the glucocorticoidogenesis under certain conditions.

1.3.3.4. ***Other cytokines***

In addition to IL-1, IL-2, a T cell-derived lymphokine which is induced by IL-1, stimulates the proliferation of T cells (221). At high doses (1-2 mg), IL-2 caused a increase in plasma ACTH in patients with cancer and acquired immune deficiency syndrome (222,223). IL-6, a molecule which mediates a host of different responses and regulates functions of many cell types, has also been shown to act as corticotrophin-releasing factor (224,225). Like IL-1, IL-6 acts at the hypothalamic CRF-containing neuron.

Several factors have been reported to produce increases in serum corticosteroid levels. Mitogen stimulation of rat spleen cells produces a corticosterone-releasing activity that can act directly on the adrenal gland (226). Thymosin peptides include thymosin fraction 5 (TF5), a complex mixture of thymic peptides, and specific thymic peptides α1. causes significant elevation in serum corticosterone after direct injection into the lateral ventricle of chronically cannulated mice (227-230). TF5 has also

Figure 1-4. Some inhibitors of steroid synthesis and their loci of action. (Adapted from Bondy PK, Rosenberg LE, Metabolic Control and Disease. 8th ed. Philadelphia: W.B. Saunders, 1980)

NAME	REACTION INHIBITED	FORMULA
Aminoglutethimide	cholesterol side-chain cleavage	
SU-9055	18-hydroxylation 17 α-hydroxylation	
SU-8000	18-hydroxycorticosterone → aldosterone 17 α-hydroxylation	
Cyanoketone	3 β-hydroxysteroid dehydrogenase	
Metyrapone	11 β-hydroxylation	
SKF 12185	11 β-hydroxylation	
Mitotane	mitochondrial damage, especially in z. fasciculata and z. reticularis	
Amphenone B	cholesterol 20 α-hydroxylase? 17 α, 11 β and 21 hydroxylases?	
Trilostane	3 β-hydroxysteroid dehydrogenase	

been found to inhibit the binding of dexamethasone to thymocytes (231). However these results could not be observed in an in vitro study using suspensions of rat adrenal fasciculata cells (232).

1.3.4. Inhibitors

Under normal circumstances, the shut-down of steroidogenesis is achieved by a direct negative feed-back loop of the final products (i.e. cortisol) on the secretion and synthesis of their secretagogues. In the absence of these stimulators, the labile protein which is absolutely required for the conversion of cholesterol to pregnenolone as the rate-limiting step in the steroidogenesis will be inactivated, therefore the steroidogenic response will be terminated rapidly. There is another mechanism utilized by the body in the negative regulation of steroidogenesis, that is, through inhibitory factors which accomplish a more precise and accurate control of these processes. There are several agents which have been found to have inhibitory effects on steroidogenesis and they will be discussed in this section.

1.3.4.1. *Chemical reagents which inhibit steroidogenesis*

There are a number of exogenous substances which are capable of inhibiting steroid synthesis by interfering with specific enzymatic reactions, as indicated in Fig.1-4. Although most of the inhibitors are only of experimental interest, among them, aminoglutethimide, metyrapone, and mitotane have been used to

treat adrenocortical hyperfunction, and metyrapone is also useful in a test of the integrity of the pituitary-adrenal control system. The reactions indicated in Fig. 1-4 are the major sites of activity. Metyrapone at high concentrations can also inhibit in vitro the hydroxylation of cholesterol at carbon 20, and could thus potentially inhibit the side-chain cleavage of cholesterol (233). It acts by competing with the substrate for binding sites on the adrenodoxin-cytochrome P-450 system. Similarly, in addition to blocking 20-hydroxylation of cholesterol, aminoglutethimide can also block 18-hydroxylation (234). In addition to cyanoketone, six related experimental blockers of 3ß-hydroxysteroid dehydrogenase have been described, providing a spectrum of activities lasting from 12 hours to seven days in vivo (235). Trilostane, an inhibitor of 3-hydroxysteroid dehydrogenase, blocks oxidation of the hydroxyl on the 3-carbon of pregnenolone to a ketone. It was tried as a treatment of hyperadrenocorticism but was found to be ineffective (236).

Recently, other chemical reagents have also been reported to have inhibitory effects on steroidogenesis. Diazepam and midazolam inhibit cortisol and aldosterone synthesis in bovine adrenal cells in vitro. Both compounds inhibit steroidogenesis at several points in the biosynthetic chain; the greatest effects were on 17 α hydroxylation and 21 hydroxylation by diazepam and midazolam respectively (237). Dantrolene,the muscle relaxant, also significantly inhibits steroid synthesis stimulated by

either Angio II or by the addition of various precursors in bovine adrenal cortex cells (238). These experimental results suggest that dantrolene inhibits the rate-limiting steps of adrenocortical steroidogenesis, i.e. the intramitochondrial conversion of cholesterol to pregnenolone and the conversion of corticosterone to aldosterone. Gossypol, an antifertility agent, shows multiple effects on adrenal function. Its effects on membrane microviscosity, adrenal steriodogenesis, cAMP and corticosterone responses to ACTH stimulation probably occur through a generalized membrane effect (239). Furthermore, a threonine analogue, D,L-ß-hydroxynorvaline, inhibits stimulated steroid synthesis by ACTH and cAMP in cells isolated from the rat adrenal cortex (240). This inhibition was found to occur in a dose-dependent manner and is reversible by a stoichiometric concentration of threonine.

1.3.4.2. ***ANF and other hormones***

In addition to extracellular messengers, several hormones exert inhibitory effects on the adrenal. The most prominent of these is ANF. ANF potently inhibits Angio II-stimulated aldosterone secretion, the mechanism of action of this atrial peptide is as yet unknown. However, recent data have suggested that ANF exerts an inhibitory effect on the calcium channel (T type) which is modulated by Angio II (241).

ANF is a potent inhibitor of aldosterone secretion stimulated by a variety of pharmacological (forskolin, phorbol

ester) and physiological (Angio II, ACTH, K^+) agonists that utilize either the Ca^{++}-phosphoinositide or cAMP messenger systems (242,243). The inhibition is dose dependent and rapid in onset (242-244). Although ANF inhibits aldosterone secretion stimulated by all known secretagogues, the type of inhibition induced varies according to the agonist. Thus, in contrast to the ANF-induced inhibition of ACTH-stimulated aldosterone secretion which can be overcome by increasing concentrations of ACTH, the ANF-induced inhibition of Angio II (or K^+)-stimulated aldosterone secretion persists in the presence of supramaximal doses of the secretagogue (242,243,245,246). Since the ANF-induced inhibition of Angio II-stimulated aldosterone secretion can be overcome by forskolin, a potent stimulator of the catalytic unit of adenylate cyclase (247), the cAMP signal many antagonize ANF at its site of action.

The primary site of ANF action in the glomerulosa cell appears to be at a step before the synthesis of pregnenolone, since ANF can inhibit pregnenolone synthesis from endogenous cholesterol (243) but not aldosterone synthesis from exogenous progesterone (3). Moreover, because ANF does not impair the production of pregnenolone from 25-OH cholesterol acetate, a polar derivative whose transport into the cell and mitochondria is not rate-limiting, ANF may inhibit aldosterone secretion by restricting the delivery of cholesterol to the side-chain cleavage enzyme complex (3). Nevertheless, at present the precise

ANF-inhibited intracellular event is unknown.

Dual effects of dopamine (DA) in rat adrenal glomerulosa cells has been suggested (248,249). There are two different DA receptors present in rat adrenal glomerulosa cells: D-1, associated with stimulation of adenylate cyclase, and D-2, coupled in an inhibitory way with the cAMP generating system. By measuring aldosterone secretion and cAMP formation in intact adrenal glomerulosa cells, a specific functional interaction has been found between D-2 receptors apparently associated with inhibition of cAMP formation and Angio II in the regulation of aldosterone production (250).

Somatostatin inhibits Angio II-stimulated aldosterone production in vitro. The mechanism is not clear, but high-affinity binding sites for somatostatin are present on glomerulosa cells (147).

Previous studies indicated that insulin at levels comparable to those present in humans during hyperinsulinemia decreased ACTH-stimulated cortisol and androstenedione secretion by bovine adrenal fasciculata-reticularis cells in primary culture (251). Very recently, this inhibitory action was found to be correlated with effects on cAMP accumulation and rates of cAMP production (252). Insulin also decreased ACTH- but not Angio II stimulated aldosterone secretion in cultured glomerulosa cells (252).

1.3.4.3. ***Peptide growth factors: TGFß, FGF, and TNFα***

As discussed in the previous section, a number of cytokines

especially IL-1 have stimulatory effects on steroidogenesis. Since glucocorticoids are the most important endogenous anti-inflammatory agents in the body, it will not be surprising that some cytokines have inhibitory effects on steroidogenesis and use it as a negative control mechanism. A detailed discussion of this topic will be found in the last chapter of this thesis.

A limited number of studies have recently been conducted to examine the effects of peptide growth factors on steroidogenesis in the adrenal gland. Macrophages secrete an impressive array of biologically active substances (253), at least one of which, TGFß, inhibits ACTH (254-256). This molecule affects cell functions such as cell morphology, cell differentiation, cell proliferation, and morphogenesis (257-268). Several studies demonstrate that TGFß inhibits the action of ACTH on the adrenal cortex in vitro but has no effect on cellular proliferation (254-256). The inhibitory effect is potent, being detectable at 10^{-13} M, is half maximal after 6 h, and reaches a plateau at about 50% inhibition between 12 and 18 h (254). Both basal and ACTH stimulated steroidogenesis are inhibited. TGFß has been shown to have high affinity receptors on the surface of the bovine adrenocortical cell (269). The number of TGFß high affinity receptors is increased by ACTH through a cAMP-dependent mechanism that does not require de novo protein synthesis; thus the TGFß receptors of the adrenal cortex are hormonally regulated. Very recently, TGFß has also been reported to be a potent inhibitor of

basal and ACTH- and cAMP-stimulated aldosterone production (270). Contrary to its effect on aldosterone, it stimulates the synthesis and release of adrenal renin and prorenin (270). TGFß may act as an autocrine or paracrine regulator of aldosterone production. It has also been found that TGFß inhibits P450c17 expression in ovine adrenocortical cells (271).

A septic shock factor has been described from peritoneal exudates which is capable of inhibiting ACTH stimulated steroid synthesis (272) and another such factor from macrophages has also been reported (273). The latter factor may turn out to be TGFß.

Fibroblast growth factor (FGF), the most potent mitogen identified for bovine adrenocortical cells, stimulates adrenal cell growth with a concomitant inhibition of steroidogenesis (6,7). In the study of factors which regulate proliferation of adrenocortical cells, FGF has been found to be a potent mitogen. The clonal bovine adrenocortical cells were selected only on the basis of their ability to growth from low density in the presence of FGF. All clones were unresponsive to ACTH, perhaps because ACTH responsiveness is a density-sensitive property in bovine adrenocortical cells and cloned cells were grown from very low density (274).

TNFα is a pleiotrophic factor that exerts a variety of effects ranging from proinflammatory and cytotoxic, to growth and immunomodulatory on a host of different cells (275). TNFα secretion from macrophages is stimulated by endotoxin and TNFα

has been implicated in the pathogenesis of septic shock. Betz and Roth have shown that TNFα inhibits ACTH stimulation of corticosterone secretion by rat adrenal cells (276). TNFα has been recently reported as a potent inhibitor of ACTH-induced cortisol production and steroidogenic P450 enzyme gene expression in cultured human fetal adrenal cells (277). This is a new, potentially clinically important interaction between the immune and endocrine systems.

Although the physiological role of these inhibitors is unknown, it is significant that the adrenal cortex is extensively infiltrated by macrophages (278), suggesting a possible paracrine interaction between the immune and endocrine cell types. Interestingly, evidence exists for a macrophage-ovarian paracrine relationship that may play a role in maintaining progesterone secretion by luteal cells (279,280). In these experiments ovarian macrophages greatly elevated steroid formation in coculture with ovarian granulosa and luteal cells through a mechanism which seemingly requires close proximity of the steroidogenic and leukocytic cell types.

Another family of immune cell peptides with anti-ACTH activity are called corticostatins. These corticostatic peptides and other related cysteine-rich peptides will be discussed in the following section.

1.4. Corticostatins and Cysteine-rich Peptides

CHAPTER 1. INTRODUCTION

Corticostatins are a group of peptides with anti-ACTH activity derived from granulocytes and monocytes, first isolated from fetal and adult rabbit lung and neutrophils. All members of this peptide family except guinea pig corticostatin 3, which will be discussed in later chapters of this thesis, have a highly conserved back-bone structure consisting of three disulphide bridges and a high arginine content. Since we are talking of consensus at the level of protein sequence and not at the level of DNA, it is reasonable to assign importance to these structures. The physiological significance of these peptides is still not fully established. The structure-activity relationship of these peptides also remains to be established and will be discussed in a later section. This thesis deals with several aspects of the biology and biochemistry of corticostatic peptides.

1.4.1. Discovery of corticostatic activity and isolation of corticostatic peptides from rabbit, human, and rat tissues.

Glucocorticoids have been known to stimulate fetal lung development in humans, rabbits (281-284), and several other species. As we mentioned above the factors that regulate adrenal gland growth in the fetus are still largely unknown. The adrenal gland of the anencephalic fetus develops normally for about 15 wk in the human (285). Fetal growth during this phase was presumed to be independent of fetal pituitary ACTH stimulation (285). Therefore the question we wanted to address at that time was

whether some peptides from the lung had effects on adrenal function. We attacked this question by isolating some adrenal trophic factors from lungs. In the course of doing so, we found a peak adjacent to the ACTH-like peak having the ability to inhibit ACTH-stimulated corticosterone production in rat adrenal cell suspensions. This peptide was called corticostatin based on its anti-ACTH activity. After repeating this experiment on a larger scale, we found more than one component with this corticostatic activity. Four corticostatic peptides have been purified, and named corticostatin I, II, III and IV respectively according to the order of elution in the RP-HPLC system (286,287). Among them corticostatin I (CSI) is the most potent with the minimum effective dose of 5 nM, and an EC_{50} of 25 nM against 33 pM of ACTH (at which ACTH reached half maximal stimulation in the rat adrenal cell bioassay system). The corticostatic activity is rapid, complete, and highly specific, since there is no effect on angiotensin II-induced steroidogenesis in rat adrenal zona glomerulosa cells (8). Because CSI could displace labelled ACTH from binding to the ACTH receptor on adrenocortical cells, the inhibitory activity appears at the receptor level (288). This conclusion was further supported by the fact that CSI had no effect on secondary stimulators such as $(Bu)_2$ cAMP, forskolin, cholera toxin, or pertussis toxin (288). CSI did depress ACTH-stimulated endogenous cAMP levels (288).

The sequences of CSI and the other three corticostatic peptides, CSII, CSIII and CSIV shows a high percentage of arginine and cysteine residues. A computer search of the Protein Identification Resource of the National Biomedical Research Foundation (Georgetown University, Washington, DC) revealed that Selsted et al. had found these same peptides in rabbit peritoneal neutrophils and had named them defensins. Two rabbit defensins MCP-1(NP-1) and MCP-2(NP-2) identical to CSIII and CSIV were initially discovered in pulmonary macrophages (289). Selsted at el. then reported the isolation of six cationic peptides (NP-1,NP-2, NP-3, NP-4, NP-5 and NP-6) from rabbit granulocytes (290). These peptides have been shown to have anti-microbial activity at high concentration (>2 μg/ml, in vitro) against Candida albicans blastospores (291), Coccidioids immitis arthroconidia (292), certain bacteria (293), fungi (294), and viruses (295).

Around that time, three human defensin peptides (HP-1,2,and 3) were also purified and sequenced in our and other laboratories (296,297). HP-1,2, and 3 were rich in cysteine, arginine, and aromatic residues, but were devoid of free sulfhydryl groups and carbohydrate moieties. They were 29-30 residues in length and identical in sequence in all but their amino terminal residues. These human peptides were homologous in sequence to rabbit corticostatic peptides, but unrelated to other neutrophil proteins of known sequence which have been isolated from human

PMN (298). HP-1 has diverse effects on mammalian cells, being cytotoxic to many cell lines, but stimulating cell growth in others (299). Since HP-1 has no corticostatic activity, we speculated that other unknown corticostatic peptides might exist in the human. In extracts of human neutrophils, a related peptide (HP-4) has been isolated and sequenced (300). Structurally this peptide resembles the rabbit peptides more closely than HP-1,2, and 3 and inhibits the action of ACTH on adrenal cell suspensions with an ED_{50} of around 7.0×10^{-7}M, and completely inhibits the ACTH induced release of corticosterone at 4×10^{-6}M. Unlike HP-1 it shows no cytotoxic effect on two cell lines, CHO-K1 and SK-MES-1, both of which were chosen because of their susceptibility to the lethal effects of HP-1.

It is highly unlikely that the inhibition of ACTH which is observed with HP-4 and the rabbit corticostatins is a nonspecific function of their positive charge as other cationic compounds are known to stimulate rather than inhibit ACTH action (301). The absence of corticostatic activity in the closely related peptide HP-1 also supports this conclusion. The comparison of the sequences of CSI with HP-4 and HP-1, HP-1 differs from CSI and HP-4 in lacking a C-terminal extension after the final cysteine and it is tempting to speculate that this may determine its lack of corticostatic activity.

Seven cationic, cysteine-rich peptides of 29-32 amino acid residues have been purified from extracts of rat bone marrow (R-

1,R-1a,R-1b.R-2,R-3,R-4, and R-5) (302). Four of them (R1,R2,R3, and R4) have also been reported as rat defensins isolated from extracts of peritoneal neutrophils (303,304). Structural analysis clearly indicated that all seven peptides belong to the corticostatin/defensin family. For R-1 to R-5, six cysteine residues were found at characteristic and highly conserved positions. R-1a and R-1b were partially characterized and appear to be structural variants of R-1. In the isolated rat adrenal bioassay, R-1,2 and 3 were shown to have no corticostatic activity but R-4 and R-5 were found to inhibit steroidogenesis. The R-4 peptides was found to be slightly less potent with an EC_{50} value of 50 nM, and the R-5 has considerably lower corticostatic potency than R-4 but because of the lower yields of this peptide, a full dose response curve was not obtained. R-4 is the only member of the corticostatin/defensin family to have potent cytotoxic, corticostatic, and antibacterial and L-type Ca^{++} channel agonist activity. This will be further discussed below

1.4.2. Cysteine-rich Peptides

In recent years, there has been an increasing interest in these cysteine-rich peptides of the corticostatin/defensin family. This is due to their diverse functions, striking consensus sequence in their basic structures, and universal existence in all mammalian species which have been examined so far. In the last few years, other cysteine-rich peptides, have also been purified and characterized, such as cryptdin, granulin,

tracheal antimicrobial peptide and sea anemone toxins. Although the biological role of these cysteine-rich peptides in steroidogenesis has not been studied, it has been speculated that they have some ancient relationship with the corticostatin family. They will be further discussed in this section.

1.4.2.1. *Cryptdin*

Cryptdin, derived from crypt Paneth cells, contains corticostatin consensus residues which constitute one-third of the mass in these 32-34 amino acid peptides (305,306). Although cryptdin shows some sequence similarity to CSIII(MCP-1,NP-1) and CSIV(MCP-2, NP-2), overall, outside of the consensus sequence, cryptdin shows little identity with other members of the corticostatin family. Since the motif has a conserved structural role (307,309), the sequence divergence in apparent domains outside of the consensus may specify function for the peptides in this family. The most noticeable phenomenon about cryptdin is that its mRNA expression is developmentally regulated in crypt Paneth cells (310,311). Five members of the cryptdin family have now been purified from the mouse small intestine, where they are present at different relative amounts in the epithelium (312).

1.4.2.2. *Tracheal antimicrobial peptide*

The peptide named tracheal antimicrobial peptide (TAP) was isolated and cloned and had potent antimicrobial activity (313). The size, basic charge, and presence of three intramolecular disulphide bonds of this 38-amino acid peptide is similar to, but

clearly distinct from the corticostatins/defensins family. The putative TAP precursor is predicted to be relatively small (64 amino acids), and the mature peptide resides at the extreme carboxyl terminus and is bracketed by a short putative propeptide region and an inframe stop codon. The mRNA encoding this peptide is more abundant in the respiratory mucosa than in whole lung tissue. This suggests that the TAP message may be produced primarily in a tracheal cell. Although it is most common to find mammalian antimicrobial peptides in myeloid-derived cells, it is worth noting that some of the corticostatin/defensin mRNAs has been found in nonmyeloid cells, the Paneth cell, which is in the small intestinal villus epithelium (314). TAP has broad-spectrum activity includes both bacteria and fungal. The activity is comparable to that of synthetic magainin 2 carboxyamide, a potent analog of an antimicrobial peptide isolated from frog skin (315). The inhibitory concentrations observed with TAP were also similar to those observed with the defensins.

Other closely related basic, cysteine-rich peptides among the animal and plant kingdoms include insect defensins (316,317), insect royalisin (318), crustacean tachypleins (319), and plant thionins (320-322).

1.4.2.3. *Granulin*

Granulins purified from human and rat inflammatory leukocytes and bone marrow (323) are cysteine-rich polypeptides with molecular weights of approximately 6Kda. The most striking

feature of their primary sequence is the high content of oxidized cysteine, over 20%. The sequence of the granulins are homologous with epithelin 1 and 2 isolated from the rat kidney. Epithelin 1 and 2 are putative cytokines that have growth inhibitory and stimulatory properties on some epithelial cell in vitro (324). The granulin precursor cDNA was isolated and sequenced from human bone marrow. The cDNA analysis shows that the prepropeptide for the human granulins is a 593-residue glycoprotein, containing seven tandem repeats of the 12-cysteine granulin domain. Northern blot analysis revealed that gene expression is observed in myelogenous leukemic cell lines of promonocytic, promyelocytic, and proerythroid lineage, in fibroblasts and was seen very strongly in epithelial cell lines. Among tissues examined, the kidney had the highest levels of granulin mRNA (325).

1.4.2.4. ***Sea anemone toxin***

Although there is little overall homology, sea anemone toxin (326,327) and corticostatin exhibit a strikingly similar distribution of the six cysteines and some amino acid conservation near the N- and C-terminal ends. It is therefore of interest to determine whether the organization of sea anemone toxin genes and the structure of their polypeptide precursors resemble those of corticostatin. A functional similarity seems unlikely , however, and it is important to point out that the two families differ with regard to other amino acids conserved within the corticostatin family.

1.5. Fetal adrenal development and parturition

As we discussed previously steroid hormones have diverse effects on almost every system in the body. Among them, most noticeably, are their regulatory effects on carbohydrate metabolism and the inflammatory process. Steroid hormones have also been found to play a pivotal role in fetal development and parturition. In normal pregnant women at or near term, daily production of steroid hormones, especially estrogen, is strikingly high (about 100 mg/d) compared with less than 1 mg/d in nonpregnant women (328). These estrogens are derived from the precursor dehydroepiandrosterone sulfate (DHAS) or 16α-hydroxy DHAS (16α-OHDHAS) predominantly synthesized by the fetal livers (100-200 mg/day). The elegant work of Liggins and colleagues (329) and other investigators, mostly in sheep, suggested that the fetus and not the mother initiates delivery. This conclusion is based on the following observations: fetal hypophysectomy or adrenalectomy delays parturition (330,331); infusion of cortisol or ACTH to the fetus induces premature delivery (329,332,333); normal delivery is preceded by an increase in the concentration of corticosteroid in the fetal blood (334,335) and an increased production of cortisol by the fetal adrenal (332). Although evidence that the fetal adrenal of the human or nonhuman primate participates in the initiation of labor appears less convincing (336-340), maturation of fetal adrenal steroidogenic enzyme systems that permit de novo synthesis of cortisol (341-343) not

only its precursor must occur, as in sheep (344,345), to ensure neonatal adrenocortical self-sufficiency in the perinatal period.

1.5.1. Development of fetal adrenal

During the course of gestation in humans (346,347) and nonhuman primates (342,348-351), the fetal adrenal gland not only undergoes extensive anatomical and biochemical changes but also exhibits a remarkable rate of growth, primarily during the final third of intrauterine development. In the baboon and rhesus monkey, and presumably the human, increased weight is associated with marked growth of the inner zone (fetal cortical zone) which comprises between 80 and 90% of the gland during the majority of gestation (352).

During normal development, the rabbit adrenal cortex is first composed on days 18-20 of pregnancy of a thin zone of irregular arcades which begins to project cords into the medulla (353). These cords then increase in size, and on day 22 they fuse laterally and constitute a continuous inner cortex still projecting cords into the medulla until birth.

1.5.2. Regulation of the fetal adrenal growth

The regulation of fetal adrenal growth and maturation is still largely unclear because it is extremely complex and seems to involve the collaborative actions of the placenta, and some fetal tissues, such as well as the pituitary gland, which are capable of producing and secreting adrenal modulators or growth factors (see section 1.3.). This conclusion is based on the

following observations. During the first 15 wk of gestation in humans, fetal adrenal growth can totally be independent of the fetal pituitary (section 1.4.1.). ACTH levels in human fetal blood decline as gestation advances (354), paradoxical to the rate of growth of the fetal adrenals. The marked growth and vascularization of the fetal cortex that occur throughout gestation does not appear to be associated with a concomitant growth of the definitive zone, which occurs only very late in gestation and continues into the perinatal period, a time during which there is marked remodelling of the fetal cortex. In the baboon, the patterns of fetal adrenal DHA/DHAS production, as well as responsivity to pituitary peptides both in vitro (355-357) and in vivo (358,359), are markedly different at midgestation and at term.

1.5.2.1. ***Placenta CRF, ACTH, cortisol and other factors***

The placenta is not only positively involved in the regulation of maternal-fetal molecular exchange but also functions as a microcosm of the entire endocrine system and plays a significant role in the function of each fetal endocrine subsystem. The steroid hormone precursor DHAS (16α-OHDHAS) produced by the fetal adrenal, is further processed in the placenta to estrogen before being secreted into maternal circulation. On the other hand, the placenta metabolizes cortisol to cortisone in order to protect the fetus from the extensive quantities of cortisol in the maternal circulation (342,360). The

placenta can also produce a vast variety of hormones and growth factors, such as hCG, human placental lactogen (hPL or hCS) (361), ACTH (362-364), TRH (365), CRF (366), IGF I, IGF II (367,368), TGF-α (369), PDGF (370) and FGF (371). The control of placental synthesis and secretion of these hormones is not well understood (372).

Placental CRF and POMC derived peptides have been suggested as factors influencing the activity of the maternal or fetal HPA axis. CRF has been localized to the placental cytotrophoblast by immunocytochemistry (373,374) whereas ACTH and POMC-derived peptides are found predominantly in syncytiotrophoblast (375). CRF mRNA is present by the seventh week of gestation in the placenta and increases in amount until 40 weeks (376). The increase in placental CRF mRNA correlates directly with an increase in placental CRF peptide content (376). POMC mRNA is also present in human placental tissue but is smaller than in the hypothalamus or pituitary (377). Its concentration does not change during pregnancy (377).

In maternal plasma, the concentration of ACTH, although lower than in nonpregnant women, rises progressively during gestation despite the elevated glucocorticoid concentration (378). It has been suggested that the rise in ACTH is due to its production in the placenta, which in turn is independent of the negative feedback control of maternal glucocorticoids. In vitro, placental ACTH secretion may be stimulated by exogenous CRF

(373,379). Since glucocorticoids also increase CRF output (380,381), it is then proposed that at term human pregnancy there is a positive cascade involving glucocorticoids-CRF-ACTH acting in a local fashion.

1.5.2.1. ***pituitary POMC-derived peptides***

In the middle and later gestational stages of fetal development, the adrenal cortex depends for its full development and functional activity upon stimulation by the fetal pituitary. The functional correlations between fetal adrenal cortex and pituitary are well documented and can no longer be doubted (382). Thus, aberrations in pituitary-adrenal function in fetal life could have important consequences on fetal development and the onset of delivery.

POMC is expressed primarily in the pituitary and in limited regions in the brain and periphery of both fetus and adult. It is processed into a large and complex family of peptides which include ACTH, α, ß, and γ-MSH, ß-LPH, α, ß, γ-endorphin, and CLIP with an array of biological activities (383) (Fig.1-5). The role of ACTH in fetal adrenal development is not very clear. As mentioned previously it does play an important role in the parturition processes. Among all these POMC derived peptides α-MSH appears to have some functions in the fetal development. α-MSH injected into rat fetuses in utero (with brain and pituitary removed) stimulates their growth. Other adenohypophysial hormones are without such an effect (384). Specific antibodies against α-

MSH, but not ACTH, induce a decrease in fetal body weight. There is also a positive correlation between fetal pituitary content of α-MSH and fetal body weight in humans. Thus α-MSH or other melanotropins may function directly or indirectly as growth-promoting hormones during fetal life and development (384).

1.5.3. Physiological function of fetal adrenal steroids

As mentioned previously, the fetal adrenal gland produces large amounts of steroids at a rate of 100-200 mg per day. The precursor steroids will be further processed in the placenta into estrogen, and released into maternal circulation. On the fetal side, in most mammalian species, products of the fetal adrenal gland appear to play an important role in regulating maturation of various organ systems (385-389), providing the fetal homeostatic mechanisms respond to stress, and initiating and/or participating in the cascade of events culminating in the birth of a newborn (390). Thus cortisol, presumably of fetal adrenal origin, is one of the chemical messengers involved in the stimuli to lung maturation (387,388), deposition of glycogen in the liver (391,392), and induction of several enzymes in the fetal brain, retina, pancreas, and gastrointestinal tract (393-398) that are normally associated with late intrauterine life.

1.5.4. Parturition

The mechanism by which labor is initiated in pregnant women is not completely understood. Several hypotheses have been formulated to explain the initiation of parturition, but none of

them seems complete.

Oxytocin has been used as a labor inducer in women at or near term for many years. This leads to the attractive hypothesis that this hormone being released by the neurohypophysis at the appropriate time plays a physiological role in the initiation of labor. Studies conducted by Chard (399) demonstrated that oxytocin only plays an important role to facilitate phase 3 of parturition but does not initiate phase 1 of parturition.

The hypothesis of a ACTH-cortisol-estrogen-PGs cascade is the most convincing. This hypothesis is based on the following observations. As shown in sheep, ACTH or cortisol infusion to the fetus will induce premature delivery and fetal hypophysectomy or adrenalectomy delays parturition. Cortisol acts on the sheep placenta to cause an increase in the activities of steroid 17α-hydroxylase and steroid 17,20 lyase (340), which in turn leads to increased estrogen production. In women, local estradiol treatment also appears to cause cervical softening and effacement and, thereafter, increased responsiveness to oxytocin (401). These elevated estrogen levels might in turn cause PG formation. The mechanism that regulates the rate of PGs formation before the initiation of parturition is not understood but twenty-four hours after estrogen administration, the concentration of PGs in uterine venous blood increases. (402). PGE2 and PGF2α, when administered in large amounts, cause uterine contractions at any stage of pregnancy in women (403,404) and effect cervical

softening and effacement (405).

The hypothesis that cytokines might be involved in the initiation of parturition is based on the fact that infection is commonly associated with the premature onset of labor. The infection can be localized in uterine or extrauterine maternal tissues or in the extraembronic fetal membranes. The role of cytokines in parturition will be further discussed in chapter 4.

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

2.1.1. Source of Peptides

Synthetic $hACTH_{1-39}$, $hACTH_{1-24}$, α-MSH, Angiotensin II, ANF and CRF were purchased from Peninsula Laboratories, Inc., Belmont, CA. Synthetic CSI was obtained from the American Peptide Company, Inc (Santa Clara, CA.)

[Nle^4, D-Phe^7]-α-MSH was a gift from Dr.A, Lerner, Dept. Dermatology, Yale University, New Haven, CT.

The amino acid compositions of peptides were checked by amino acid analysis using a high performance amino acid analyzer, Beckman System 6300, prior to use.

2.1.2. Source of Tissues

Fetal and maternal tissues and plasma were obtained from pregnant rabbits (New Zealand) at day 24, 27 and 30 of gestation and normal female rabbit tissues from animals at ages 4 to 6 months. These pregnant rabbits and normal female rabbits were obtained from Reimer Fur Ranch, Ste. Agathe, Ont.

Guinea pig lung and bone marrow tissues were obtained from Hartley male guinea pigs which were purchased from Charles River Breeding Laboratories, St-Constant. Que.

Rats (Sprague-Dawley male) weighing from 150 to 250 grams were purchased from Charles River Breeding Laboratories, ST-

Constant, Que.

Anolis carolinensis (lizards) were purchased from a local pet store.

2.1.3. Source of Reagents and Instruments

^{125}I (100 μCi/ml in NaOH pH 7-10) and ^{125}I-Protein A (100 μCi with Bolton and Hunter reagent buffered aqueous solution) were purchased from Amersham, Oakville, Ont.

D-[1,2,6,7,-^{3}H(N)]-Aldosterone, (93.80 Ci/mmol) in ethanol solution, [1,2-^{3}H (N)]-Corticosterone (58.0 Ci/mmol) in ethanol solution and [1,2-^{3}H (N)]-cortisol (41.9 Ci/mmol) in ethanol solution were purchased from Du Pont Canada Inc. Mississauga, Ont.

Aldosterone, corticosterone, and cortisol antisera were purchased from Endocrine Sciences, Tarzana, CA.

Aldosterone, Bovine Serum Albumin (BSA, fraction V), Charcoal, Corticosterone, cortisol, DAB (diaminobenzidine), DNase (deoxyribonuclease I), DTT(dithiothreitol), Leupeptin, PABA (para-aminobenzamidine), Gentamicin, Glutaraldehyde, Glycogen, Guanidine, Keyhole Limpet Haemocyanin, Polylysine (4K) and Trypsin Inhibitor were purchased from Sigma, St. Louis, MO.

Acetonitrile (HPLC grade), Methanol (HPLC grade), Methylene Chloride (HPLC grade) and Xylene were purchased from Fisher Scientific, Fair Lawn, N.J.

Heptafluorobutyric Acid (HPLC grade) and Hydrochloric acid 6 N (Sequanal grade) were purchased from Pierce Chemicals,

Rockford, Illinois.

Trifluoroacetic Acid, 99 +% (suitable for protein sequencing) was purchased from Aldrich Chemical Company, Inc., Milwaukee Wis.

2-Mercaptoethanol (Baker Grade) was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J.

Medium 199 with Earle's Salts & L-Glutamine, Ham's F-12 medium, Dulbecco's modified Eagle's medium, HEPES, Horse serum and Fetal calf serum were purchased from Gibco Laboratories, Grand Island, N.Y.

Heparin 1,000 iu/ml was purchased from Leo Laboratories Canada LTD, Pickering Ont.

Collagenase and Hyaluronidase was purchased from Boehringer Mannheim, Montreal, Que.

Deoxyribonucleic acid (phenol extracted) from salmon testes was purchased from P-L Biochemicals, Inc (Milwaukee, Wis.)

Dextran T-70 was purchased from Pharmacia, Uppsala, Sweden

Sodium Pentobarbital (for veterinary use) was purchased from M.T.C. Pharmaceuticals, Mississauga, Ont.

RIBI adjuvant (0.5 mg MPL, 0.5 mg TDM and 2 μl of Tween-80) was purchased from RIBI Immunochem Research Inc., Hamilton, Montana.

Saline was purchased from Travenol Canada Inc., Mississauga Ont.

Xylazine (20 mg/ml) (Rompun) was purchased from Bayvet

Division Chemagro Limited, Etobicoke, Ont.

Ketamine, 57.5 mg/ml, (Ketalar) was purchased from Parke-Davis Canada Inc., Scarborough, Ont.

Goat serum, Biotinylated anti-guinea pig IgG (H+L) immunoglobulin, ABC (Vectastain ABC Kit: Avidin DH Reagent B, Biotinylated Horseredish peroxidase H) were purchased from Vector Laboratories, Inc., Burlimgame, CA.

Ammonium persulfate, Acrylamide (2x cryst.), Bisacrylamide (2x cryst.), Sucrose, SDS (Sodium dodecyl sulfate), TEMED (N,N,N`,N`,-tetra-methylethylenediamine), Tris and Urea were purchased from Bethesda Research Laboratories, Gaithersburg, MD.

Borosilicate glass disposable culture tubes (12 x 75 mm and 16 x 100 mm) were purchased from Fisher Scientific Co. Limited, Pittsburgh, PA.

μBondapak 3.9mm x 30cm and 7.8mm x 30 cm stainless steel columns, Protein-Pak-125 (old name, I-125) 7.8mm x 30 cm stainless steel columns, C_{18} Sep-Paks, NorganicTM Water Purification System and the HPLC system were purchased from Waters Associates, Milford, MA.

Vydac 3.9 mm x 30 cm C_{18} column was purchased from Cole Palmer, Chicago, IL.

High performance amino acid analyzer, System 6300, was purchased from Beckman Instruments, Inc., Palo Alto, CA.

Fraction Collector FRAC-100 was purchased from Pharmacia, Baie d'Uefe, Que.

LKB-WALLAC 1277 Gammamaster Automatic Gamma Counter (WALLAK Oy Turku Finland) was purchased from Fisher Scientific Co. Montreal, Que.

Savant Speed Vac concentrator was purchased from Savant Instruments Inc., Farmingdale N.Y.

The Dubnoff Metabolic Shaking incubator, was purchased from Precision Scientific Co. Chicago.

The Spectrophotometer: Spectronic 7000, was purchased from Bausch & Lomb and the U-2000 was purchased from Hitachi.

The Millipore Multiscreen Assay System and Durapore(PVDF) membrane (0.22 μm) were purchased from Millipore Corporation, Bedford, MA.

The Model 3000xi Computer Controlled Power Supply and Model 1000/500 Power Supply and the Modular Mini Electrophoresis System were purchased from Bio-Rad Laboratories Canada Ltd. Mississauga, Ont.

Light Orthoplan Microscopy was purchased from LEITZ. Leica, Welzlar,Germeny.

Black and white photographs were made using Kodak Technical Pan film.

2. Isolation and Purification of Peptides

2.2.1. Collection of tissues

2.2.1.1. *Collection of guinea pig bone marrow and lung*

The lungs and femurs were collected from fifty adult guinea pigs weighing 500 to 750 g immediately after decapitation following complete ether anaesthesia. The lungs were frozen on dry ice and stored at -80°C. Bone marrow from the femurs was first suspended in saline and centrifuged at 2000 x g. The cell pellet was resuspended in the same buffer containing 0.15M ammonium chloride, 0.01M Tris and incubated at 37°C for 40 min. After centrifugation at 2000 x g for 10 minutes, the cell pellet was suspended in the red blood cell lysis buffer and purified one more time. The bone marrow cells were then extracted in acidic medium by sonication (see section 2.2.2.1.).

2.2.1.2. ***Collection of rabbit tissues, plasma and bone marrow***

Normal female and pregnant rabbits (3.2 to 4 Kg) were sacrificed by administration of an overdose of sodium pentobarbital (100mg/Kg, i.v.). Blood was collected from adult rabbit hearts using a 30 cc syringe and transferred into a tube containing heparin (50 i.u./ml) and another tube containing EDTA (1 mg/ml). The fetuses were decapitated under sodium pentobarbital (20 mg/kg, i.p.) anaesthesia and the blood was collected into a tube containing EDTA (1 mg/ml). These tubes were stored at 4°C for 4 to 6 h, and then centrifuged at 2000 x g for 10 min. After centrifugation, the supernatant (plasma) was collected and stored at -80°C. The lungs, adrenals, intestines, spleen, placenta, liver and kidneys were collected on dry ice, and the pituitary, hypothalamus, pons oblongata, thalamus,

cerebellum, cerebrum and corpus collosum were dissected from the brain and frozen on dry ice immediately after the rabbits were sacrificed. The fetal organs from one pregnant rabbit were pooled. All tissues were stored at -80°C before use. The rabbit bone marrow was collected using the same method as described in section 2.2.1.1.

2.2.2. Extraction of Tissues

2.2.2.1. *Acidic medium extraction*

Frozen tissues were homogenized at 4°C in an acidic medium consisting of 1M HCl/5% formic acid/1% NaCl (wt/vol)/1% trifluoroacetic acid (286). The ratio of the tissues and the extraction medium was kept at about 1:1 (wt/vol) except bone marrow which was extracted in 10 volumes of extraction medium using ultrasonic homogenization (5 min). After centrifugation (3000 x g for 15 min), the pellet was re-extracted twice in the same volume of extraction medium and the peptide enriched supernatants were combined for ODS-silica cartridge extraction (2.2.2.2.).

2.2.2.2. *ODS silica cartridge extraction*

ODS-silica cartridges (C_{18} Sep-Pak) were prepared using a slightly modified procedure (8). A set of five C_{18} Sep-Paks were connected in series using headless pipet tips (i.e. the bottom 1 to 1.5 inch of 2 inch of 2-200 μl polypropylene pipet tip). The Sep-Paks were first conditioned by wetting with 20 ml of 80%

acetonitrile containing 0.1% TFA followed by 30 ml of 0.1% TFA. Each 150 ml of extracts of guinea pig and rabbit bone marrow extract (see section 2.2.1.1. and 2.2.1.2.) was passed through one set of the cartridges. The cartridges were then washed with 50 ml of 0.1% TFA to remove unbound material. Peptides bound to the ODS-silica resin were eluted with 10 ml of 80% acetonitrile containing 0.1% TFA. The eluates were stored at -20°C for later use or directly subjected to reversed-phase HPLC (see section 2.2.3.).

2.2.3. HPLC Purification

2.2.3.1. *General methods*

HPLC separations were carried out on a Waters HPLC system consisting of two M-45 pumps and a Model 680 Automated Gradient Controller. Eluates from the column were monitored at 280 and 215 nm using a Waters Model 441 and a Model 481 variable wavelength detector connected in series.

HPLC grade water was made from deionized, glass-distilled water by filtration through a Norganic Water Purification System (Waters Associates, Milford, MA). All reagents used for HPLC purification procedures were HPLC grade, except TFA which was prepared as a stock solution of 1% (v/v, 0.13M) and purified by passing through a C_{18} Sep-Pak. Prior to use, the Sep-Pak was conditioned as described in section 2.2.1.2.. Acetonitrile and water were degassed under water vacuum for about 20 minutes

immediately before use.

2.2.3.2. ***Reversed-phase HPLC purification***

Reversed-phase HPLC purifications were carried out as previously described (286,287). The method used is as follows. The Sep-Pak eluates or HPLC fractions were concentrated in the Speed Vac to evaporate acetonitrile, and loaded onto a C_{18} μBondapak column (Waters Associates, Milford, MA) via an injector (Beckman Instruments Inc.) or an HPLC pump (Milton Roy Inc.) Two solvent systems were used, one employing TFA as the hydrophobic counter-ion pairing reagent at a concentration of 0.1% (v/v) and the other employing HFBA as the hydrophobic counter-ion pairing reagent at a concentration of 0.13% (v/v). In both solvent systems, 80% acetonitrile was used as the organic modifier (Buffer B). Buffer A (0.1% TFA) was prepared with HPLC-grade water. The concentration of the corresponding hydrophobic counter-ion pairing reagent was the same in both buffer A and buffer B. Buffer A and buffer B were delivered by separate pumps, pump A and pump B which were controlled by a automated gradient controller (Waters Model 680). Bound peptides were eluted from the column with linear gradients of solvent B. Fractions were collected in glass or plastic 12 x 75 mm test tubes, using an LKB Ultrarak Fraction Collector (Fisher Scientific Co., Montreal, Quebec.)

2.2.3.3. ***Gel permeation HPLC purification***

Gel permeation HPLC separations were performed isocratically

at a flow rate of 1.0 ml/min on two I-125 Waters protein analysis columns connected in series with the solvent system of 40% acetonitrile containing 0.1% TFA (287). Samples from reversed-phase HPLC were either dried to a small volume or to complete dryness and then dissolved in 50 to 100 µl of 40% acetonitrile containing 0.1% TFA for application to the columns.

2.3. Characterization of Peptides

2.3.1. Amino Acid Analysis

2.3.1.1. *Acid hydrolysis*

Aliquots of 100 to 1000 picomoles from samples purified by HPLC (see section 2.2.3) were routinely taken for amino acid analysis. They were dried in the Speed Vac in 6 x 50 mm KIMAX culture tube and hydrolsized at 105°C for 16 hours using the vapour of constant boiling HCl containing a small amount of phenol. HCl was evaporated under vacuum prior to analysis as described in section 2.3.1.2..

2.3.1.2. *Amino acid analysis*

Amino acids derived from acid hydrolysis as described in section 2.3.1.1. were separated and quantified on a high performance amino acid analyzer (System 6300, Beckman Instruments, Inc.) consisting of an automatic sample loading turn table, ion exchange HPLC and ninhydrin colour reaction. The ninhydrin derivatives were monitored by an on line

spectophotometer at 440 and 570 nm and the sum of both peak areas was integrated by a Hewlett Packard 3390A Reporting Integrator. The program was set up according to instructions supplied by the manufacturer.

2.3.2. Gas Phase Sequencing of Peptides

2.3.2.1. *Reduction and pyridylethylation*

A total of about 40 µg of purified peptide was dissolved in 200 µl of the denaturant buffer (6M Guanidine-HCl, 0.25 M Tris, 1 mM EDTA pH 8.5) and incubated with 10 µl of 1.4 M 2-mercaptoethanol at room temperature in the dark. After 2 hours, 10 µl of 4-vinylpyridine (undiluted) was added and the mixture was incubated for another 2 hours under the same conditions. The linear pyridylethylated peptide was then purified by RP-HPLC as described in section 2.2.2.2.

2.3.2.2. *Amino acid sequence analysis*

The amino acid sequence of the purified peptides was determined by automated Edman degradation in an Applied Biosystems 470 A gas-phase sequenator with a trifluoroacetic acid conversion program. The PTH (phenylthiohydantoin) amino acids were monitored using an Applied Biosystem 120 A on-line analyzer. All sequence analysis was carried out in the Institut de Recherches Clinique de Montreal, by Dr. Claude Lazure.

2.3.3. Enzyme digestion

2.3.3.1. *Trypsin and chymotrypsin*

A total amount of 20 nmoles of GPCS3 was digested using a

mixture of 2 μg chymotrypsin and 2 μg trypsin in 50 mM Tris HCl buffer (pH 7.5) at 37°C for 18 h. The fragments obtained were separated by HPLC on a $C_{18}\mu$ Bondapak column , and each fragment was subjected to amino acid analysis.

2.3.3.2. ***Carboxypeptidase B***

About 100 nmoles of the synthetic CSI was digested using carboxypeptidase B, at a enzyme to substrate ratio of approximately 1 to 31.5 by weight. Digestions were performed at 37°C in 0.6 M Tris buffer (pH 8.5) for 4 to 8 h (406). The truncated peptides were separated by HPLC on a Vydac C_{18} reversed-phase column, and the identity of each peptide was determined by both amino acid analysis and ion-spray mass spectroscopy. Ion-spray mass spectroscopy was performed by Dr. S. Konishi of the Biotechnology Research Institute, Montreal.

2.3.4. Edman degradation

Approximately 100 nM of a mixture of GPCS1 and GPCS2 were prepared by two cycles of Edman degradation (407). At the end of the second cycle, 2 ml of 1% TFA was added to lower the pH to about 4, prior to purification by reversed-phase HPLC. The identity of these peptides was also confirmed by both amino acid analysis and ion-spray mass spectroscopy.

2.4. Radioimmunoassays

2.4.1. Radioimmunoassays of Peptides

2.4.1.1. ***Iodination of peptides***

Labelled peptides for radioimmunoassay were prepared using a modification of the chloramine-T method, as described by Browne *at al* (408). 2 μg of peptide dissolved in 10 μl of 2 M phosphate buffer pH 7.4 and 1 mCi of $^{125}I^{-}$ in 10 μl of NaOH solution pH 8-11 were mixed with 10 μg of chloramine T in 20 μl of PBS buffer in a 1.5 ml polypropylene microfuge tube for 15 seconds. The reaction was then terminated by adding 50 μg of sodium metabisulphite in 20 μl of PBS buffer.

The ^{125}I-peptide and a small amount of unlabelled peptide were separated from iodide by chromatography on a C_{18} reversed phase cartridge. A preconditioned ODS-silica cartridge was prepared as described previously (see section 2.2.1.2.). The mixture of the products of iodination were passed through the cartridge which was then washed with 50 to 100 ml of 0.1% TFA to remove unbound free iodide. Iodinated peptides were eluted with 3 ml of 80% acetonitrile containing 0.1% TFA. The eluates were stored at 4°C for use as tracer in the corresponding radioimmunoassay.

2.4.1.2. ***Antisera***

Antisera to ACTH, and α-MSH were generated in our laboratory by Dr. C. A. Browne (408).

CSI antiserum was raised in guinea pigs with conjugates of CSI and keyhole limpet haemocyanin I.M.. A total of 5 mg of synthetic CSI and 5 mg of keyhole limpet haemocyanin dissolved in

5 ml of PBS buffer were conjugated by using glutaraldehyde to a final concentration of 0.25% v/v. After 1 hour incubation at room temperature, the reaction was terminated by addition of lysine to a final concentration of 0.2 M. A total of 0.8 ml of conjugate containing 0.8 mg CSI was added to RIBI adjuvant reconstituted in 1.2 ml of saline per vial. Booster injections contained 0.4 ml of conjugate and 1.6 ml of adjuvant reconstituted in saline. Immunization was performed in guinea pigs as follows: a total of 0.2 ml of the mixture of the conjugate and adjuvant was injected I.M. into each side of the hind legs and 0.1 ml of the mixture was injected I.P.. Boosters were given at 3, 6, 9 and 12 weeks. Blood was taken by cardiac puncture using Xylazine (1mg/kg) and Ketamine (40 mg/kg) as anaesthetics two weeks after the booster shots. The serum was stored at -70°C.

2.4.1.3. ***Specificity of antisera***

2.4.1.3.1. Radioimmune cross-reactivity

The ACTH antiserum was directed towards amino acid 17-20, and cross-reacted equally well with $ACTH_{1-24}$, $ACTH_{17-39}$, $ACTH_{16-27}$ and CLIP (408). The antiserum did not cross-react significantly (<0.05%) with α-MSH (408).

The α-MSH antiserum was directed toward the C-terminal amino acid residues (10-13 amide), and cross-reacted poorly (<0.1%) with $ACTH_{1-39}$, $ACTH_{1-24}$ and $ACTH_{1-18}$ (408).

The CSI antiserum cross-reacted very weakly with CSII, CSIII, and CSIV (<1%) and had no detectable cross-reactivity with

other peptides including human corticostatin HP-4, rat corticostatin R-4, ANF, ACTH and α-MSH (Fig.3-13(B)).

2.4.1.3.2. SDS-PAGE and Western blot analysis

Samples from tissue extracts were subjected to electrophoresis on a SDS-PAGE system using a 16.5 % running gel with 6 M urea and a 4 % stacking gel according to Schagger et al. (409). After electrophoresis, the gel was stained with Ponceau S (0.5 % Ponceau S in 7.5 % Trichloroacetic acid). Excess dye was washed away with double distilled H_2O.

Blotting of proteins from polyacrylaminde gels onto nitrocellulose paper was carried out according to the directions provided by the manufacter (Bio-Rad). Briefly, electrotransfer took place using 100V at 4°C for 10 to 14 hours in 50 mM Tris-glycine, pH 8.3, containing 20% methanol. The nitrocellulose blot was incubated first with poly-clonal antiserum against CSI and then with ^{125}I-protein A using the protein A method as described by Lindmark et al. (410).

2.4.1.4. *Radioimmunoassays*

A 0.02 M Barbitone buffer containing 1% (w/v) BSA, 0.18% (w/v) NaCl, and 0.4% mercaptoethanol pH 8.6 was used for the radioimmunoassays for ACTH and α-MSH, and PBS buffer containing 0.5% BSA was used for CSI.

Standard curves were constructed using known amounts of unlabelled peptide (10 to 4,000 pg of ACTH, or 50'to 12,000 pg of α-MSH, or 25 to 10,000 pg of CSI) in a volume of 100 µl of

buffer. Aliquots of the samples from HPLC were dried in the Speed-Vac concentrator prior to assay. In order to obtain accurate RIA data, a second or third RIA were performed and serial dilutions of samples were sometimes prepared in order that the amount of peptide measured fell on the linear portion of the standard curve.

A total of 100 μl of appropriate tracer containing 10,000 to 20,000 cpm was added to each of the tubes, followed by the addition of 100 μl of antiserum solution to all tubes except for those used to determine total counts and non-specific binding, (100 μl of buffer was added instead). The tubes were incubated at room temperature for 2 hours for the ACTH assay, 3 hours for α-MSH assay and at 4°C for 12-16 hours for CSI assay. At the end of incubation, 750 μl of dextran-charcoal buffer containing 0.25% charcoal and 0.05% dextran T-70 at 4°C, was added to each tube except for those used to determine total counts, and vortexed. The tubes were then incubated for 6 min at 4°C and centrifuged at 2000 x g for 15 min. The supernatants were decanted into 12 x 75 mm test tubes, stoppered with corks and radioactivity determined in a gamma counter for 1 to 2 min.

2.4.2. Radioimmunoassay of Steroids

The radioimmunoassay for corticosterone, cortisol and aldosterone were carried out according to the instructions supplied by the manufacturer (Immunocorp, Montreal, Que.).

2.5. DNA assay

Tissues were collected as described in section 2.2.1.2. The tissues were homogenized in 10 volumes of saline using a Dounce homogenizer. The DNA content of the tissues was determined using the diphenylamine reaction, as reported previously (411) and the tissue content was ultimately expressed as ng/mg DNA. The Student's t-test was used for determining statistical significance between two means.

2.6. In Vivo Study of the effect of CSI on the Length of Gestation

Pregnant rabbits were anaesthetized on days 24 to 29 of gestation using the following drugs: Ketamine (50-60 mg/kg), Xylazine (5 mg/kg) and atropine (0.4 mg/kg). Acetyl promazine (0.5 mg/kg) was used in addition to the above drugs when necessary. Laparotomy was performed using a midline insertion. The uterus was exteriorized, and CSI (100 μg/0.1ml/fetus) or CSI antiserum (0.1ml of diluted Antiserum/fetus) were injected into each fetus (I.P.). The uterus was placed back into the abdominal cavity, the muscles and skin were sutured. The entire surgical procedures were done under sterile conditions. After the recovery period the animals were returned to large cages with nest boxes and the animals were allowed to deliver (412). During this period animals were observed six times during the 24 hour period and the time of delivery was noted. Upon delivery, in each series of

experiments, the mothers were sacrificed with an overdose of pentobarbitol (100 mg/kg given i.v. very rapidly). The pups were injected with pentobarbitol (20 mg/kg I.P.) then decapitated. Newborn and maternal blood and tissues were used for extraction and measurement of CSI and determination of other hormones by RIA (see sections 2.4.1.4. and 2.4.2.).

2.7. Immunocytochemistry

Normal female and pregnant rabbits (2.5-3.5 kg) were killed by administration of sodium pentobarbital i.v. Rabbit tissues were removed rapidly, placed in the Bouin's solution (picric acid :formalin:acetic acid;15:4:1) before dehydration, and then embedded in paraffin as described previously (413). Paraffin sections affixed to glass slides were stained immunocytochemically according to an indirect immunoperoxidase procedure (413). Sections were first blocked with 10% normal goat serum for 10 min at room temperature, and then incubated overnight at 4°C with preimmune serum or immune antiserum, either untreated or preabsorbed with 0.02 and 2 μg CSI/100ul for 24 and 48 hours at 4°C. The concentration of antiserum used in these experiments was 1:100. Sections were washed with PBS for 10 min and treated with biotinylated goat anti-guinea pig IgG secondary antiserum (1:100 dilution) for 45 min at room temperature and washed again. This was followed by reaction with avidin- biotin peroxidase (ABC, Vector Laboratories, 1:100 dilution) for 45 min

at room temperature. After a 10 min wash with PBS, the sections were treated for 6-8 min in a solution containing 30 mg DAB and 10 μl fresh 30% hydrogen peroxide in 100 ml PBS. In order to identify the cell types, sections were counterstained with methylene blue (0.12%) in water. The results were analyzed and photographed under light microscopy, and this work was done in collaboration with Dr. Serge Jothy, Department of Pathology.

2.8. *In Vitro* Bioassay

2.8.1. Rat Adrenal Cell Bioassay

The dispersed rat adrenal cell bioassay was a modification of the method of Sayers (414-418). Prior to use, all media used in the bioassay were prewarmed to 37°C in a shaking water bath in an atmosphere of 95% O_2/5% CO_2. A total of 10-26 Sprague-Dawley male rats weighing 150 to 200 grams were sacrificed by decapitation. Adrenals were decapsulated, removed immediately, and quartered in Ham's F-12 medium containing 0.5% BSA. The tissue was then incubated for 1 hr in 10 ml of medium containing 2 mg/ml collagenase and 250 μg/ml DNase. By the end of this incubation, dispersal of cells was completed by pipetting the medium up and down 50 times using a Pasteur pipette. The cell suspension was centrifuged at 150 x g for 10 min, and the cell pellet was resuspended and washed twice with 12 ml of the same medium. Finally, the cell pellet was suspended in 2 ml of medium

and filtered through pre-wetted nylon gauze (100 μm) and layered on top of 8 ml of Ham's F-12 medium containing 2.5% BSA prior to gradient centrifugation. After centrifugation at 150 x g for 10 min, 6 ml of the upper layer was aspirated and the cells in the lower 4 ml were diluted with 10 ml of incubation medium (Ham's F-12 medium containing 0.5% BSA and 7 mM Ca^{2+}) before determining the cell number.

The viability of the cells was determined by the Trypan Blue exclusion method. Only cells which contained lipid droplets and excluded Trypan Blue were counted. The cell suspension was adjusted to a final concentration of 400,000 cells per ml and 0.5 ml was added to the incubation tubes. The cells were preincubated for 60 min at 37°C in a shaking water bath under an atmosphere of 95% O_2/5% CO_2. Then, a 0.5 ml aliquot of incubation medium or incubation medium containing either synthetic human ACTH ($ACTH_{1-39}$) alone or ACTH plus test material was added. The mixture was incubated for an additional 2 hr. After incubation the tubes were centrifuged at 120 x g for 10 min, and the supernatant was decanted into borosilicate culture tubes. Steroids were extracted with 2 ml of methylene chloride and corticosterone in the extract was determined by radioimmunoassay which was done in duplicate.

2.8.2. Rat Adrenal Zona Glomerulosa Cell Bioassay

Zona glomerulosa cells were prepared essentially as described by Douglas *et al.* (419). A total of 15-20 Sprague-Dawley male rats weighing between 175 and 200 grams were

sacrificed by decapitation. The adrenals were freed from fat tissue before removal and decapsulation. The capsules were minced and the resulting mince was incubated for 45 min at 37°C in 10 ml of medium 199 containing 2 mg/ml collagenase and 250 μg/ml DNase in a shaking water bath in an atmosphere of 95% O_2/5% CO_2 as described in section 2.8.1.. The tissue was mechanically dispersed and centrifuged at 150 x g for 10 min and then washed with medium 199. The resulting cells were resuspended in 2 ml of the medium and filtered through nylon gauze (100 μm) onto a bed of 8 ml of the medium containing 2.5% of BSA prior to gradient centrifugation as described in section 2.8.1.. The cells (400,000) contained in 1 ml of medium 199 which was supplemented with 0.5% BSA and 5 mM K^+, were incubated together with either Angio II, ANF, or α-MSH alone or corticostatin I plus Angio II, ANF, or α-MSH for 2 h under the same conditions as previously described in section 2.8.1. Aldosterone output was measured by radioimmunoassay following extraction of supernatant with methylene chloride as described in section 2.8.1.

2.8.3. Anolis skin bioassay

The assay was preformed as previously described by Tilderes et al(420). The skin of uniform green colour was removed from decapitated lizards (Anolis carolinensis) and cut into square pieces of approx.5 mm. The pieces of skin were equilibrated in medium (8.3 g NaCl, 0.33 g KCl, 0.16 g $CaCl_2$-$2H_2O$, 0.21 g $MgCl_2$-$6H_2O$, 0.1 g $NaHCO_3$ and 10 mg BSA per liter of double distilled

water). Serial dilutions of α-MSH and the test peptides were placed in different wells of 96-multiwell culture plates and the fragments of skin were introduced. A positive result was indicated by a green to brown colour change and the results were assessed by two different investigators.

2.8.4. Rat Pituitary Cell Culture Bioassay

The rat pituitary cell culture bioassay was carried out essentially as previously described (421) with a few modifications. Prior to use, all media employed in the culture were pre-warmed to 37°C in a shaking water bath in an atmosphere of 95% O_2/5% CO_2. A total of 20-22 Sprague-Dawley male rats weighing 150 to 200 grams were sacrificed by decapitation, and pituitaries were removed immediately, and quartered in Dulbecco's Modified Eagle's medium containing 0.5% BSA. The tissue was then incubated for 1 hr in 10 ml of the medium containing 20 mg/ml collagenase, 10 mg hyaluronidase, 100 mg BSA and 0.1 ml of 1M HEPES buffer. At the end of the digestion, dispersal of cells was completed by pipetting the medium up and down 50 times using a Pasteur pipette and filtered through nylon gauze (100 μm). The cell suspension was centrifuged at 150 x g for 10 min, and the cell pellet was resuspended and washed twice with 12 ml of Dulbecco's modified Eagle's medium containing 10% horse serum, 5% fetal calf serum and 0.002% Gentamicin. Following the final wash, the cells were suspended in 5 ml of medium and the viability of the cells was determined by Trypan Blue exclusion method. Only

cells which contained lipid droplets and excluded Trypan Blue were counted. Cells were plated in 1 ml aliquots of 5 x 10^5 cells per well in 24 well tissue culture plates and cultured for 72 h in a 37°C incubator gassed with air and 5% CO_2. After incubation the cells were washed twice with 1 ml of serum-free Dulbecco's modified Eagle's medium containing 0.5% BSA and 0.002% Gentamicin, and then treated with CRF alone or CSI plus 2 ng/ml CRF for 4 and 24 h beginning with 1.25 ml volume. A total of 300 µl aliquots were taken at the 4 h incubation time, and the entire cell culture procedure was performed under sterile conditions.

2.9. Radioligand Binding Assay

2.9.1. Preparation of Labelled α-MSH and CSI Ligand

The fully biologically active α-MSH analogue ([Nle^4,D-Phe^7]α-MSH) and CSI were freshly iodinated and purified for each experiment. A total of 2 µg of [Nle^4,D-Phe^7] α-MSH or CSI in 10 µl of 2 M phosphate buffer pH 7.4 and 1 mCi of ^{125}I were mixed with 2 µg of chloramine T in 20 µl of PBS buffer in a 1.5 ml polypropylene microfuge tube for 15 seconds. The reaction was terminated by adding 25 µg of sodium metabisulphite in 20 µl of PBS buffer. The ^{125}I-α-MSH analogue or CSI was separated from iodide and non-iodinated α-MSH analogue or CSI respectively by reversed-phase HPLC using a pre-column (422). After loading, the pre-column was first washed with 0.1% TFA for 60 min to wash away

unbound free iodine and then eluted with two linear gradients of 1-15% acetonitrile (for the first 10 min) and 16-35% acetonitrile for α-MSH and 16-40% acetonitrile for CSI (for the next 50 min). Fractions were collected every minute, and 10 μl aliquots were taken from each fraction and counted in a LKB-WALLAC 1277 Gammamaster Automatic Gamma Counter . The efficiency of the gamma counter was estimated from a known dpm standard ^{129}I supplied by LKB-WALLAC company at 55% (31865 CPM = 58300 DPM). The [^{125}I]iodo-[Nle^4,D-Phe^7]α-MSH concentration was determined by its ability to activate α-MSH-sensitive colour change in skin fragments of the lizards (see section 2.8.3.), and to stimulate aldosterone production in a rat adrenal zona glomerulosa cell bioassay (see section 2.8.2) using a calibration curve mode with authentic [Nle^4,D-Phe^7]α-MSH. The [^{125}I]iodo-CSI concentration was determined based on its ability to inhibit ACTH-stimulated corticosterone production in an isolated rat adrenal cell bioassay (see section 2.8.1.). The specific radioactivity of the labelled peptides were approximately 2000 μCi/nmol.

2.9.2. α-MSH Receptor Binding Studies

α-MSH binding studies were carried out as previously reported (423) except that the Millipore Multiscreen assay system was used instead of the Millipore sampling manifold system.

The rats were killed by ether anaesthesia, and the lacrimal glands and the capsular layer of adrenal glands were removed, collected and dispersed in a Dounce homogenizer in 10 volumes of

ice-cold homogenization medium (0.3 M sucrose, 20 mM Tris acetate, pH 7.6, 0.1 mM EDTA, 1 mM PABA), using 5 strokes with a loose pestle and 5 strokes with a tight pestle. The homogenate was then filtered through a nylon gauze (100 μm) and centrifuged for 10 min at 12000 X g. The pellet was discarded and the supernatant was centrifuged further for 60 min at 100,000 X g. The resulting pellet was resuspended in 10 mM Tris acetate pH 7.4, 1 mM DTT and frozen in liquid nitrogen in aliquots that were thawed once shortly before using for the α-MSH receptor binding assay.

The binding assay was carried out in PBS buffer contained 0.1 ml of calcium (1 mM $CaCl_2$) and magnesium (1 mM $MgCl_2$) supplemented with 0.1% BSA and some protease inhibitors (10 μg/ml leupeptin, 1mM PABA, 10 μg/ml soya bean trypsin inhibitor) in non-sterile 96-well filtration plates. [^{125}I Iodo-[Nle^4,D-Phe^7] α-MSH (1.0-2.0 X 10^5 c.p.m.), and varying concentrations of unlabelled [Nle^4,D-Phe^7] α-MSH, α-MSH, ACTH and CSI as specified in the individual experiments were coincubated with 18 to 20 μg of membrane protein for 20 min at 30°C. Binding reactions were terminated by the addition of 0.2 ml ice-cold washing buffer (0.1% BSA in PBS). The membrane suspension was filtered through 0.22 μm Durapore (PVDF) Millipore filter, and washed twice with 0.2 ml of ice-cold washing buffer. The filters were counted in an autogamma counter. All binding data are reported as specific binding of α-MSH after subtraction of the non-specific binding

value observed in the presence of an excess 1 μM unlabelled [Nle^4,D-Phe^7] α-MSH. Specific binding (>80% of total binding) is expressed as moles of [Nle^4,D-Phe^7] α-MSH bound/mg membrane protein, means ± S.D. of quadruplicate determinations from three separate expriments (n=3).

2.9.3. CSI Receptor Binding Studies

CSI binding studies were prepared as described for the α-MSH binding studies (see section 2.9.2) except that radiolabelled CSI was used instead of radio labelled [Nle^4, D-phe^7]-α-MSH and 10 μM of 4K polylysine has been added to the binding incubation buffer to minimize non-specific binding (426).

CHAPTER 3: RESULTS

3.1. Isolation and Characterization of Corticostatic Peptides from Guinea Pig and Rabbit Bone Marrow

As mentioned in the first chapter, the corticostatic peptides and defensins belong to a family of cysteine-rich, cationic peptides of low molecular weight which have been recently purified from rabbit lung (8) and from cells of the immune system of the rabbit, human (424,300) and rat (302). The basic amino acids and the cysteines in these peptides are highly conserved. Most members of this family have been found to have antimicrobial activity possibly by a non-oxygen dependent mechanism (289). Some members of the family have been found to be corticostatic (anti-ACTH) and act by competing for the binding of ACTH to its receptor (288). However the motifs of these molecules which determine their biological function are still unknown. In order to gather more information about this peptide family we decided to study the guinea pig peptides and to further investigate rabbit bone marrow. In preliminary studies with guinea pig lung we noted that there were peptides eluted from a RP-HPLC column that were corticostatic. We then turned to bone marrow to obtain sufficient amounts of the peptides for isolation and sequence analysis.

CHAPTER 3 RESULTS

3.1.1. Isolation of GPCS1, GPCS2 and GPCS3

The first RP-HPLC purification step used in the isolation of guinea pig corticostatin is shown in Fig.3-1. There were three peaks eluted with corticostatic activity. The first peak (fractions 48-55, Fig.3-1A) which was also the most abundant, was further purified on a second HPLC run using 0.13% HFBA as the counter-ion (Fig.3-1B). This peak proved to be homogenous from its UV absorbance and the rat adrenal cell bioassay also indicated one main bioactive peak. This corticostatic material was further purified on a C_{18} Vydac column using 0.1% TFA as the counter-ion (Fig. 3-1C). The fractions constituting the front of the peak (Fig. 3-1C) were collected separately from those at the tail of the peak and both were rechromatographed twice more until homogeneous materials were obtained (data not shown). Amino acid analysis (Table 3-1) of the material from both peaks indicated that two distinct peptides (GPCS1 and GPCS2) were present that differed only by two amino acids, leucine and isoleucine. A total of 0.5 nM of the pyridylethylated GPCS1 and GPCS2 were submitted for sequence analysis and the sequences obtained are shown in Table 3-2.

Figure 3-1. Isolation of GPCS1 and GPCS2. HPLC purification of bone marrow extract from guinea pig. Bone marrow from fifty guinea pigs was extracted as described in the methods(section 2.2.1.1 & 2.2.2.1.). (A) The extract was loaded onto a Waters μBondapak reversed-phase column which was eluted using a linear gradient of 0 to 48% acetonitrile in 0.1% TFA for the first 90 min and 48 to 80% acetonitrile in the last 30 min. One minute fractions were collected and bioassayed. (B) Fractions 48-55 from (A) were combined and applied onto the same column as above and eluted with a linear gradient of 0 to 48% acetonitrile in 0.13% HFBA over the first 90 min and 48-65% acetonitrile in the last 10 min. One minute fractions were collected and bioassayed. (C) Fractions 67-74 from (B) were loaded onto a Vydac reversed-phase HPLC column and the column was eluted using a linear gradient of 15-30% acetonitrile in 0.1% TFA in water throughout. Fractions were collected by hand. Fraction 40 and 43, which contained material from the beginning and the end of the major peak in (C) respectively, were chromatographed separately using the same Vydac column and a gradient of 20-27% acetonitrile in 0.1% TFA for 5 to 40 min. The material in each peak was rechromatographed twice using the same system until both were homogeneous. The material in fraction 40 was characterised as a mixture of GPCS1 and GPCS2.

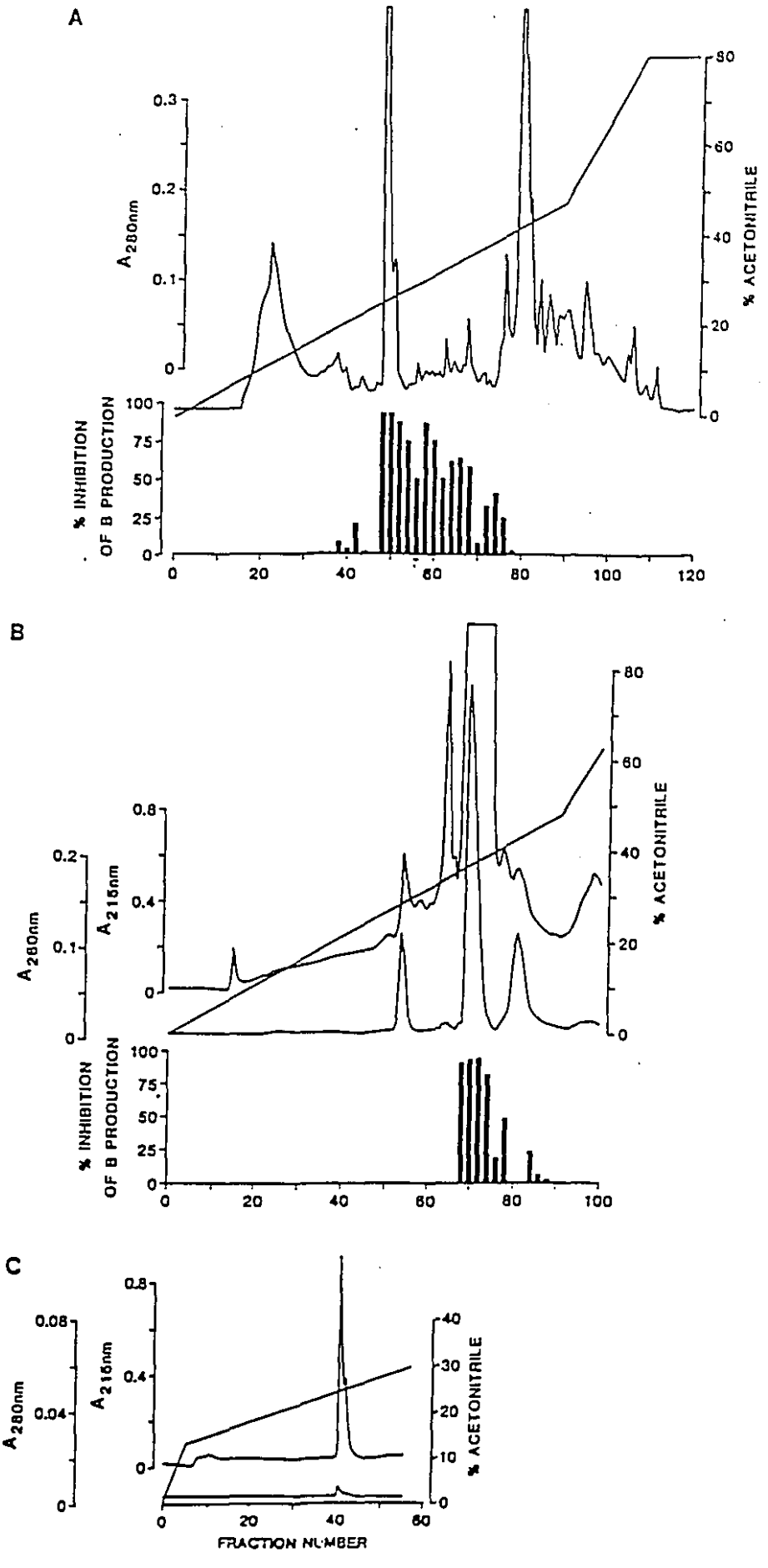

A
A_{280nm}
% ACETONITRILE
% INHIBITION OF B PRODUCTION
B
A_{280nm}
A_{215nm}
% ACETONITRILE
% INHIBITION OF B PRODUCTION
C
A_{280nm}
A_{216nm}
% ACETONITRILE
FRACTION NUMBER

Table 3-1. Amino acid composition of GP Corticostatins after hydrolysis.

	CORTICOSTATIC PEPTIDES		
AMINO ACID	GPCS1	GPCS2	GPCS3
Aspartic acid	1.2 (0) *	1.2 (0)	
Asparagine	(1)	(1)	
Threonine	4.7 (5)	4.7 (5)	
Glutamic acid	1.1 (0)	1.1 (0)	
Glutamine	(1)	(1)	
Proline	1.0 (1)	1.0 (1)	1.1 (1)
Glycine	1.2 (1)	1.2 (1)	
Cysteine	5.4 (6)	4.7 (6)	3.5 (4)
Valine	1.2 (1)	1.1 (1)	
Isoleucine	2.0 (2)	1.0 (1)	
Leucine	1.2 (1)	2.1 (2)	1.1 (1)
Tyrosine	2.0 (2)	2.0 (2)	
Phenylalanine	2.7 (3)	2.9 (3)	1.1 (1)
Histidine			0.9 (1)
Arginine	7.4 (7)	7.2 (7)	4.8 (5)

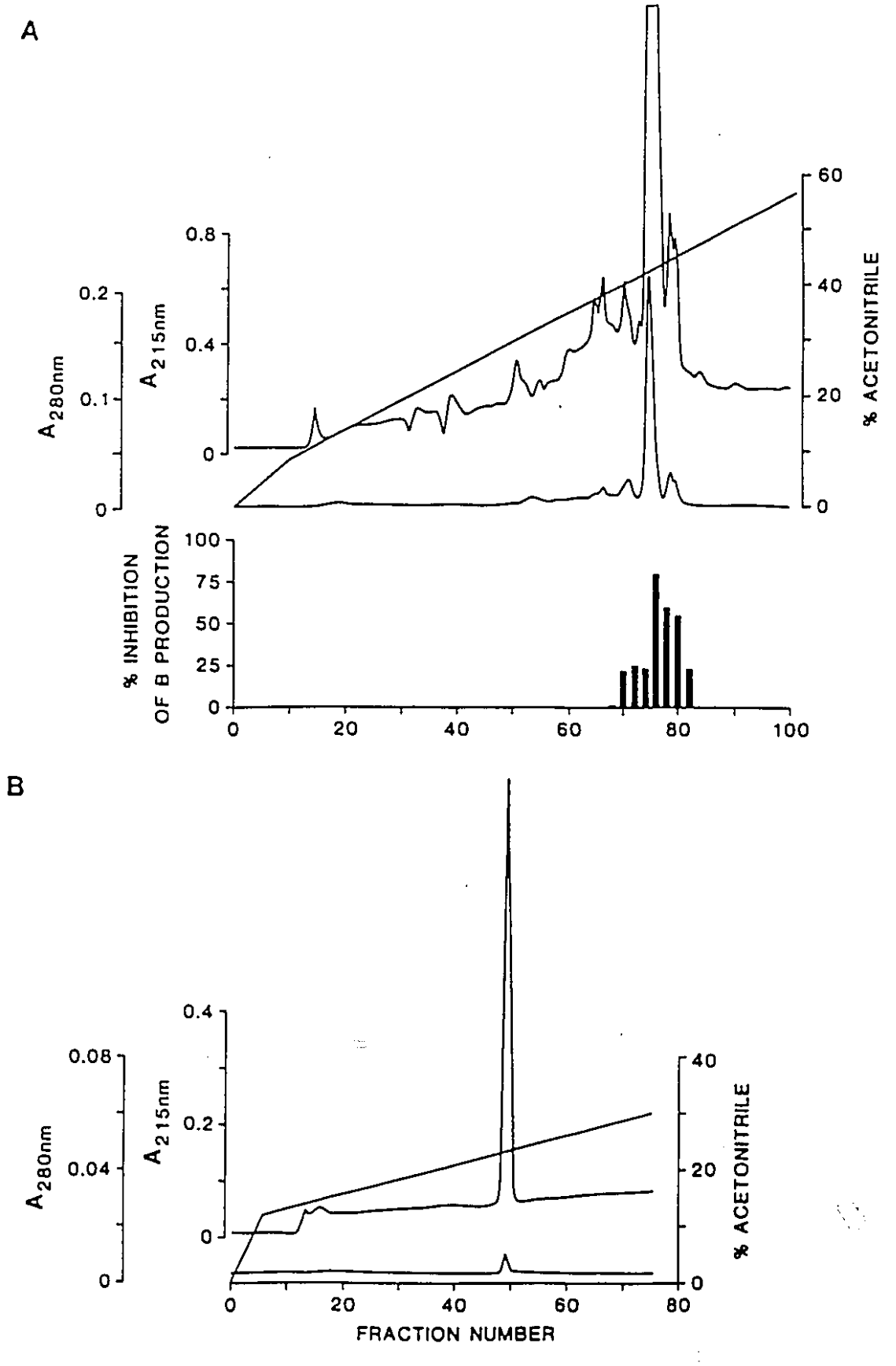
* Numbers in parentheses are values prediated from sequence analysis data.

The second corticostatic peak (Fig. 3-1A) (fractions 56-63) was also chromatographed on RP-HPLC as shown in Fig.3-2A and the bioactive material eluted (fractions 75-76) was rechromatographed on RP-HPLC to yield homogeneous material (Fig. 3-2B). The amino acid composition of this material revealed an abundance of cysteine and arginine as shown in Table 1 and it was named GPCS3. When 0.5 nM of the pyridylethylated GPCS3 was submitted for sequence analysis the following sequence was obtained:

RRPRCFCRLHCRC

Gel permeation HPLC analysis indicated that the mass of GPCS3 was

Figure 3-2. Isolation of GPCS3. (A) Fractions 56-63 from Fig.1A were combined and the material was applied onto a Waters reversed-phase μBondapak column which was eluted using a linear gradient of 0-56% acetonitrile in 0.13% HFBA in water over 100 min. One minute fractions were collected and bioassayed. (B) Corticostatic fractions 75-76 from (A) were subjected to another reversed-phase HPLC step using a Vydac column eluted with a gradient of 15-30% acetonitrile in 0.1% TFA. 1.5 ml fractions were taken over an 80 min period. One minute fractions were collected and bioassayed.


A
A_{280nm}
A_{215nm}
% ACETONITRILE
% INHIBITION OF B PRODUCTION
B
A_{280nm}
A_{215nm}
% ACETONITRILE
FRACTION NUMBER

too high for this peptide to be composed of a monomer of 13 residues. In Fig. 3-3, the elution position of GPCS3 was between α-MSH (1.6 Kd) and insulin (6 Kd). From the chromatographic evidence it seemed that this peptide could exist as a dimer. Ionspray Mass Spectroscopy performed by Dr. Konishi of the Biotechnology Research Institute, Montreal indicated a mass of 3403.74 (charge of 6) and 3403.46 (charge of 5) and the molecular weight of the monomer was calculated to be 1702.0986. The calculated mass of the dimer was 3404.1972. The slight discrepancy in mass is possibly due to small alignment problem with the instrument when the sample was run. In addition GPCS3 was subjected to endoprotease digestion using a mixture of chymotrypsin and trypsin as described in section 2.3.3.1. The fragments obtained were subjected to reversed-phase HPLC which is shown in Fig. 3-4. Three major fragments were obtained which had the following amino acid compositions (molar ratios shown in parentheses). Fragment A, Pro (1), Arg (3); Fragment B, Cys (2), Phe (1) and Fragment C, Cys (2), Leu (1), His (1), Arg (2). All of this data suggests that GP-CS3 is a dimer having an anti-parallel configuration (Table 3-2).

Figure 3-3. Molecular weight comparison of GPCS3, α-MSH and insulin by gel filtration HPLC. The peptides were loaded onto two gel filtration columns connected in series under conditions described in section 2.2.3.3. Fractions were collected every half minute and subjected to amino acid analysis as described in the methods section (2.4.1.2.).

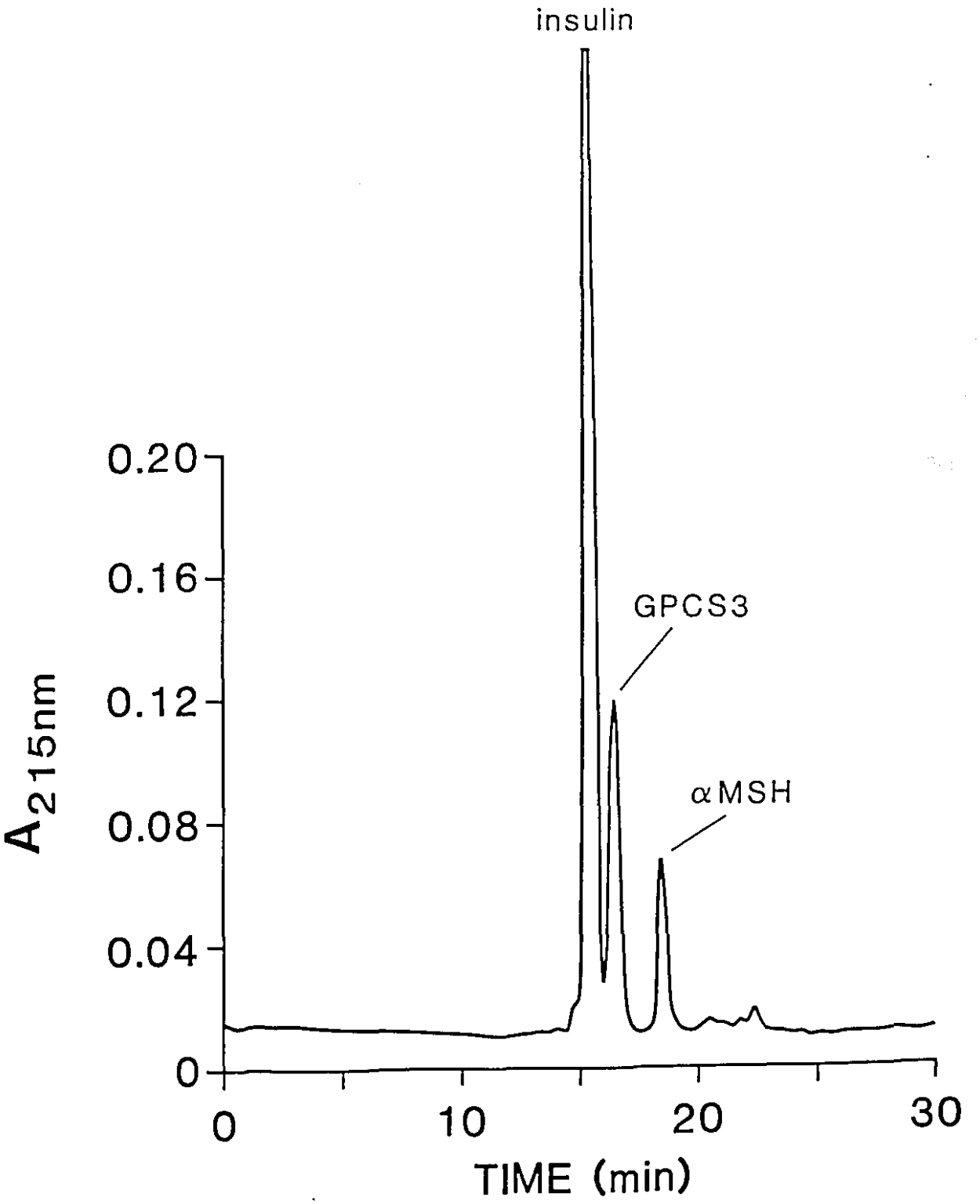
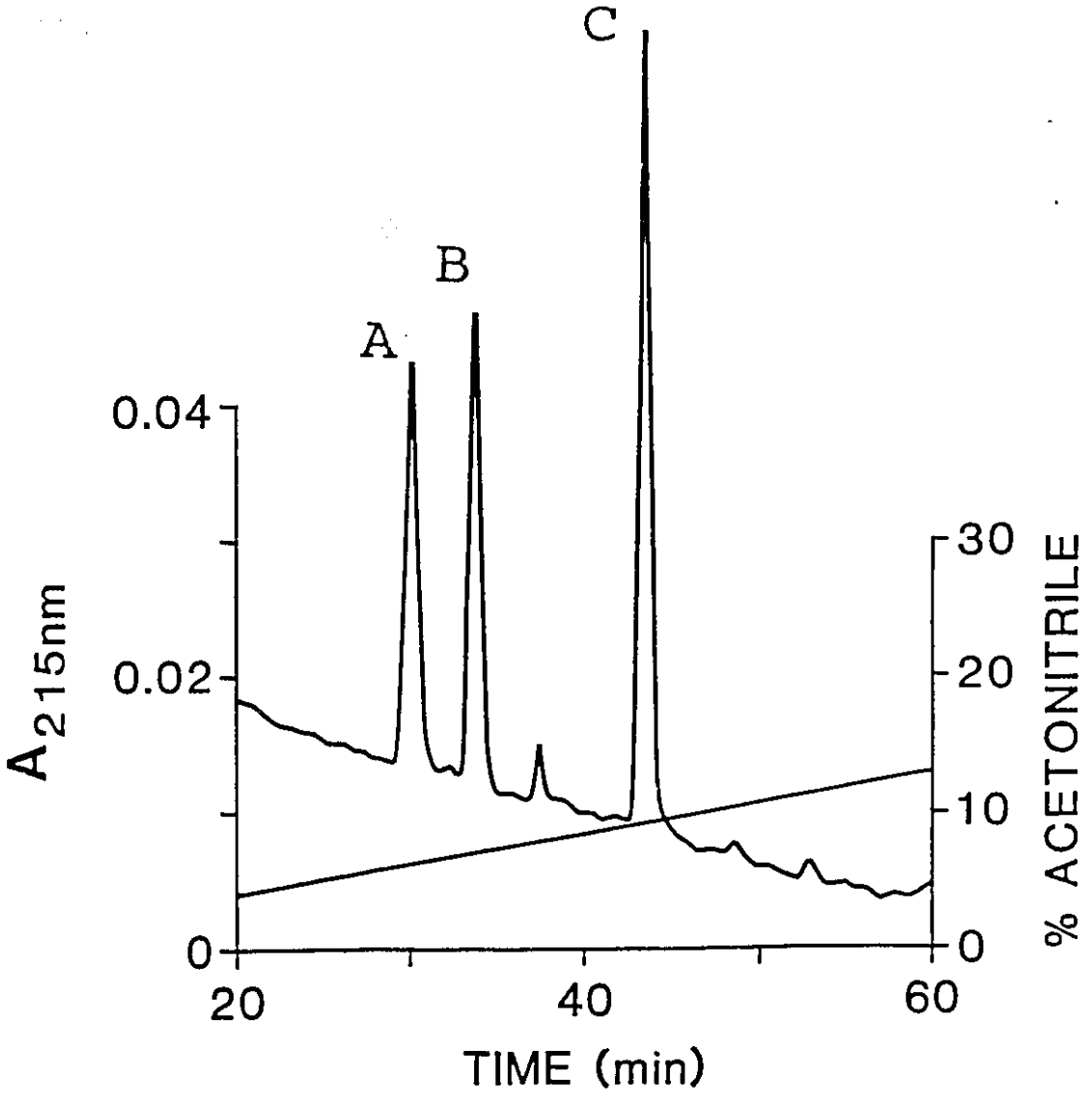

insulin
GPCS3
αMSH
0.20
0.16
0.12
0.08
0.04
0
A_{215nm}
0
10
20
30
TIME (min)

Figure 3-4. Trypsin and chymotrypsin digestion maps of GPCS3. A total of 10 nM of GPCS3 was digested with trypsin and chymotrypsin as described in the methods (section 2.3.3.1.). At the end of the incubation, 1 ml of 1% TFA was added to lower the pH to approximately 3 prior to purification by reversed-phase HPLC using 0% acetonitrile for the first 10 minutes, followed by a linear gradient to 15% acetonitrile over the next 80 minutes. The flow rate was 1.5 ml/min, and 0.1% TFA was used as the counter-ion. The upper panel shows UV absorbance at 215 nm for CPCS3. The lower panel shows the structure of GPCS3 and the structure of GPCS3 digestion products A,B and C which were confirmed by amino acid analysis.




```
GPC3    RRPRCFCRLHCRC
              CRCHLRCFCRPRR
A       RRPR
B            CF
             C
C               CR
               RCHL
```

Table 3-2. Guinea pig corticostatins and their $I.D._{50}$ for the inhibition of ACTH induced corticosterone production in rat adrenal cell suspensions.

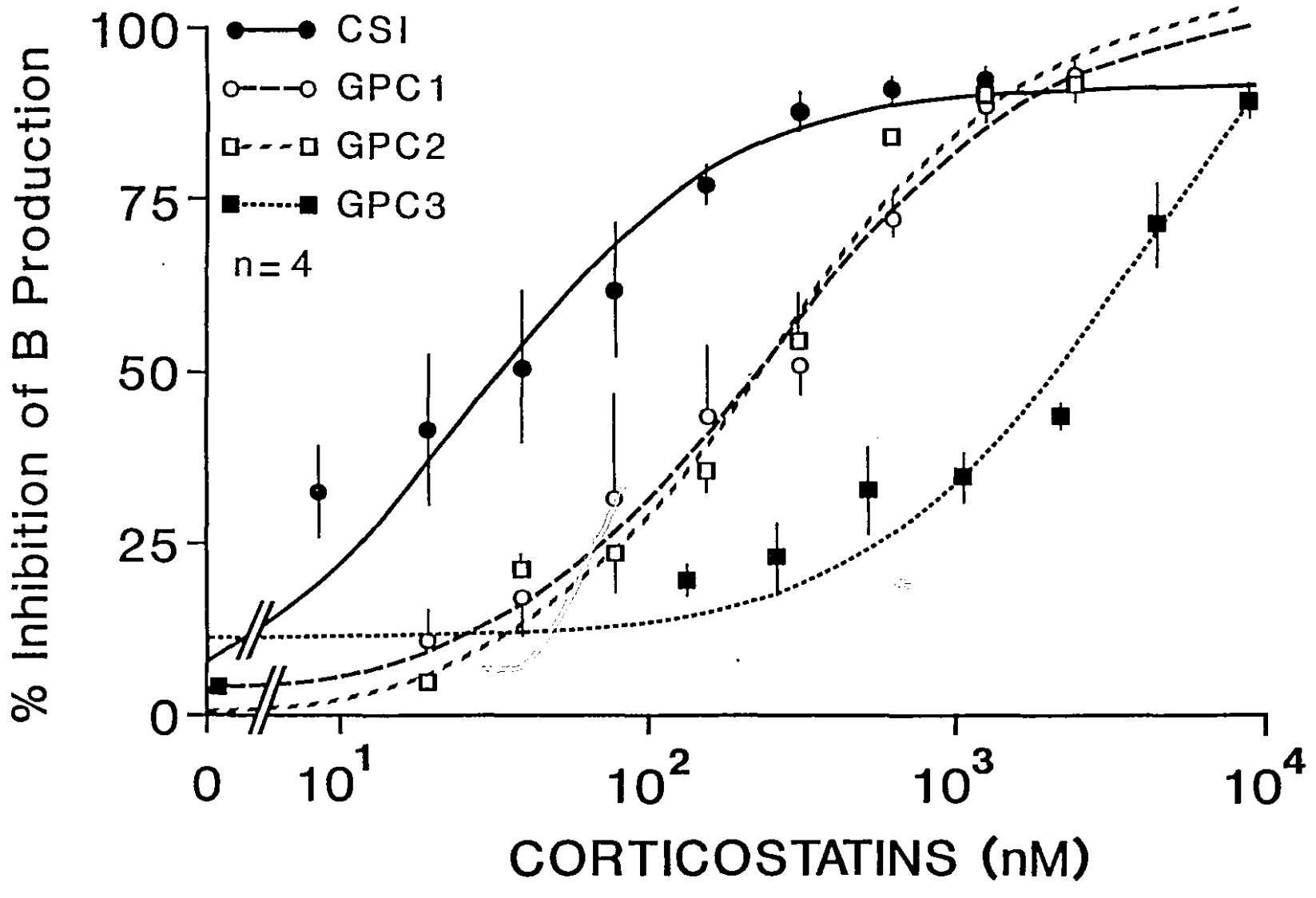
Peptide	Sequence	$I.D._{50}$ (nM)
CSI	G-I-C-A-C-R-R-R-F-C-P-N-S-E-R-F-S-G- Y-C-R-V-N-G-A-R-Y-V-R-C-C-S-R-R	27
GPCS1	R-R-C-I-C-T-T-R-T-C-R-F-P-Y-R-R-L-G- T-C-I-F-Q-N-R-V-Y-T-F-C-C	250
GPCS2	R-R-C-I-C-T-T-R-T-C-R-F-P-Y-R-R-L-G- T-C-L-F-Q-N-R-V-Y-T-F-C-C	250
GPCS3	R-R-P-R-C-F-C-R-L-H-C-R-C : : : : C-R-C-H-L-R-C-F-C-R-P-R-R	2000

In Fig. 3-5 is shown the comparison of the biologic activity of the three guinea pig corticostatins with the most active of the rabbit corticostatins, CS1. It can be seen from this data GPCS1 and GPCS2 have approximately 1/10 of the activity and GPCS3 has approximately 1/80 of the activity of CS1. Most interestingly, GPCS3 is the first corticostatic peptide found which does not belong to the corticostatin/ defensin peptide family.

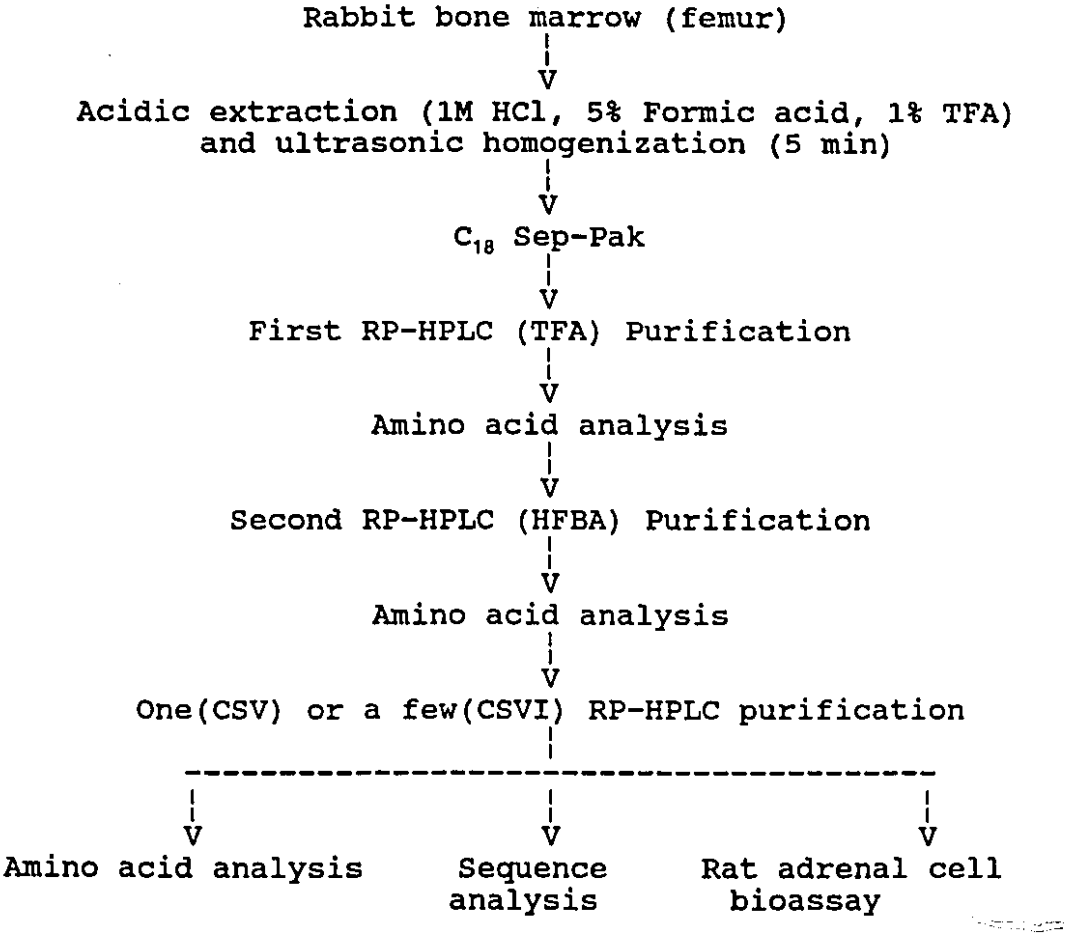
3.1.2. Isolation of rabbit CSV and CSVI

Although corticostatins CSI to CSIV were first isolated from fetal rabbit lungs, it has been shown that immune cells in the tissue were the major cell type where these peptides were

Figure 3-5. A comparison of the corticostatic activity of GPCS1, GPCS2,GPCS3 and rabbit CSI. A total of 33 pM (150 pg/ml) of ACTH was used to stimulate isolated rat adrenal cells at a concentration of 200,000cells/ml, in the presence of increasing concentrations of CSI(solid circles), GPCS1(open squares),GPCS2(open circles) and GPCS3(solid squares). Levels of corticosterone was measured by RIA. Values shown are the mean ± standard deviation of the mean of four separate experiments.


100
75
50
25
0
% Inhibition of B Production
CSI
GPC1
GPC2
GPC3
n = 4
0
10^1
10^2
10^3
10^4
CORTICOSTATINS (nM)

localized (section 3.4.). In addition, neutrophils (424,300) and bone marrow (302) have been used as alternative sources for the isolation of these peptides. Two new corticostatic peptides CSV and CSVI have been purified from bone marrow extracts. The experimental procedure for the purification of the corticostatic peptides from rabbit bone marrow is outlined below:



The HPLC profile of a typical extract of rabbit bone marrow is shown in Fig. 3-6A. In addition to CSI, CSII, CSIII and CSIV, two other corticostatic peptides are present. These peptides were

then further purified separately using a HFBA solvent system which is shown in Fig. 3-6B and Fig. 3-7A. CSV reached homogeneity after a third RP-HPLC (Fig. 3-6C) but CSVI required a further three RP-HPLC purification steps (Fig. 3-7B to E). Purity was judged by both the shape of the UV absorbance curve and by amino acid analysis. Amino acid compositions from the last purification step are given in Table 3-3 and their primary structures and corticostatic activity compared to CSI is given in table 3-4 and Fig. 3-8. In terms of corticostatic activity, CSV is about 16 times, and CSVI is about 200 times less potent than the most potent peptide, CSI.

Table 3-3. Amino acid composition of Rabbit Corticostatins after hydrolysis.

	CORTICOSTATIC PEPTIDES		
AMINO ACID	CSI	CSV	CSVI
Aspartic acid	2.1 (0)*	1.1 (0)	1.2 (0)
Asparagine	(2)	(1)	(1)
Threonine		2.8 (3)	2.6 (3)
Serine	2.7 (3)	3.5 (4)	2.5 (3)
Glutamic acid	1.2 (0)	1.1 (0)	1.3 (0)
Glutamine	(1)	(1)	(1)
Proline	1.0 (1)		
Glycine	2.8 (3)	4.3 (4)	4.7 (5)
Alanine	2.2 (2)	1.1 (1)	1.1 (1)
Cysteine	5.2 (6)	4.9 (6)	5.4 (6)
Valine	1.9 (2)	2.9 (3)	1.8 (2)
Isoleucine	1.0 (1)		1.0 (1)
Leucine		1.0 (1)	2.0 (2)
Tyrosine	1.9 (2)		

Phenylalanine	1.9 (2)	1.8 (2)	1.7 (2)
Histidine		0.9 (1)	
Arginine	8.9 (9)	5.8 (6)	3.9 (4)

* Numbers in parentheses are calculated from sequence analysis data.

Table 3-4. Rabbit corticostatins and their $I.D._{50}$ for inhibition of ACTH induced corticosterone production in rat adrenal cell suspensions.

Peptide	Sequence	$I.D._{50}$ (nM)
CSI	G-I-C-A-C-R-R-R-F-C-P-N-S-E-R-F-S-G-Y-C-R-V-N-G-A-R-Y-V-R-C-C-S-R-R	27
CSV	V-S-C-T-C-R-R-F-S-C-G-F-G-E-R-A-S-G-S-C-T-V-N-G-V-R-H-T-L-C-C-R-R	650
CSVI	V-F-C-T-C-R-G-F-L-C-G-S-G-E-R-A-S-G-S-C-T-I-N-G-V-R-H-T-L-C-C-R-R	6000

3.1.3. Structure Activity Studies of Corticostatic peptides

After comparison of the structures of corticostatic peptides with the noncorticostatic defensins of the same family, we noticed that the absence of the C-terminal basic amino acid which was common to all known noncorticostatic defensins except the guinea pig corticostatic peptides GPCS1 and GPCS2 which have two N-terminal arginines instead of the usual C-terminal arginines. This observation suggested that these terminal arginines may play some biological roles in the determination of their corticostatic activity. In order to understand further the biological function of these terminal basic residues, the effect of removing either

Figure 3-6. Isolation of CB5. HPLC purification of bone marrow extracts from four rabbits. (A) The extract, prepared as described in the Methods section 2.2.1.2, & 2.2.2.1. was loaded onto a Waters C_{18} μBondapak reversed-phase column which was eluted using a linear gradient of 0 to 80% acetonitrile in 0.1% TFA over 100 min. One minute fractions were collected and submitted for amino acid analysis. (B) Fractions 30-35 from (A) were combined and applied onto the same column as above and eluted with a linear gradient of 25-55% acetonitrile in 0.13% HFBA. One minute fractions were collected and subjected to amino acid analysis. (C) Fractions 38 to 40 from (B) were loaded onto a C_{18} Vydac reversed-phase HPLC column and the column was eluted using a linear gradient of 0-27% acetonitrile in 0.1% TFA in water over 80 min. 1.5 ml fractions were collected.

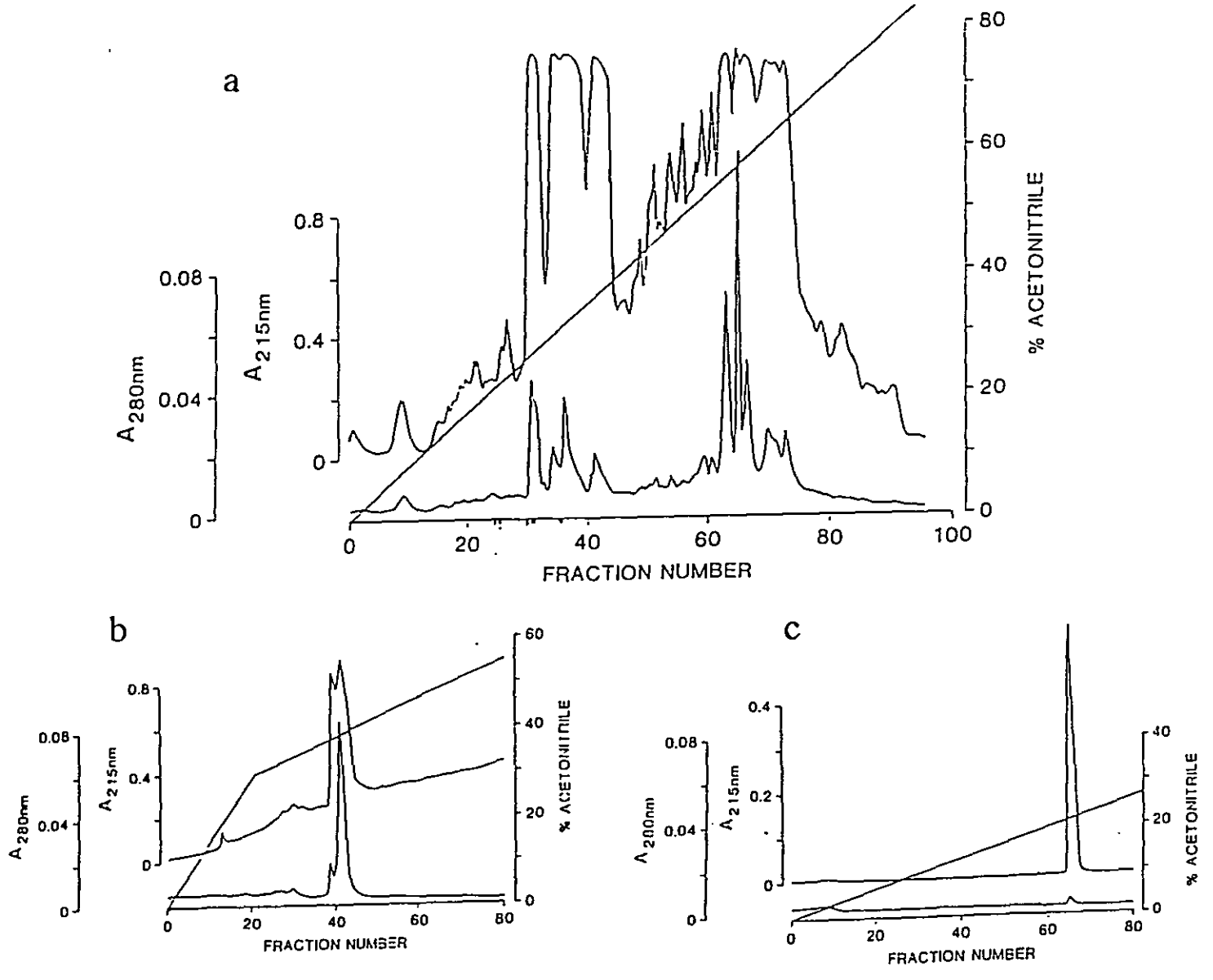

a
b
c
A_{280nm}
A_{215nm}
% ACETONITRILE
FRACTION NUMBER
0.08
0.04
0
0.8
0.4
0.2
80
60
40
20
0
100
20
40
60
80

Figure 3-7. Isolation of C86. (A) Fractions 36 to 45 from Fig.3-6(A) were combined and applied onto a Waters reversed-phase μBondapak column which was eluted using a linear gradient of 20 to 58% acetonitrile in 0.13% HFBA in water over 80 min. One minute fractions were collected and subjected to amino acid analysis. (B) Fractions 33 to 36 from (A) were subjected to another reversed-phase HPLC step using the same column eluted with a gradient of 0 to 33% acetonitrile in 0.1% TFA. One minute fractions were collected and subjected to amino acid analysis. (C) Fractions 57 to 73 of (B) were combined and applied onto the same column as above and eluted with a linear gradient of 30 to 55% of acetonitrile in 0.13% HFBA in water for 80 min. One minute fractions were collected and subjected to amino acid analysis. (D) Fractions 35 to 39 from (C) were subjected to another reversed-phase HPLC step using a Vydac column eluted using gradient of 10 to 28% acetonitrile in 0.1% TFA. One minute fractions were collected and subjected to amino acid analysis. (E) Fractions 42 to 44 of (D) were loaded onto the same Vydac column using a linear gradient 15-25% acetonitrile in 0.1% TFA over 50 min. 1.5 ml fractions were collected.

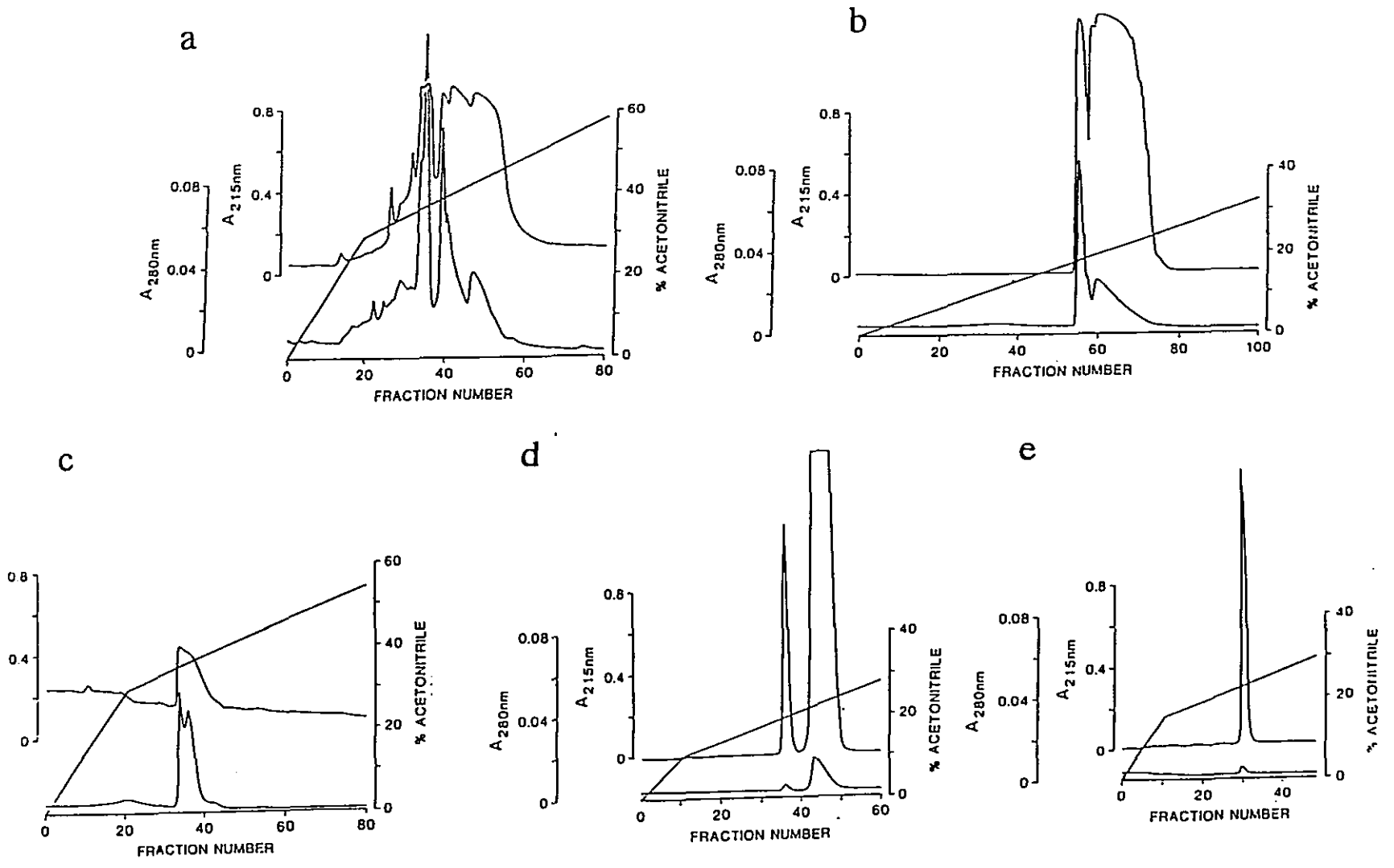
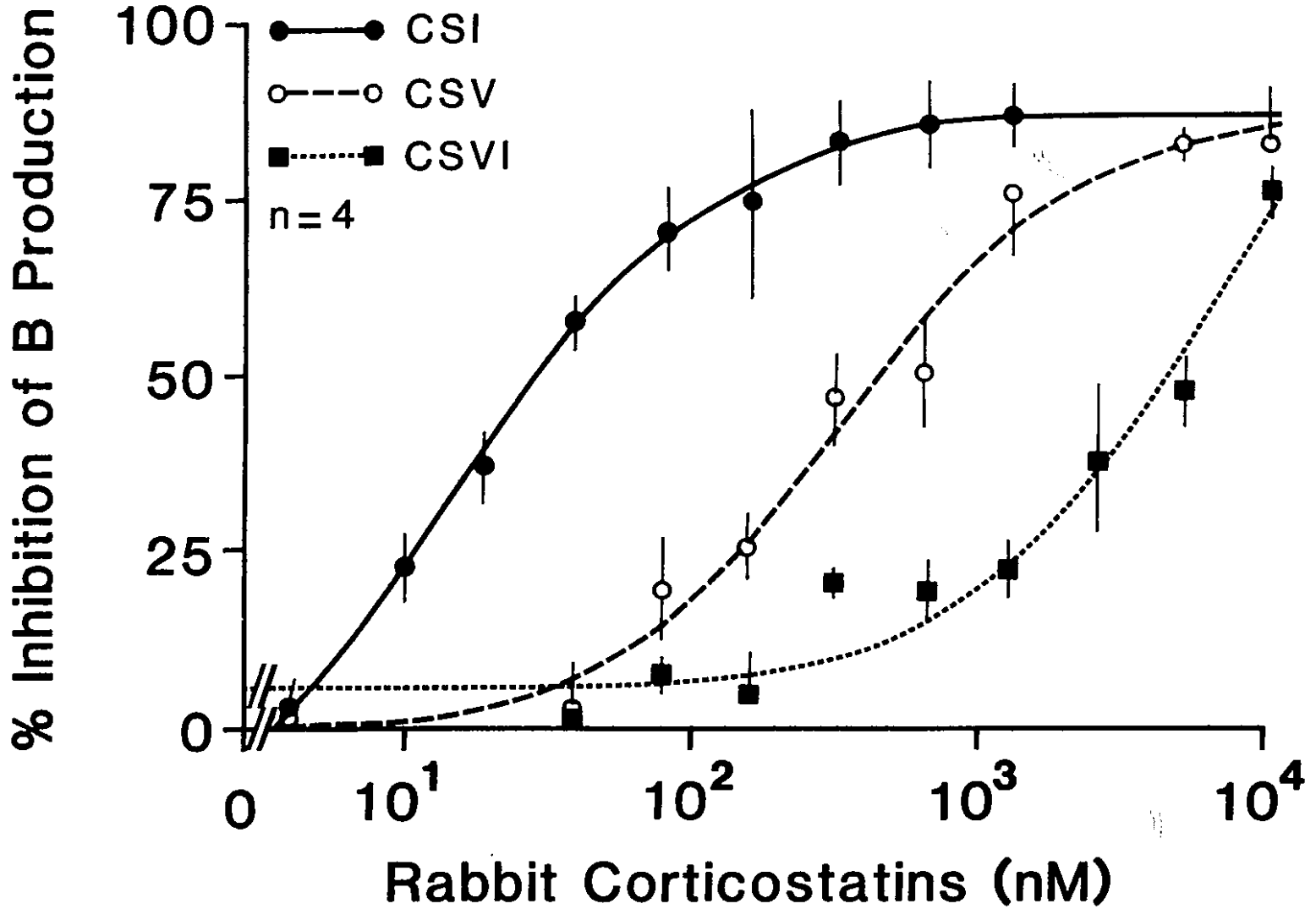

a
b
c
d
e
A_{280nm}
A_{215nm}
% ACETONITRILE
FRACTION NUMBER

Figure 3-8. A comparison of the corticostatic activity of rabbit CSI, CSV and CSVI. A total of 33 pM (150pg/ml) of ACTH was used to stimulate isolated rat adrenal cells at a concentration of 200,000 cells /ml, in the presence of increasing concentrations of rabbit CSI(solid circles), CSV(open circles) and CSVI (solid squares). Corticosterone was measured by RIA. Values shown are the mean ± standard deviation of the mean of four separate experiments.


% Inhibition of B Production
100
75
50
25
0
CSI
CSV
CSVI
n = 4
0
10^1
10^2
10^3
10^4
Rabbit Corticostatins (nM)

the carboxy- or amino-terminal arginine(s) from GPCS and CSI was examined.

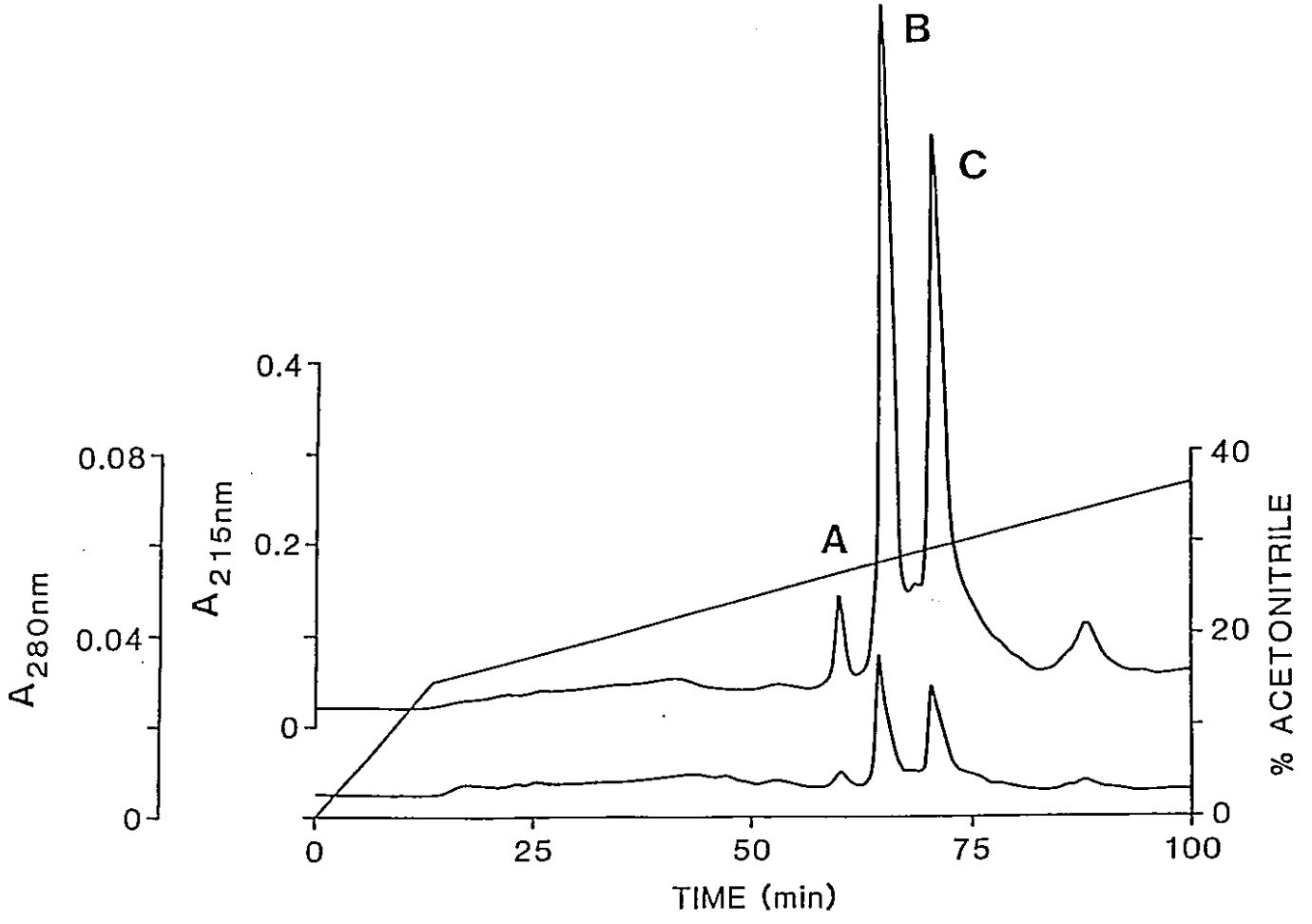
3.1.3.1. *Removal of the two arginines from the N-terminal of GPCS1 & GPCS2*

Using a mixture of GPCS1 and GPCS2 as starting material, analogues of guinea pig corticostatin were prepared by sequential Edman degradation as described in section 2.3.4. In the Fig. 3-9, the upper panel shows the RP-HPLC profile of the UV absorbance at 215 and 280 nm for the mixture of GPCS1 and GPCS2. The structures of GPCS1 & GPCS2 and their analogues are shown on the lower panel. In contrast to what we expected, the removal of one or both of the amino-terminal arginines in this manner generated peptides whose corticostatic activity differed little from the guinea pig corticostatins themselves (ID_{50} approximately 200 nM) as shown in Fig. 3-10.

3.1.3.2. *Removal of the two arginines from the C-terminal of CSI*

About 50 nM of CSI was subjected to carboxypeptidase B digestion as described in the methods, section 2.3.3.2. The fragments were isolated by RP-HPLC which are shown in the upper panel of Fig. 3-11, and a one-fiftieth aliquot analyzed by amino acid analysis and ion-spray mass spectroscopy. Fig. 3-11 shows the structure of CSI and its analogues. After removal of one or both of the carboxyl-terminal arginines, these resulting

Figure 3-9. HPLC profile of a two cycle Edman degradation of GPCS1 and GPCS2. Approximately 100nM of a mixture GPCS1 and GPCS2 were prepared for two cycles of Edman degradation. At the end of the two cycles, 2 ml of 1% TFA was added to lower the pH value to about 4, prior to purification by reversed-phase HPLC using a gradient of 0-15% acetonitrile in 0.1% TFA for the first 15 min, followed by a linear gradient to 38% acetonitrile in 0.1% TFA over the next 85 min. The flow rate was 1.5 ml/min. The upper panel shows UV absorbance at 215 and 280 nm for the mixture of GPCS1 and GPCS2. The structures of A, B and C were confirmed by both amino acid analysis and ion-spray mass spectrometry and are shown in the lower panel.



A GPC1&2 RRCICTTRTCRFPYRRLGTCI(L)FQNRVTFCC
B GPC1&2 RCICTTRTCRFPYRRLGTCI(L)FQNRVTFCC
C GPC1&2 CICTTRTCRFPYRRLGTCI(L)FQNRVTFCC

Figure 3-10. A comparison of the corticostatic activity of GPCS1 & GPCS2, GPCS1 & GPCS2-R and GPCS1 & GPCS2-RR. A total of 33 pM (150 pg/ml) of ACTH was used to stimulate the isolated rat adrenal cells used at a concentration of 200,000 cells /ml, in the presence of increasing concentration of GPCS1 & GPCS2(solid circles), GPCS1 & GPCS2-R(open circles) and GPCS1 & GPCS2-RR(solid squares). The level of corticosterone was measured by RIA. Values are the mean ± standard deviation of mean of four separate experiments.

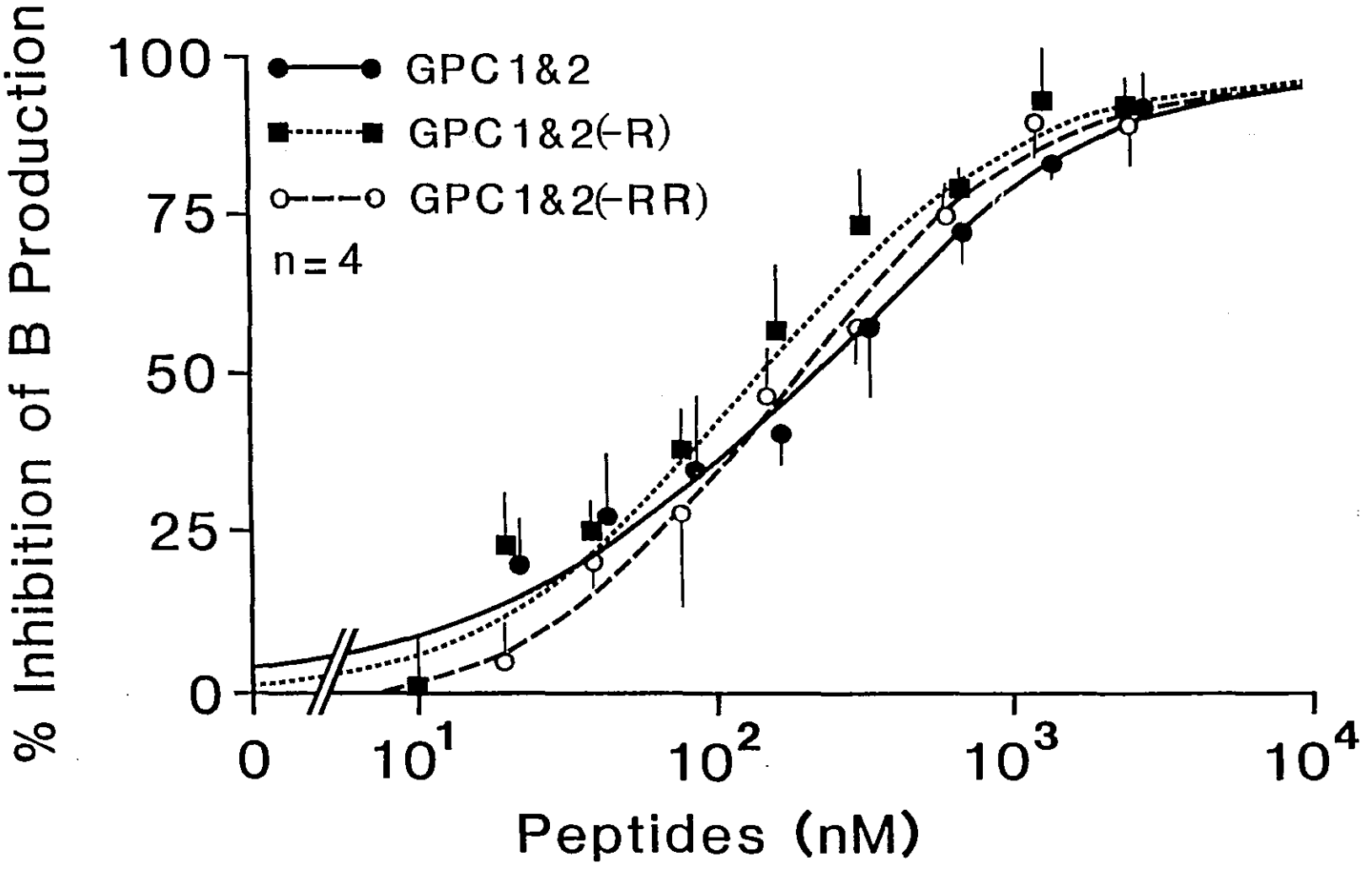
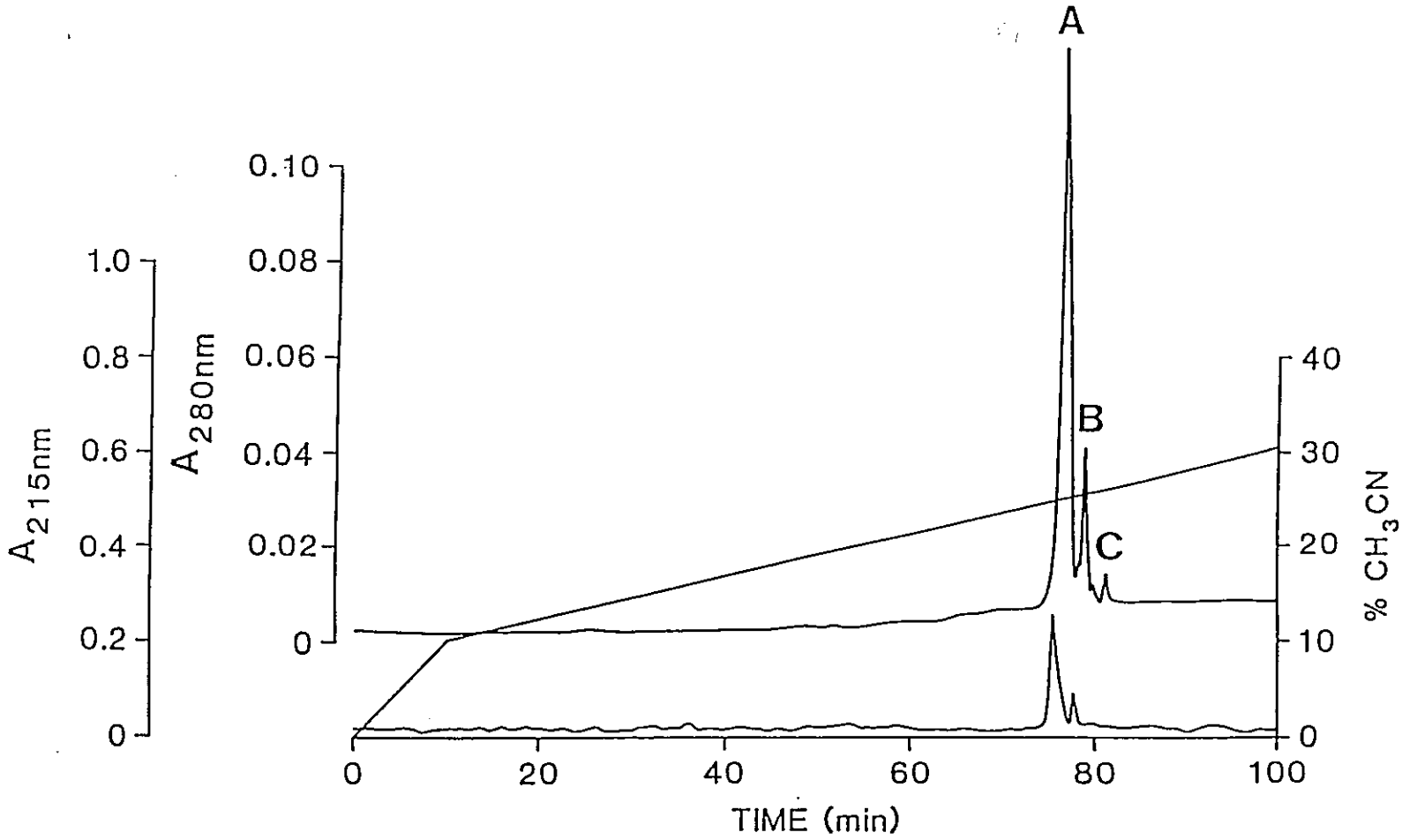

% Inhibition of B Production
100
75
50
25
0
GPC1&2
GPC1&2(-R)
GPC1&2(-RR)
n=4
0
10^1
10^2
10^3
10^4
Peptides (nM)

Figure 3-11. Digestion maps of CSI with Carboxypeptidase B. A total of 50 nM of CSI was digested with carboxypeptidase B as described in the Methods (section 2.3.3.2.). At the end of the incubation 1 ml of 1% TFA was added to lower the pH to approximately 3 prior to purification by reversed-phase HPLC using 0-10% acetonitrile for the first 10 minutes, followed by a linear gradient to 30% acetonitrile in 0.1% TFA in water over the next 90 minutes. The upper panel shows UV absorbance at 215 nm and 280 nm for CSI and analogues. The structure of CSI digestion products A,B and C were confirmed by both amino acid analysis and ion-spray mass spectrometry and are shown in the lower panel.



A	CSI	G-I-C-A-C-R-R-R-F-C-P-N-S-E-R-F-S-G-Y-C-V-N-G-A-R-Y-
B	CSI-(R)	G-I-C-A-C-R-R-R-F-C-P-N-S-E-R-F-S-G-Y-C-V-N-G-A-R-Y-
C	CSI-(RR)	G-I-C-A-C-R-R-R-F-C-P-N-S-E-R-F-S-G-Y-C-V-N-G-A-R-Y-
	CSI	V-R-C-C-S-R-R
	CSI-(R)	V-R-C-C-S-R
	CSI-(RR)	V-R-C-C-S

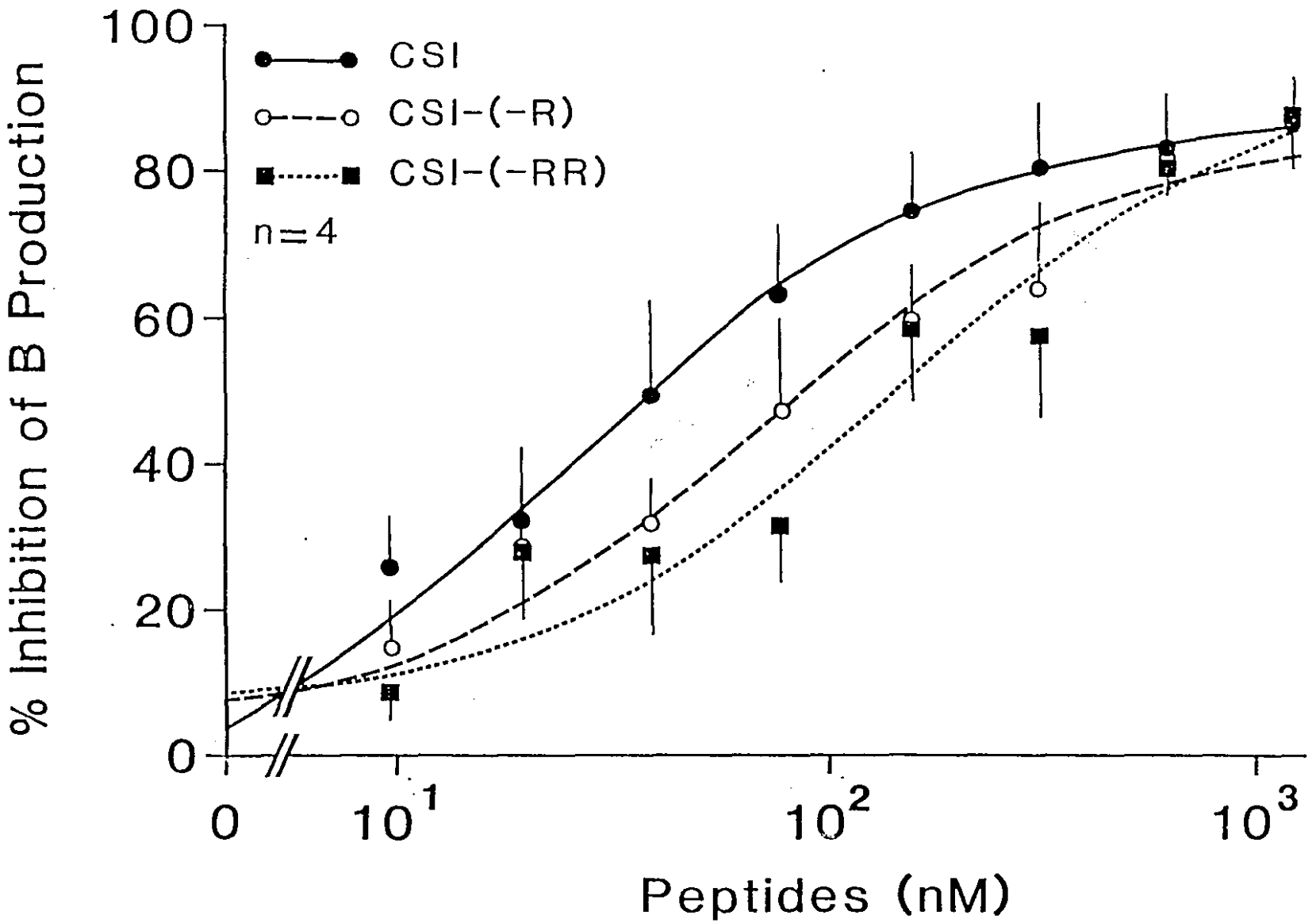
truncated peptides were still able to inhibit ACTH action in the rat adrenal bioassay but to a lesser extent (Fig. 3-12). The peptide lacking one arginine is about 1.7 times less potent than the intact CSI, and the one lacking two arginines is about 2.5 times less potent compared to CSI. These results will be further discussed in the last chapter of this thesis.

3.2. Distribution and Quantification of CSI in Rabbit Tissues

As mentioned previously, corticostatin(s) are peptides which inhibit ACTH-stimulated glucocorticoid synthesis in vitro and belong to the corticostatin/defensin family, which possess broad antimicrobial activities. Although defensins are reported to be found in mammalian phagocytic cells, i.e., lung macrophages, bone marrow cells and small intestinal mucosa, little is known as to how they exist in the organs which directly or indirectly regulate adrenal glucocorticoid synthesis. To obtain more information on the distribution of CSI in immunocell rich tissues and in the hypothalamic-pituitary-adrenal axis, the following experimental protocol was developed.

Tissue extraction
(1M HCl, 5% Formic acid, 1% NaCl, 1% TFA)
↓
C_{18} Sep-Pak
↓
RP-HPLC
(linear gradient, TFA solvent system)
↓
RIA for CSI

Figure 3-12. A comparison of the corticostatic activity of CSI, CSI-R,CSI-RR. A total of 33 pM (150 pg/ml) of ACTH was used to stimulate isolated rat adrenal cells at concentration of 200,000 cells/ml, in the presence of increasing concentrations of CSI(solid circles), CSI-R(open circles) and CSI-RR(solid squares). The concentration of corticosterone was measured by RIA. Values are the mean ± standard deviation of the mean of four separate experiments.


100
80
60
40
20
0
% Inhibition of B Production
CSI
CSI-(-R)
CSI-(-RR)
n=4
0
10^1
10^2
10^3
Peptides (nM)

CHAPTER 3 RESULTS

3.2.1. The specificity of CSI antisera

A guinea pig polyclonal antiserum was raised against a synthetic CSI as detailed in section 2.4.1.2.. Western blots of CSI in extracts of several tissues demonstrated a 4-KDa CSI band in spleen, lung, adrenal, and intestine, but not in liver (Fig. 3-13A). The immuno cross reactivity of the antiserum with non-rabbit corticostatins (R-4 and HP-4) as well as ACTH shows lack of activity. In addition, HP-1, R1, R3, ANF, and αMSH shows no cross reactivity. The cross reactivity with other rabbit corticostatins (CSII, CSIII and CSIV) is less than 1%. The specificity of the antiserum is shown in Fig. 3-13A & B.

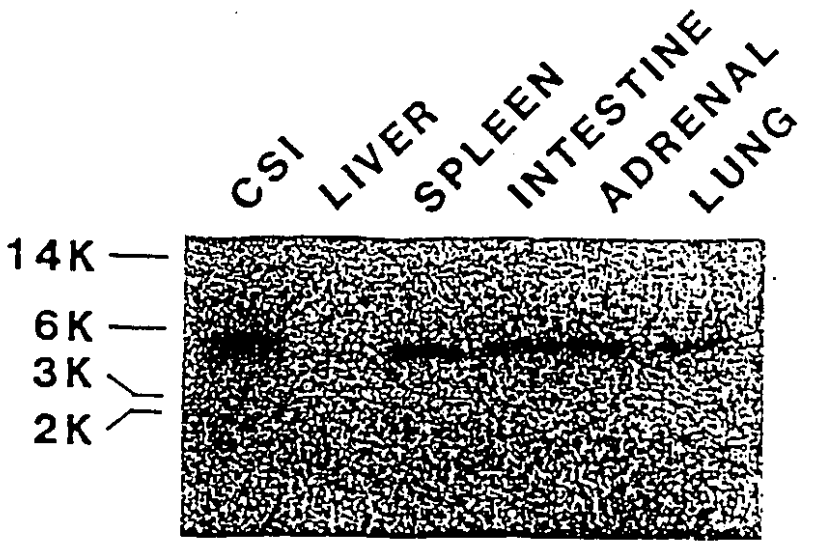
The sensitivity of the RIA for CSI is from 6.25 fmoles to 1.25 pmoles. The inter- and intra-assay coefficient of variations of the RIA for CSI are 13.3 ± 6.5% and 5.1 ± 3.3% respectively.

3.2.2. CSI Level in Normal Female Rabbit Tissues

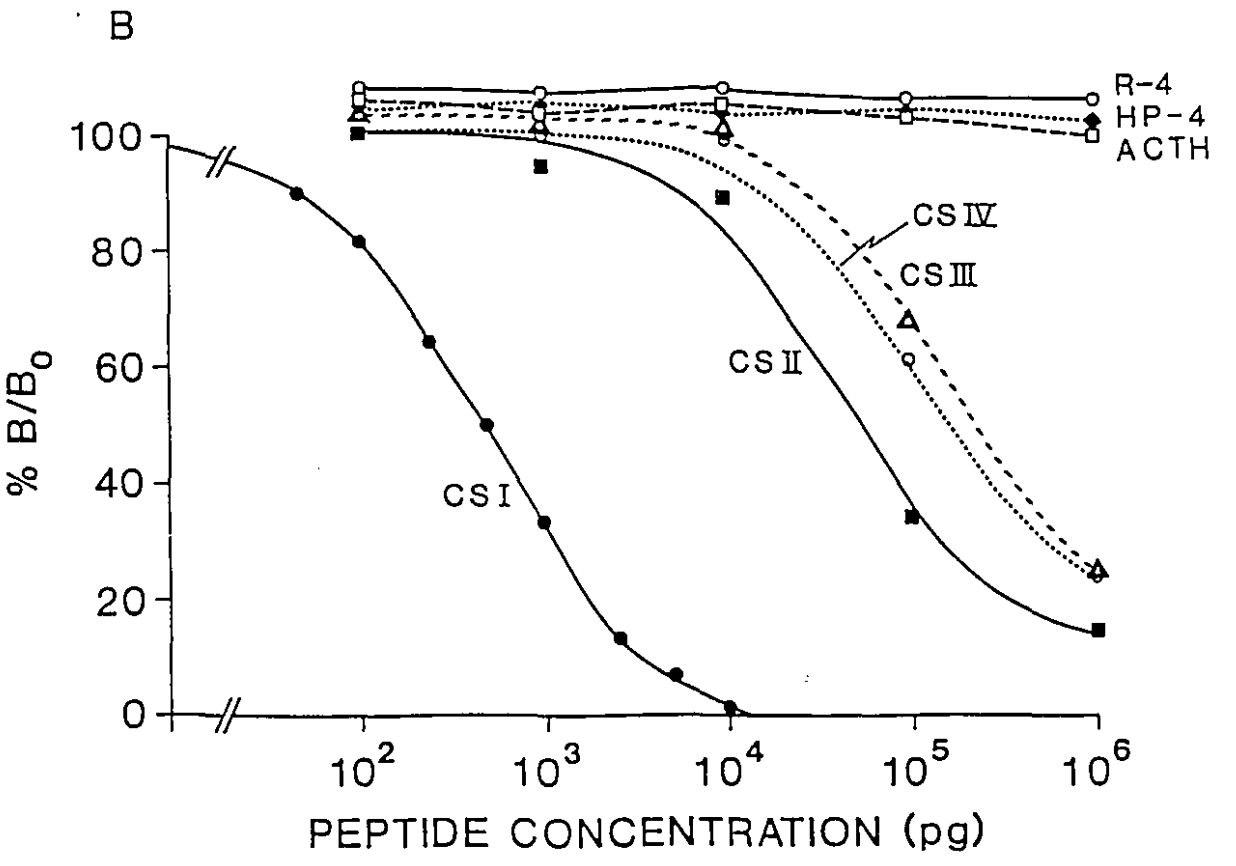
The amount of CSI in different organs of normal female rabbits was quantitated by RIA after one step of RP-HPLC purification. The results are shown in Fig. 3-14. Lung contained the highest amount of CSI (4130±720 ng/mg DNA, N=3) among the tissues tested and small amount of the order of 23±4 ng/mg DNA (N=4) were present in the adrenal glands. In the small intestine, the level of CSI was about 97±4 ng/mg DNA (N=3). From the results of the Western blot studies and RIA, the level of CSI was found not to be detectable in liver and kidney (Fig.3-13 & 14). Fig. 3-

Figure 3-13. The specificity of CSI antisera. (A) Western blot of CSI using polyclonal antisera against CSI. Autoradiograph of the blots of purified CSI from rabbit bone marrow (marked CSI) and tissues extracts from lung, spleen, adrenal, intestine and liver with anti-CSI antisera shows a single 4-KD band except in liver where there is no positive signal. (B) Standard curve for CSI-specific radioimmunoassay and the immuno cross reactivity of the antisera with a number of rabbit corticostatins and other related peptides. B/B_0 represents the bound over free ratio.

A


CSI
LIVER
SPLEEN
INTESTINE
ADRENAL
LUNG
14K
6K
3K
2K

B


R-4
HP-4
ACTH
CS Ⅳ
CS Ⅲ
CS Ⅱ
CS I
100
80
60
40
20
0
% B/B0
10^2
10^3
10^4
10^5
10^6
PEPTIDE CONCENTRATION (pg)

14 shows that spleen contains large amounts of CSI, there are several CSI immunoreative peaks with different retention times. Further purification of these fractions, revealed that CSI could form complexes with other components from spleen observed under the condition of the first HPLC but most of these peaks dissociated in later purification steps and only CSI itself could be observed.

Distribution of CSI in the normal rabbit brain was also studied by RIA. Surprisingly the pituitary contains a much higher amount of CSI (1100±120 ng/mg DNA, N=5) followed by hypothalamus (308± 18ng/mg DNA, N=5) compared to other parts of brain, as shown in Fig. 3-15. CSI was undetectable in other parts of brain such as pons oblongata, thalamus cerebellum, cerebrum and corpus collosum (Fig. 3-16 and Fig. 3-17).

3.2.3. CSI Levels in Fetal and Maternal Rabbit Tissues at Days 24, 27 and 30 of Gestation

In mammals, parturition is under the control of neural and endocrine factors. The available evidence shows that the rise in fetal glucocorticoids acts as a trigger for the onset of parturition. As mentioned previously CSI is the inhibitor of ACTH-stimulated release of glucocorticoids. We therefore hypothesized that CSI might play an important role in controlling

Figure 3-14. Purification and quantification of CSI in normal rabbit tissues. Lung, adrenal, intestine, spleen, kidney and liver were extracted using the acidic extraction medium method, and the extracts were first purified by one step RP-HPLC and then quantified by a specific RIA as described in the methods (section 2.4.1.4.).

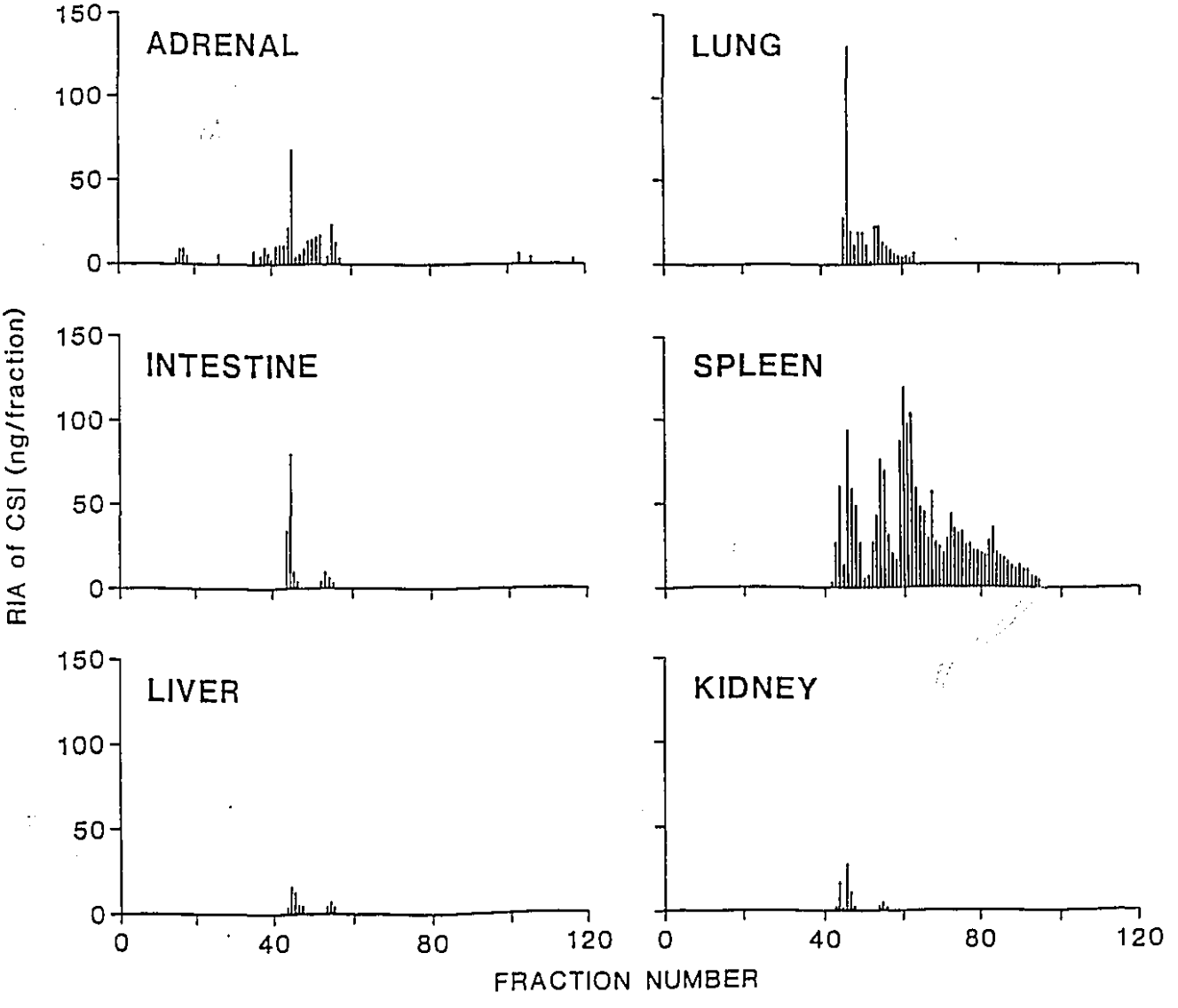

ADRENAL
LUNG
INTESTINE
SPLEEN
LIVER
KIDNEY
150
100
50
0
0
40
80
120
RIA of CSI (ng/fraction)
FRACTION NUMBER

Figure 3-15. Purification and quantitation of CSI in normal rabbit hypothalamus and pituitary. The profiles of the HPLC in the upper panels and the RIA in the lower panels using the same methods as described in the legend of Fig.3-14.

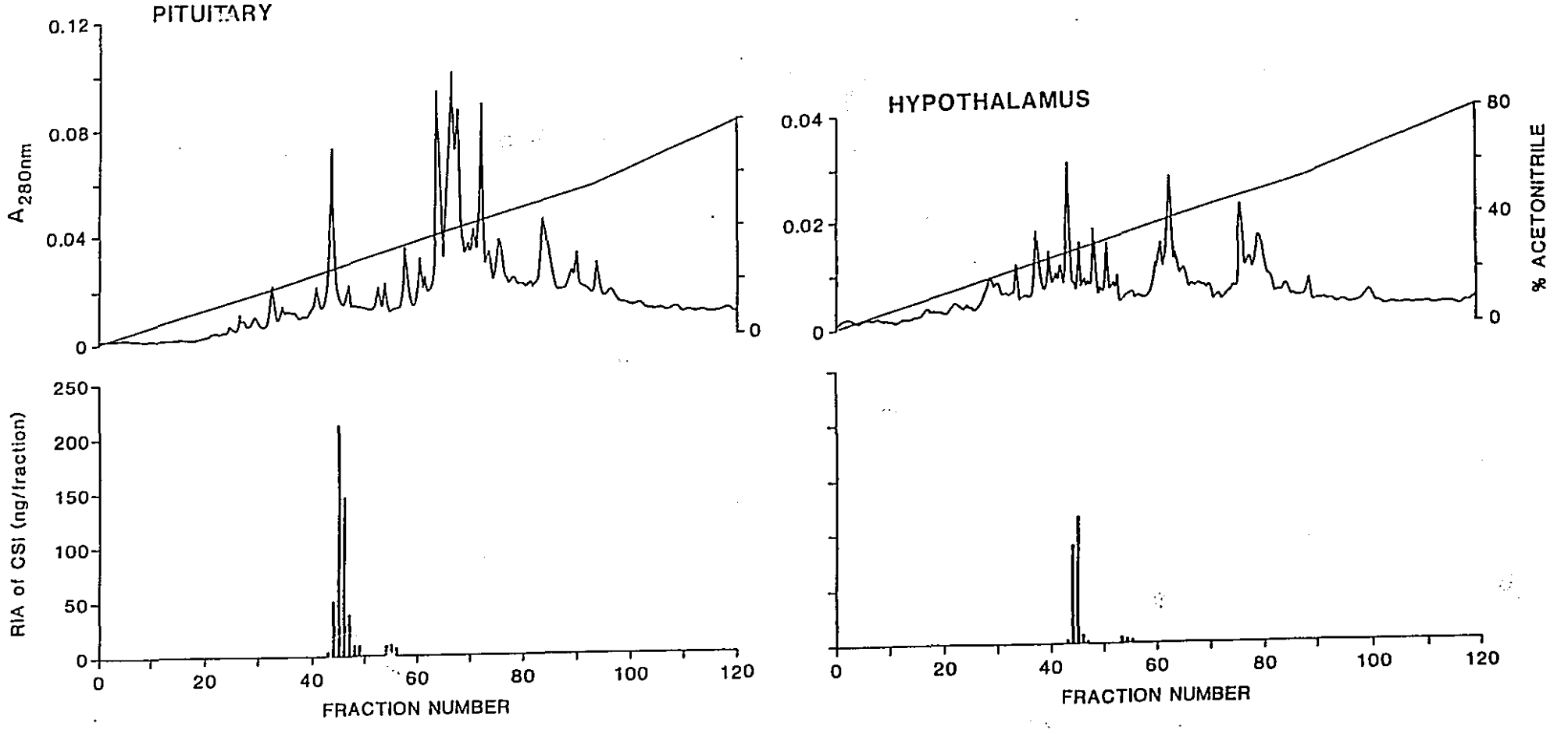

PITUITARY
HYPOTHALAMUS
A_{280nm}
% ACETONITRILE
RIA of CSI (ng/fraction)
FRACTION NUMBER
FRACTION NUMBER
0.12
0.08
0.04
0
0.04
0.02
0
80
40
0
250
200
150
100
50
0
0
20
40
60
80
100
120

Figure 3-16. CSI in fetal, maternal and normal female rabbit brain tissues. The tissues of pituitary, hypothalamus, pons oblongata, and thalamus on day 24, 27 and 30 of gestation are examined using the same methods as described in the legend of Fig.3-14. Vertical bars represent the standard error of the mean of five seperate experiments.

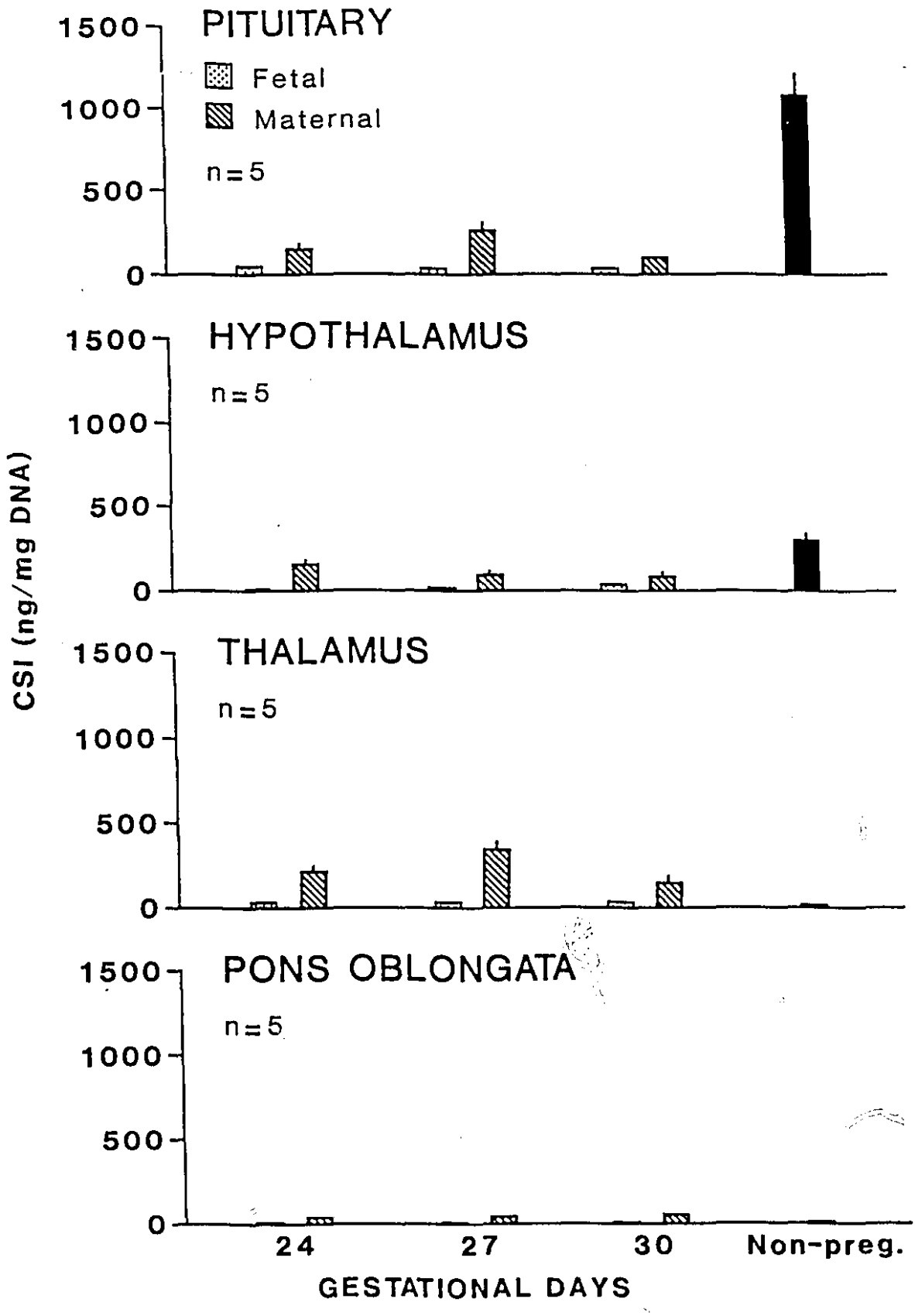
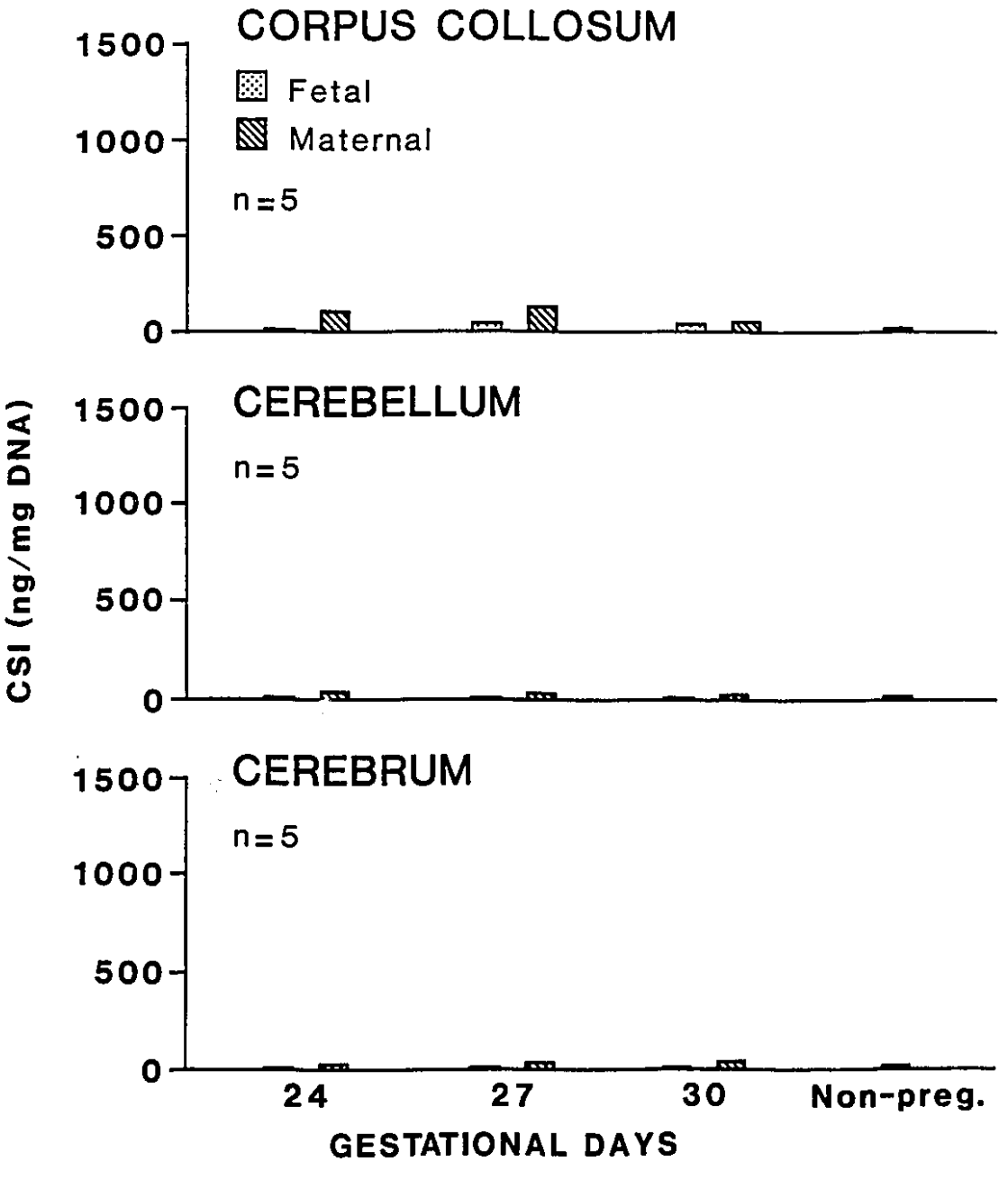

PITUITARY
Fetal
Maternal
n=5
HYPOTHALAMUS
n=5
THALAMUS
n=5
PONS OBLONGATA
n=5
1500
1000
500
0
CSI (ng/mg DNA)
24
27
30
Non-preg.
GESTATIONAL DAYS

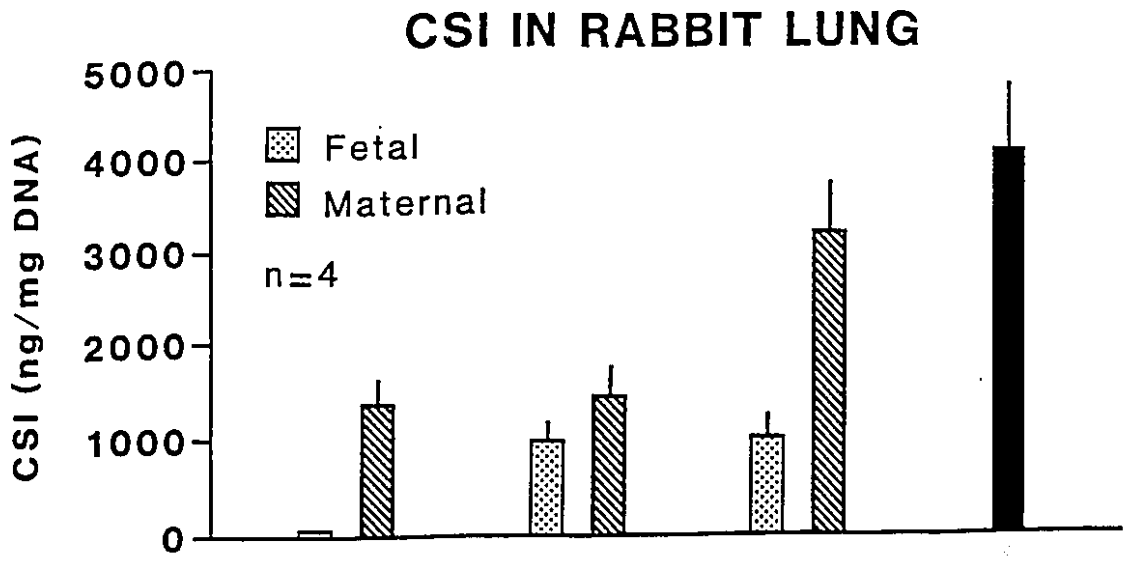
Figure 3-17. CSI in fetal, maternal and normal female rabbit brain tissues. The tissues of cerebellum, cerebrum and corpus callosum on day 24, 27 and 30 of gestation are examined using the same methods as described in the legend of Fig.3-14. Vertical bars represent the standard error of the mean of five separate experiments.


CORPUS COLLOSUM
Fetal
Maternal
n=5
1500
1000
500
0
CEREBELLUM
n=5
CEREBRUM
n=5
CSI (ng/mg DNA)
24
27
30
Non-preg.
GESTATIONAL DAYS

the fetal adrenal in late gestation. To test this hypothesis we first determined the blood and tissue levels of CSI in the pregnant rabbit. Using an acidic extraction medium, RP-HPLC and a specific RIA, the levels of CSI were determined in brain, lung, adrenal, placenta and plasma on days 24, 27 and 30 of gestation in the pregnant rabbit.

The distribution of CSI in different regions of fetal and maternal brain was measured by RIA. Fig. 3-16 and Fig. 3-17 show that during gestation, the pituitary of both fetus and mother contained the higher amount of CSI as compared to the hypothalamus. The levels of CSI in the maternal thalamus were much higher on day 27 of gestation compared with the nonpregnant rabbit. Furthermore normal female rabbit pituitary contained the highest amount of CSI compared with other parts of the brain in both the non-pregnant and pregnant states. A large amount of CSI has been found in both maternal and fetal lungs (Fig. 3-18 upper panel), and increase from 40 to 1100 ng/mg DNA in the fetuses and from 1400 to 3200 ng/mg DNA in mothers on gestational days 24 to 30 respectively. The lower panel of Fig. 3-18 shows that the amounts of CSI in the maternal adrenal were much lower compared with fetal adrenal during gestational days 24 to 30. In the fetal adrenal the levels decreased from 1800 to 400 ng/mg DNA, and in maternal adrenal gland the levels also decreased from 100 to 40 ng/mg DNA. Fig. 3-19 shows there are large amounts of CSI also present in the placenta. The level changes from 500 to 900 ng/mg

Figure 3-18. Quantification of CSI in adrenal and lung. The tissues were examined using the same method described in the legend of Fig.3-14. Each bar represents the mean of CSI concentrations detemined in these tissues from four (N=4) pregnant rabbits. Fetal tissues from the same pregnant rabbit were pooled to obtain one determination value.


CSI IN RABBIT LUNG
CSI (ng/mg DNA)
5000
4000
3000
2000
1000
0
Fetal
Maternal
n=4

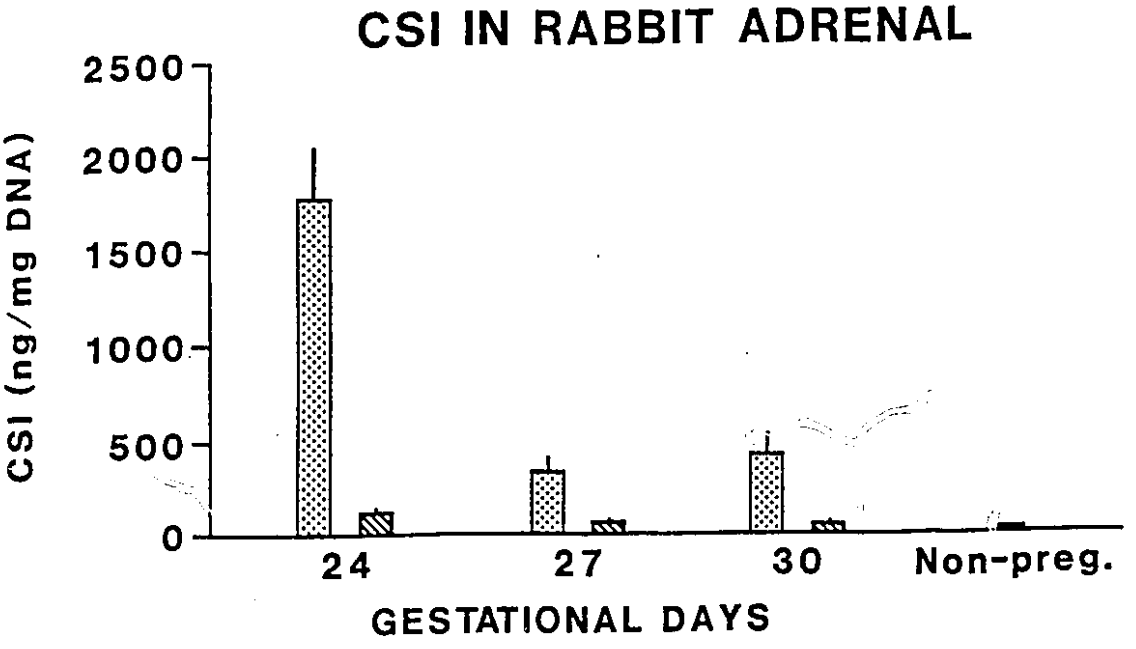
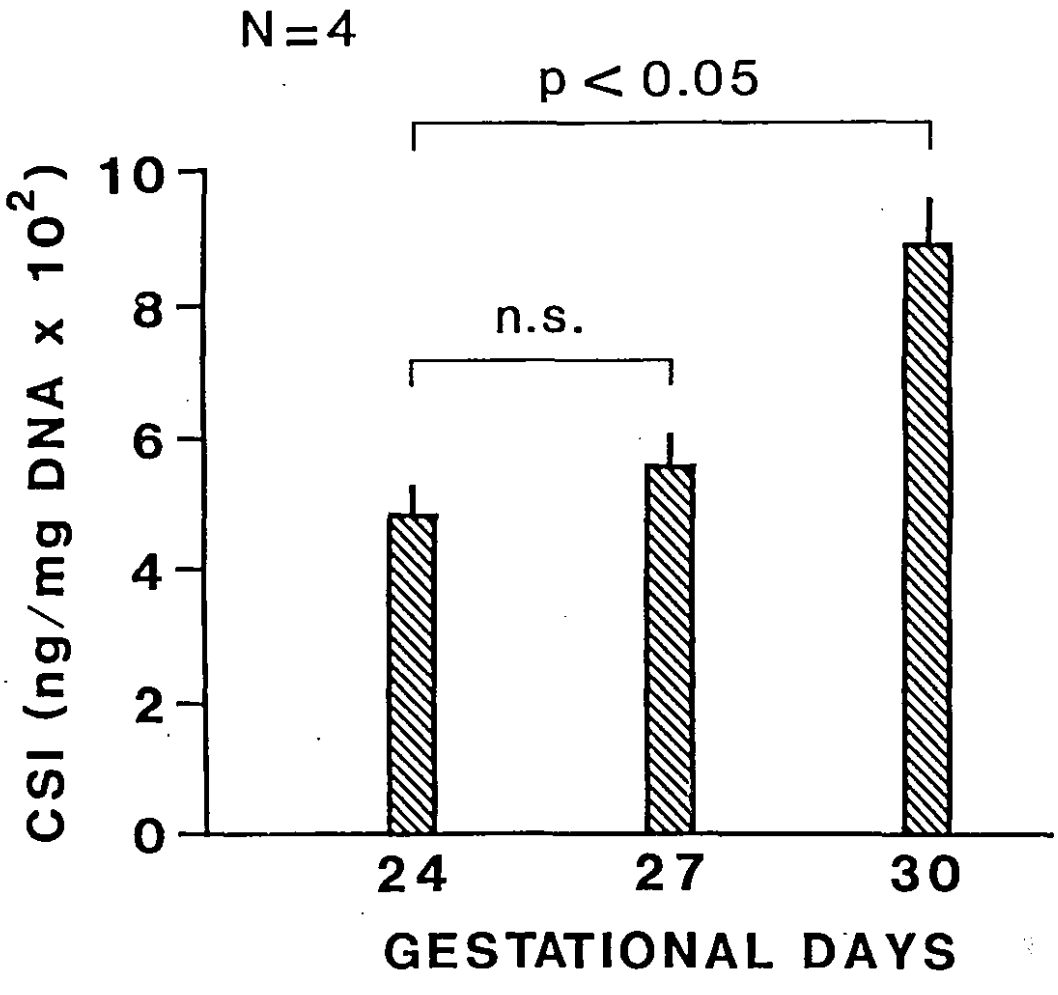

CSI IN RABBIT ADRENAL
CSI (ng/mg DNA)
2500
2000
1500
1000
500
0
24
27
30
Non-preg.
GESTATIONAL DAYS

Figure 3-13. Quantitation of CSI in placenta. The tissues were examined using the same method described in the legend of Fig.3-14. Each bar represents the mean of CSI concentrations detemined in these tissues from four (N=4) pregnant rabbits. Fetal tissues from the same pregnant rabbit were pooled to obtain one determination value.

CSI IN RABBIT PLACENTA



DNA on gestational days 24 to 30.

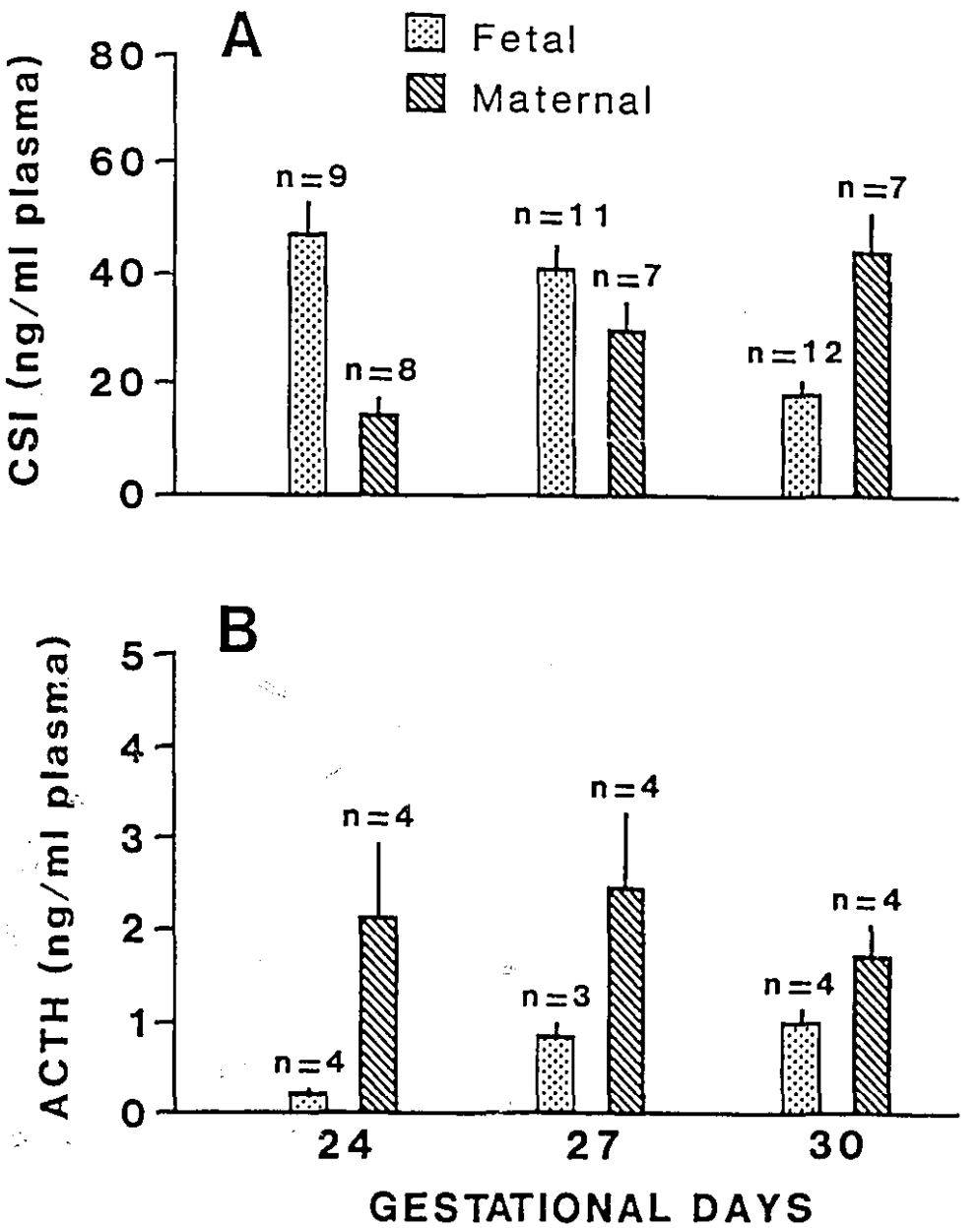
3.2.4. ACTH and CSI Levels in Fetal and maternal Rabbit Plasma at Days 24, 27 and 30 of Gestation

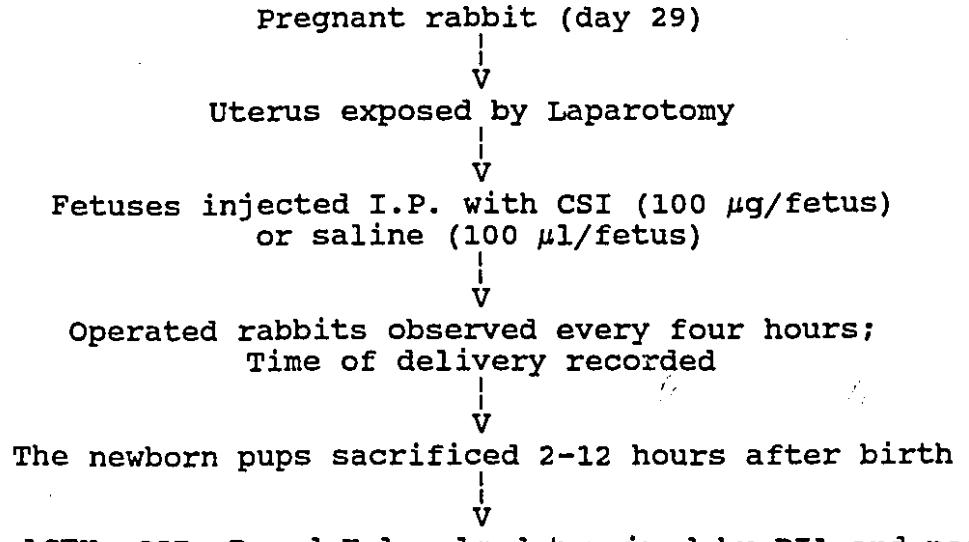
The concentration of CSI in plasma of nonpregnant female rabbits was 40 ng/ml, but was 15±2.9, 30±4.9, 45±6.7 ng/ml on gestational days 24, 27 and 30 in maternal plasma and 48±5.1, 41±4.5, 18±2.4 ng/ml in fetal plasma respectively (Fig. 3-20A). In contrast, maternal ACTH remains constant throughout this period but there is a four-fold increase in fetal plasma ACTH (Fig. 3-20B). The rise in the CSI in the maternal compartment and the fall in the fetal circulation may explain the increase in concentration of B and F prior to parturition in the rabbit.

3.3. Study In Vivo Effects of CSI on Length of Gestation

Based on the results shown above (see section 3.2.), we then tried to determine whether exogenous administration of CSI delayed the onset of parturition and conversely administration of CSI antiserum accelerated parturition. To accomplish this, 100 μg/0.1 ml/ fetus of CSI was injected into each fetus via the I.P. route under sterile conditions at day 29 of gestation. The experimental protocol used is as follows:

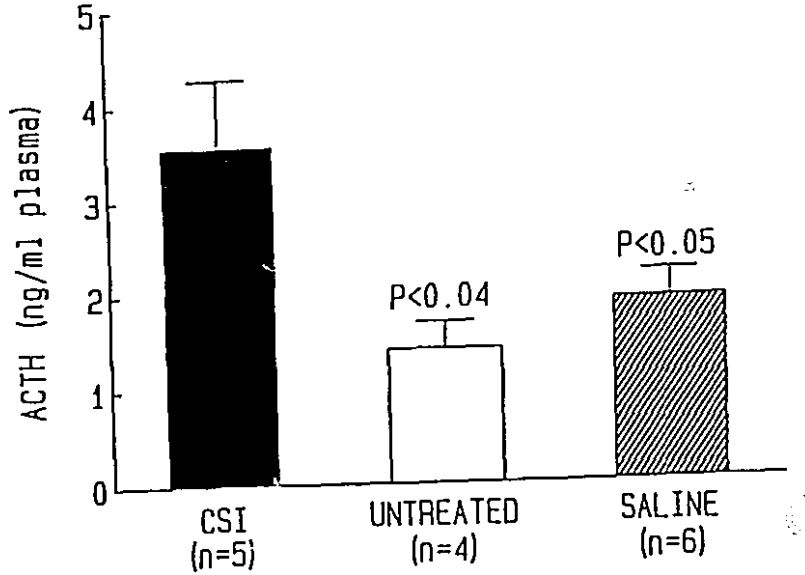
Figure 3-20. Quantification of CSI and ACTH in fetal and maternal plasma at gestational days 24, 27 and 30. The levels of CSI were measured using the same methods as described in Fig.3-14. The ACTH levels were examined by RIA as described in the methods (section 2.4.1.4.). The student's t-test was used for determining statistical significance between two means. N on the top of each bar represents the number of pregnant rabbits used in that group.

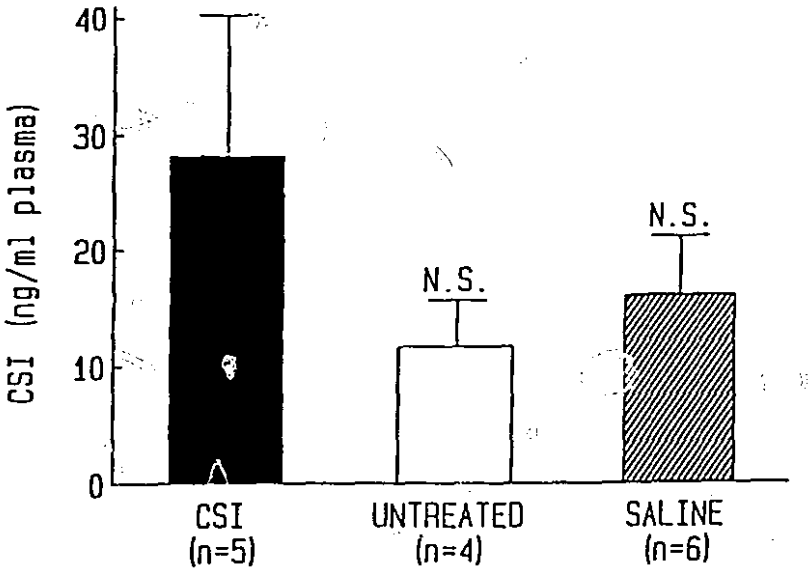

A
Fetal
Maternal
CSI (ng/ml plasma)
80
60
40
20
0
n=9
n=8
n=11
n=7
n=12
n=7
B
ACTH (ng/ml plasma)
5
4
3
2
1
0
n=4
n=4
n=3
n=4
n=4
n=4
24
27
30
GESTATIONAL DAYS

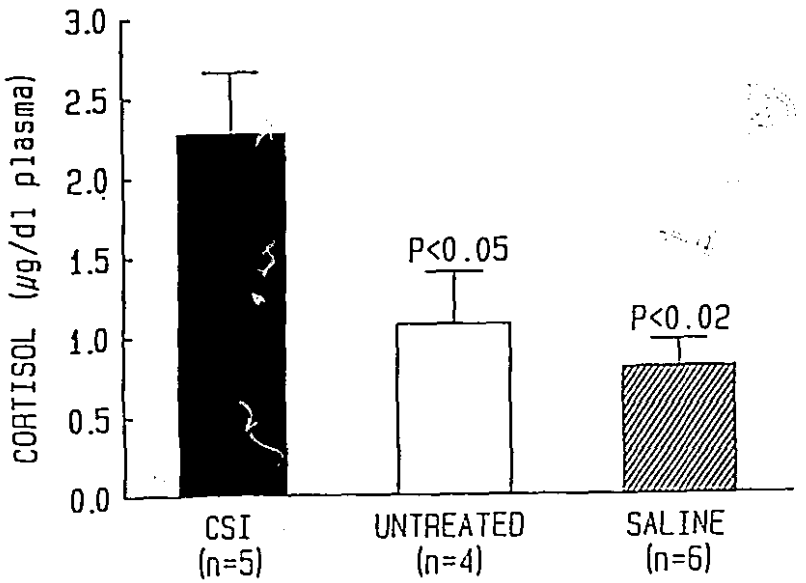


The data obtained indicated that there was no delay in the length of parturition (Table 3-5), however, in the plasma the levels of ACTH and F both increased but CSI and B remained the same in newborns after injection of CSI (Fig. 3-21). In the same experiments, we found no difference in the levels of CSI in the lung but the levels of CSI increased in the adrenal (Fig. 3-22). With another group of fetuses 100 μl of CSI antiserum at a final dilution of 1:5 was also injected I.P. at 29 days of gestation. The effects of CSI antiserum on the level of ACTH, CSI, B and F were measured in the plasma of the newborn. Similarly no acceleration occurred in parturition and also there was no significant difference in the levels of ACTH, CSI, B and F after injection of CSI antiserum (Fig. 3-23).

Figure 3-21. ACTH, CSI, B and F levels in plasma from pups injected with CSI in utero. Groups of fetuses were injected with CSI (solid bar, five pregnant rabbits), with saline (hatch bar, five pregnant rabbits), and untreated (open bar, three pregnat rabbits) controls at gestational day 29. Plasma from two to three pups were pooled using for one determination value. The standard error (N > 10) of the mean is shown (vertical bar). All hormones were measured by RIA. The student's t-test was used for determining statistical significance between two means.


ACTH (ng/ml plasma)
5
4
3
2
1
0
P<0.04
P<0.05
CSI (n=5)
UNTREATED (n=4)
SALINE (n=6)


CSI (ng/ml plasma)
40
30
20
10
0
N.S.
N.S.
CSI (n=5)
UNTREATED (n=4)
SALINE (n=6)


CORTISOL (µg/dl plasma)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
P<0.05
P<0.02
CSI (n=5)
UNTREATED (n=4)
SALINE (n=6)

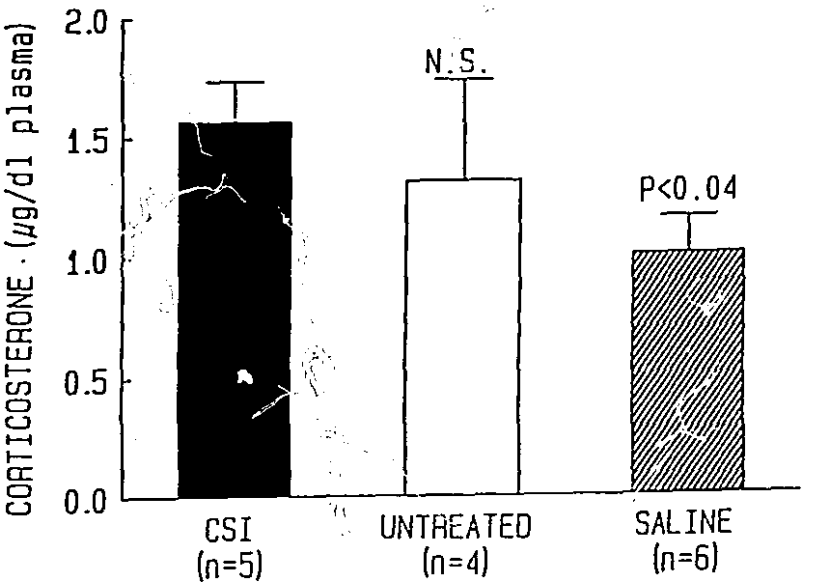
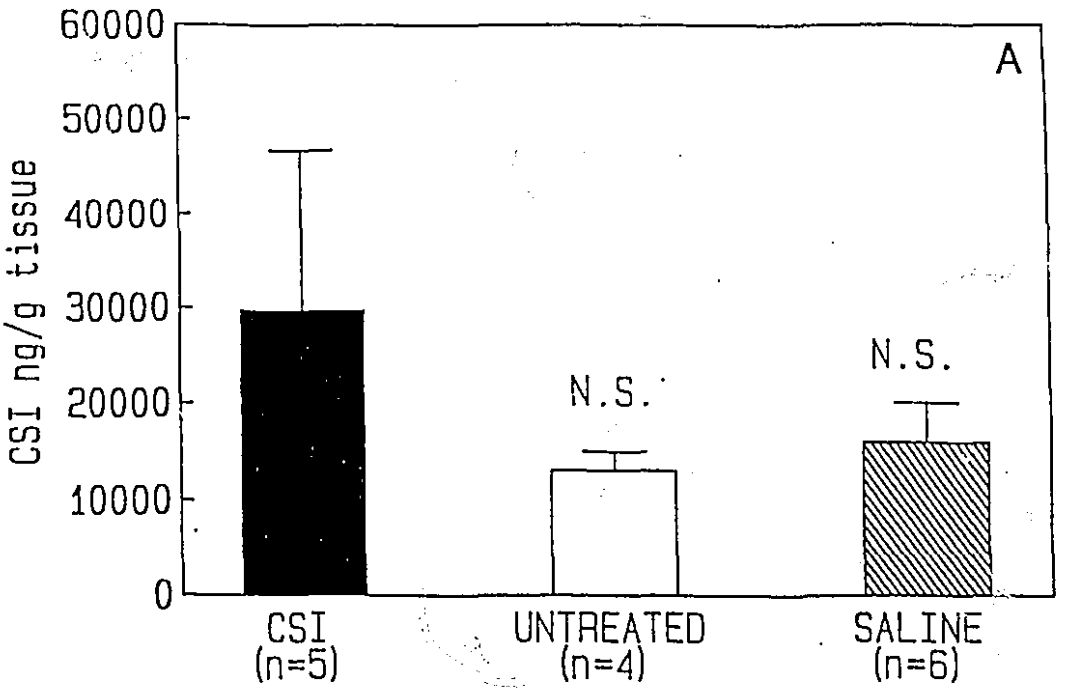

CORTICOSTERONE (µg/dl plasma)
2.0
1.5
1.0
0.5
0.0
N.S.
P<0.04
CSI (n=5)
UNTREATED (n=4)
SALINE (n=6)

Figure 3-22. CSI levels in lungs (A) and adrenals (B) from pups injected with CSI in utero. Groups of fetuses were injected with CSI (solid bar, five pregnant rabbits), with saline (hatch bar, five pregnant rabbits), and untreated (open bar, three pregnant rabbits) controls at gestational day 29. Tissues from three pups were pooled and extracted for one determination value. CSI was measured by RIA using the same method described in the legend of Fig.3-14. Each bar represents the mean of CSI concentrations detemined in these tissues from four (N=4) pregnant rabbits. The standard error (N > 9) of the mean is shown as the vertical bar. The student's t-test was used for determining statistical significance between two means. The standard error of mean is shown as the vertical bar.


A
60000
50000
40000
30000
20000
10000
0
CSI ng/g tissue
N.S.
N.S.
CSI
(n=5)
UNTREATED
(n=4)
SALINE
(n=6)

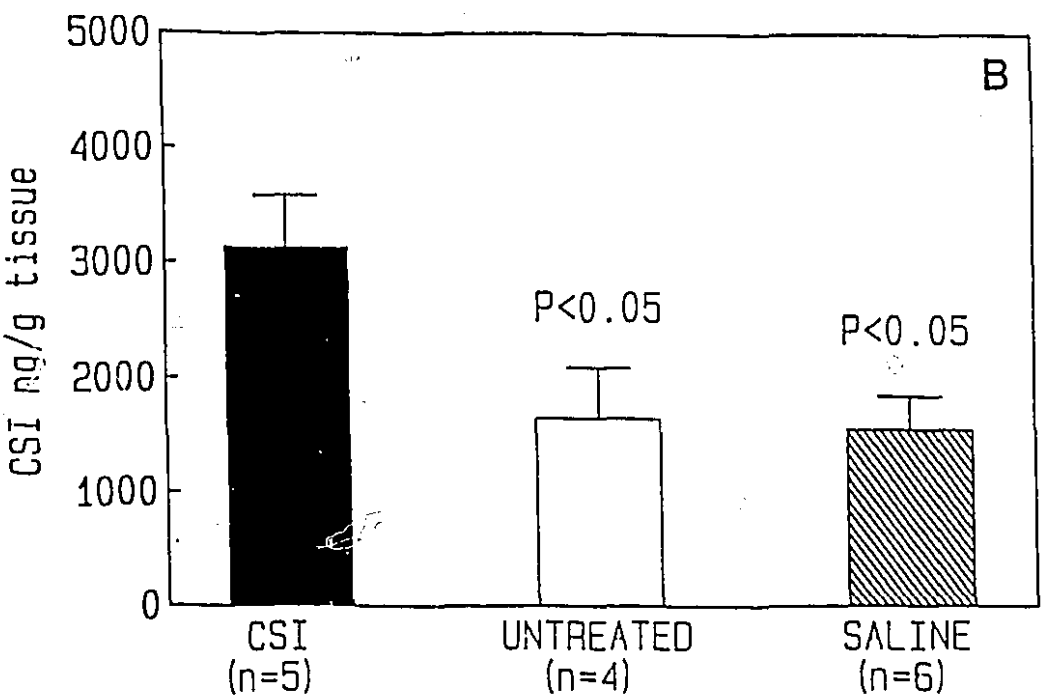
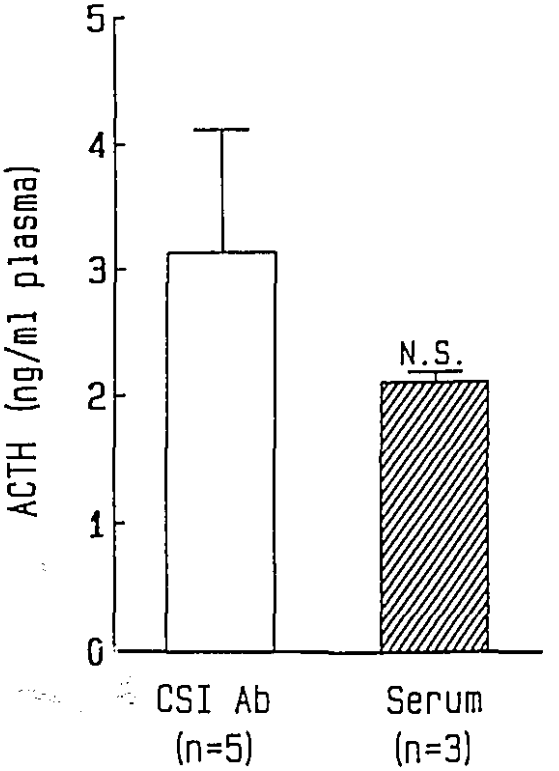
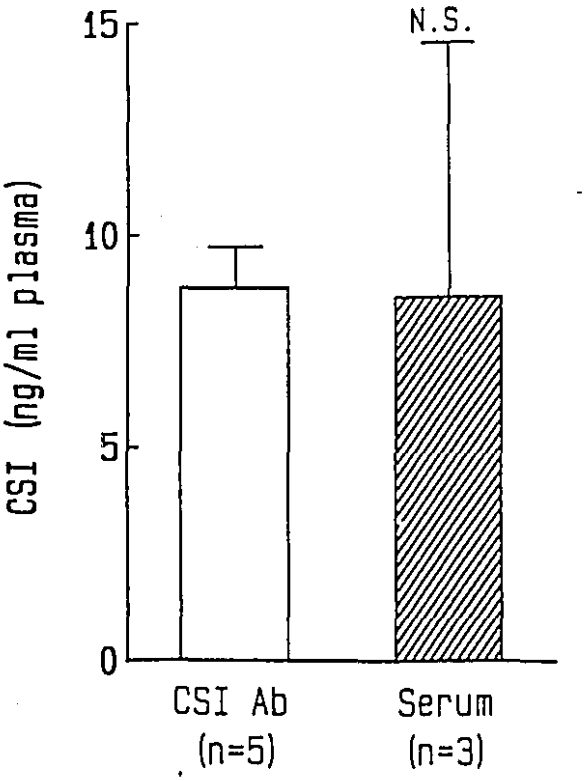
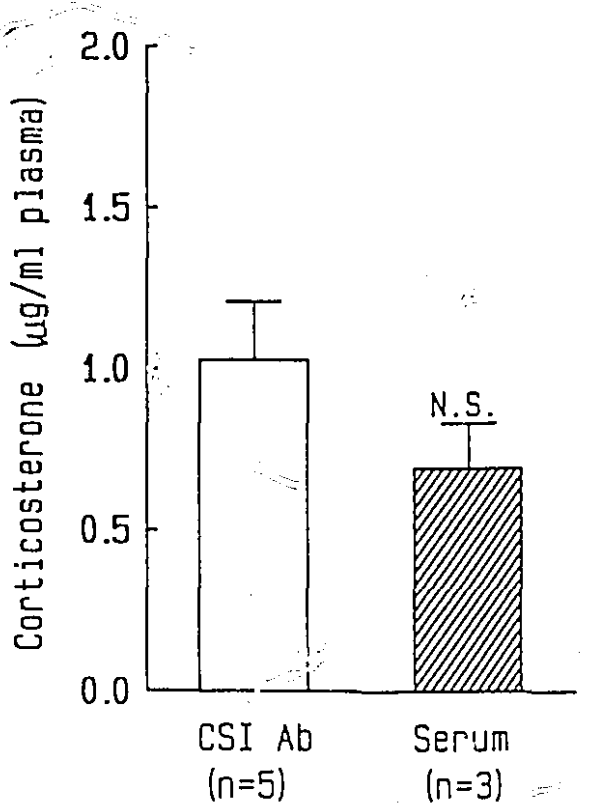

B
5000
4000
3000
2000
1000
0
CSI ng/g tissue
P<0.05
P<0.05
CSI
(n=5)
UNTREATED
(n=4)
SALINE
(n=6)

Figure 3-23. ACTH, CSI, B and F levels in plasma from pups injected with CSI-Ab in utero. Groups of fetuses were injected with CSI (solid bar, five pregnant rabbits), with saline (hatch bar, five pregnant rabbits), and untreated (open bar, three pregnat rabbits) controls at gestational day 29. Plasma from two to three pups were pooled using for one determination value. The standard error (N > 10) of the mean is shown as the vertical bar. All hormones were measured by RIA. The student's t-test was used for determining statistical significance between two means.


ACTH (ng/ml plasma)
5
4
3
2
1
0
N.S.
CSI Ab
(n=5)
Serum
(n=3)


CSI (ng/ml plasma)
15
10
5
0
N.S.
CSI Ab
(n=5)
Serum
(n=3)


Corticosterone (µg/ml plasma)
2.0
1.5
1.0
0.5
0.0
N.S.
CSI Ab
(n=5)
Serum
(n=3)

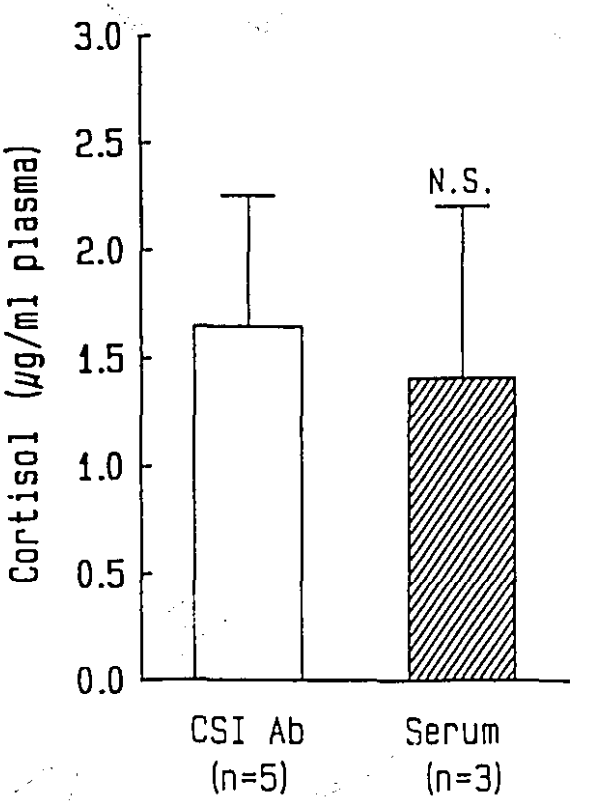

Cortisol (µg/ml plasma)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
N.S.
CSI Ab
(n=5)
Serum
(n=3)

Table 3-5. Length of gestation, plasma CSI, ACTH, B and F levels in Newborn rabbits after the in utero administration of CSI or its antiserum

Treatment	ACTH ng/ml	CSI ng/ml	B ng/ml	F ng/ml	Gestation
No treatment	1.4±0.3	11.7±4.0	13.2±4.2	10.8±3.3	32.0±0.3
Saline	1.9±0.3	16.1±5.0	10.1±1.5	8.0±1.7	31.6±0.4
CSI	3.5±0.7	28.2±12.7	15.7±1.7	22.8±3.8	31.4±0.7
GP serum	2.1±0.1	8.6±7.0	7.0±1.4	14.1±8.7	31.4±0.6
CSI Ab	3.1±1.0	8.8±1.0	10.3±1.8	16.5±6.1	32.3±0.3

3.4. Immunocytochemical Localization of CSI in Rabbit Tissues

Because we measured the endogenous concentrations of immunoreactive CSI in tissues, the next step was to focus on the localization of CSI to various cell types in the tissues where it is present in large amounts using the experimental protocol shown below.

Rabbit tissue
↓
Fixation in Bouin's fluid
(picric acid:formalin:acetic acid; 15:4:1)
↓
Dehydration in ethyl alcohol
↓
Clearing in xylene
↓
Embedding in melted paraffin at 60°C
↓
Staining using the immunoperoxidase technique
↓
Light microscopy

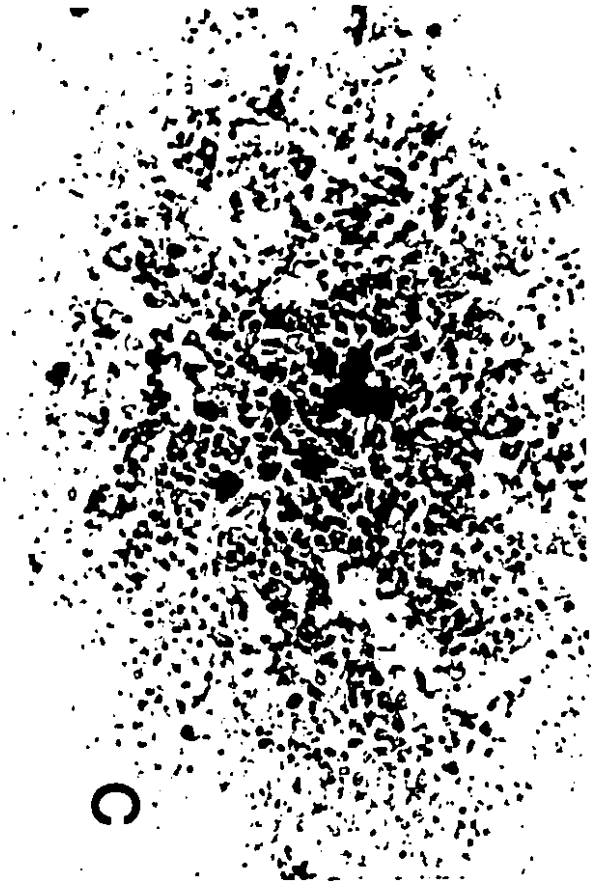
3.4.1. Specificity of Immunocytochemical Reaction

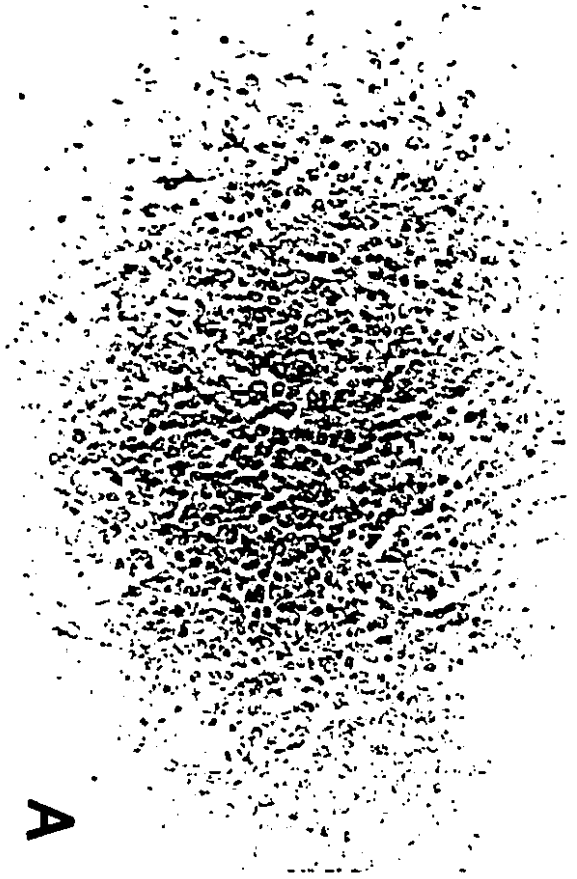
The immunoabsorption assay in most tissues, except the adrenal, confirmed the specificity of the immunocytochemical staining by a decrease in the intensity of staining when increasing concentrations of antigen were added (Fig. 3-24). At a competing concentration of CSI higher than 2 μg/100 μl, the staining was almost completely abolished (Fig. 3-24D). When pre-immune serum was substituted for anti-CSI antisera, no specific staining was observed (Fig. 3-24A). The control tissue, liver, lacked positive immunostaining for CSI (data not shown). A similar result was also obtained using another antiserum against CSI (data not shown). Below are described results using either of the two antisera. Essentially the same results were obtained with either antisera.

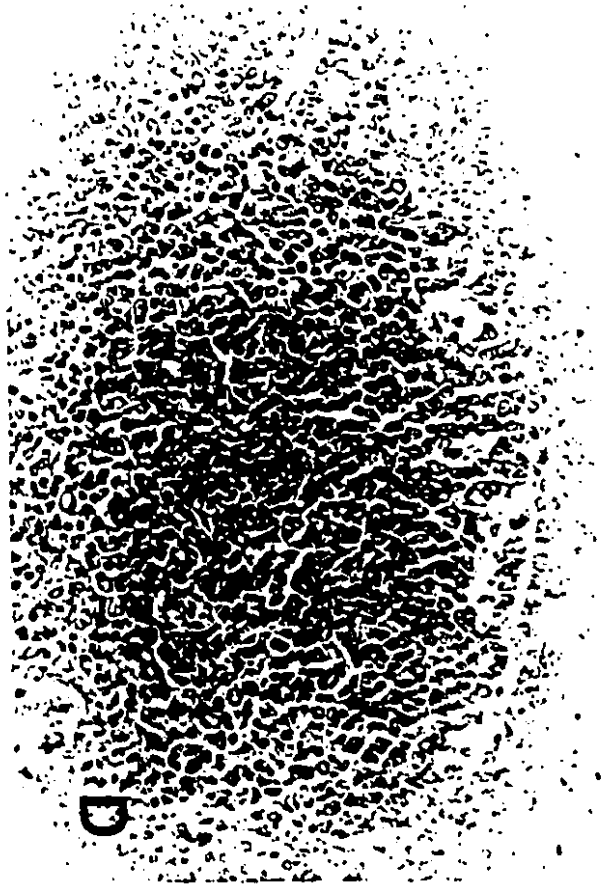
3.4.2. Localization of CSI in Rabbit tissues

In the adult spleen (Fig. 3-25A) there are numerous heavily stained cells with morphological features consistent with macrophages. A similar result has also seen in the fetal spleen (Fig. 3-25B) where strong CSI immunostaining was observed in large cells with a distribution consistent of macrophages. At high magnification (Fig. 3-25C & D), in both fetal and maternal lung, the staining was evident within large cells in the interstitium, consistent with macrophages. In addition, occasional smaller cells (arrowheads) of alveolar epithelial lining are also positive for CSI immunostaining. In the placenta

Figure 3-24. Demonstration of the immunocytochemical staining reaction in serial sections of normal female rabbit spleen tissue following immunoabsorbtion of anti-CSI antiserum with CSI. In (A) the section was stained with 1:100 dilution of guinea pig preimmune serum; (B) with same dilution of CSI antiserum; (C) with 0.02 μg CSI /100 μl immunoabsorped CSI antiserum and (D) with 2 μg CSI /100 μl immunoabsorbed CSI antiserum.(x340)


C


A


D

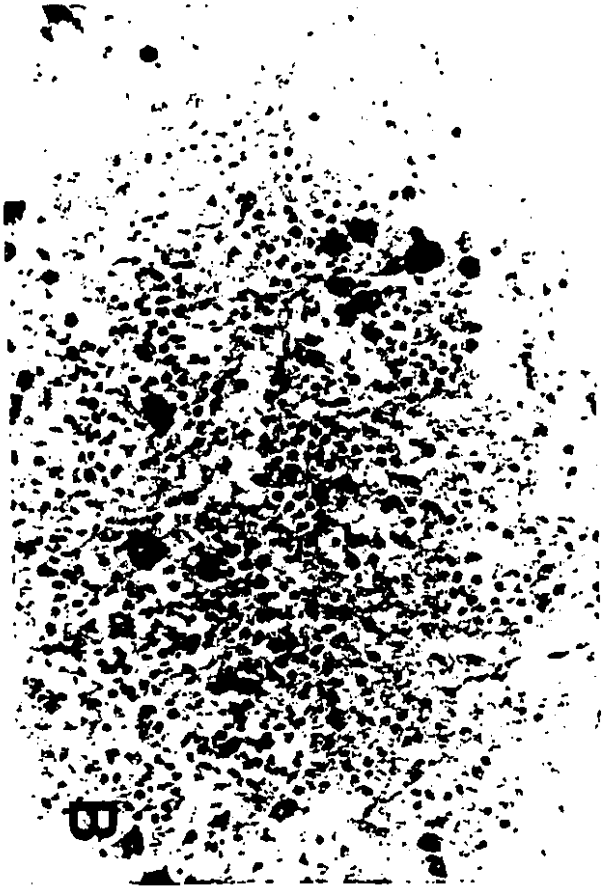

B

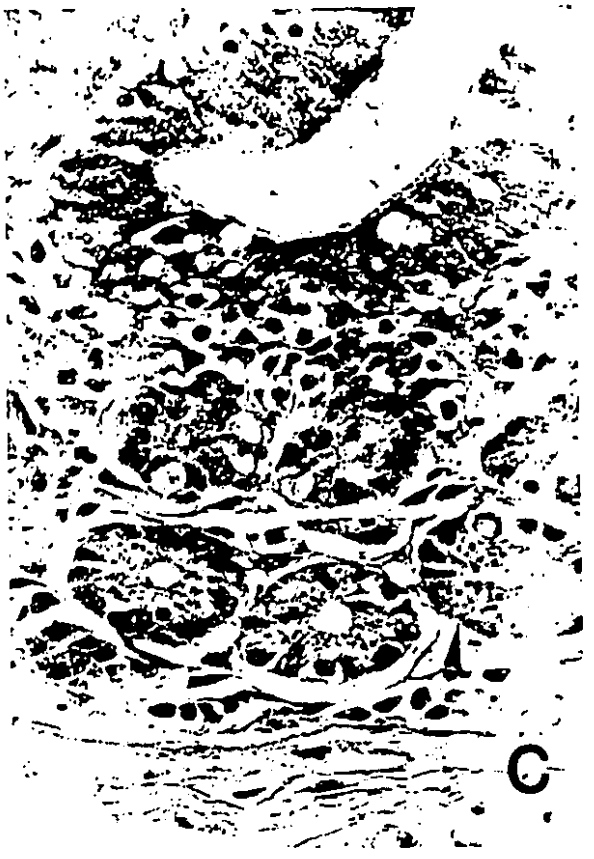
Figure 3-25. Expression of CSI in rabbit adult and fetal spleen and lung. In adult spleen(A),large cells with morphological features consistent with macrophages display a high level of cytoplasmic CSI. (x544). In fetal spleen(B), strong CSI immunostaining was observed in large cells with distribution consistent with macrophages. (x136). In adult and fetal lungs of the rabbit (C and D), strong immunostaining is observed in large cells in the interstitium, consistent with macrophages. In addition, occasional smaller cells (arrowheads) of the alveolar epithelial lining are also positive for CSI immunostaining. (Cx544 and Dx340).

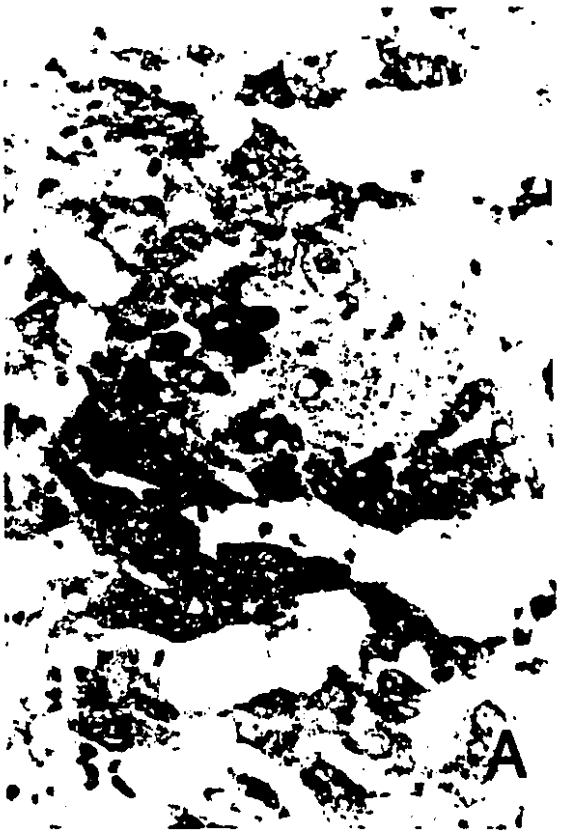
C

D

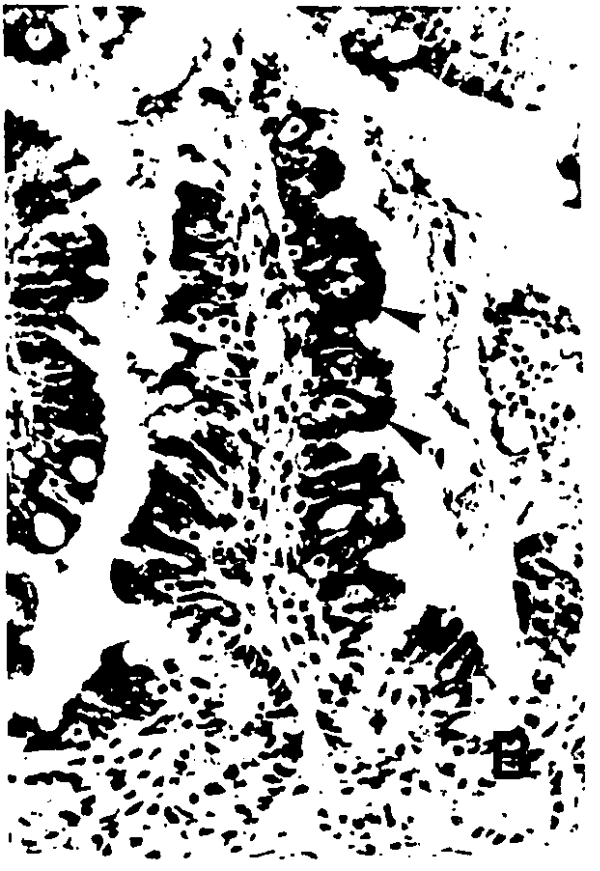
B

Figure 3-26. Expression of CSI in rabbit mature placenta, adult small intestine and duodenum. In the placenta(A), cells in stroma below the cytotrophoblastic cells express a high level of CSI. Their morphological features are consistent with macrophages.(x340). In adult small intestine, CSI immunostaining present in the surface epithelium (arrowheads,Bx272), but not in Paneth's cells(arrowhead) of the lower part of villus (Cx544). CSI immunostaining is present in the apical part of the enterocyte cytoplasm of duodenum.(Dx136).


C


A


D


B

(Fig. 3-26A) at day 27 of gestation, cells in stroma below cytotrophoblastic cells express a high level of CSI, their morphological features are consistent with macrophages. Similar results have also been observed in the placenta at days 24 and 30 of gestation. Immunostaining revealed the presence of CSI in the epithelum of the upper half of the villi of the adult small intestine (Fig.3-26B). However, there is no CSI present in the Paneth cells which are located at the bottom of the villi (Fig. 3-26C). Furthermore, CSI immunostaining also appears in the apical part of the cytoplasm of enterocytes of the duodenum (Fig. 3-26D). In the adult and maternal adrenal, the staining is only seen in the zona reticularis and fasciculata cells but not in the zona glomerulosa and medulla (Fig. 3-27A and B). The cells of the zona fasciculata express CSI at a high level, and inner compartment and zona reticularis are weakly positive. However, the distribution of CSI was undetectable in the same region of the fetal adrenal. In the adult and maternal rabbit brains, immunostaining for CSI was observed in the cytoplasm of the anterior pituitary gland with one third of the cell population (Fig. 3-27C) being positive. The blood vessel of hypothalamus contained cells which were histologically identical to macrophages and were positively stained for CSI. No CSI immunostaining was detectable in other compartments of the brain. There was also no CSI immuno staining in the fetal brain (Fig. 3-27D).

Figure 3-27. Expression of CSI in rabbit adult adrenal and brain. In the adrenal(A), cells of the zona fasciculata express CSI at high level.(x34). Inner compartment and zona reticularis are weakly positive. Medulla (star) does not express detectable levels of CSI which is shown in B.(x136). Sections of the rabbit anterior pituitary gland showing cytoplasmic CSI immmunostaining in approximately one third of the cells(Cx544). There is no CSI immunostaining in the fetal brain (Dx34).

A
B
C
D

CHAPTER 3 RESULTS

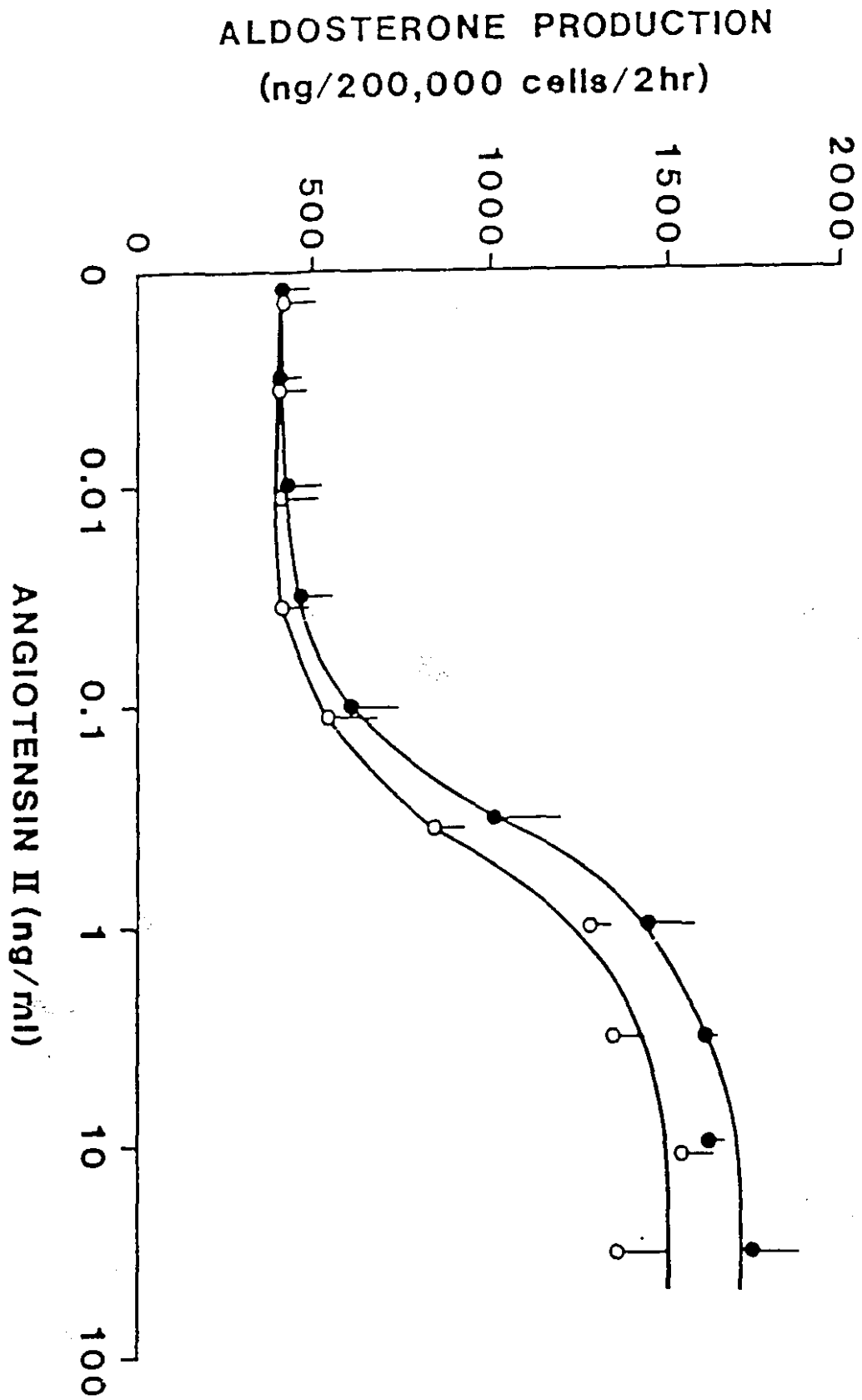
3.5. The Effects of CSI in the Zona Glomerulosa of the Rat Adrenal

CSI, the most potent anti-ACTH peptide in the corticostatic family, inhibits steroidogenesis in rat adrenal cells by displacing the binding of ACTH (288). In the dispersed rat adrenal cell system (not including zona glomerulosa) α-MSH ($ACTH_{1-13}$ acetoamide) can increase B production at high concentration (10^{-5}M). This action of α-MSH was not inhibited by CSI although the stimulatory activity of $ACTH_{1-18}$ amide was inhibited (288). Because the effects of α-MSH, ACTH and other modulators of steroid synthesis in zona glomerulosa cells seem to be different in different cell types of the adrenal, we initiated a series of studies in dispersed rat adrenal zona glomerulosa cells to determine the effects of α-MSH and CSI.

3.5.1. CSI, Angio II, ANF and α-MSH action in zona glomerulosa cells

Angio II is the most important regulator of aldosterone production in the zona glomerulosa of the rat adrenal gland. Rat zona glomerulosa cells respond to Angio II with increased aldosterone production and this action is not inhibited by CSI as is shown in Fig. 3-28. The effective dosage range of Angio II was from 10^{-11} to 10^{-9} M, and the maximal aldosterone production by the adrenal cells in response to Angio II was about 10^{-9} ng/$2x10^5$ cells/2h. There was no significant difference between the action of Angio II in the absence or presence of CSI, even at

Figure 3-28. The effect of CSI on the Angiotensin II stimulated-aldosterone production in the dispersed zona glomerulosa cell bioassay. Aldosterone log-dose-response to angiotensin II in the absence (open circles) or presence(closed circles) of 2 μg/ml CSI using rat adrenal zona glomerulosa cells at concentration of 200,000 cells/ml. The results are the mean of four separate experiments, each measured in duplicate. The vertical lines represent the standard deviations of the mean.


ALDOSTERONE PRODUCTION
(ng/200,000 cells/2hr)
0
500
1000
1500
2000
ANGIOTENSIN II (ng/ml)
0
0.01
0.1
1
10
100

concentrations of 2 μg/ml of CSI, at which CSI was able to completely abolish the steroidogenic response to 150 pg/ml of ACTH under the same assay conditions. ANF was found to be very active in inhibiting aldosterone synthesis in adrenal glomerulosa cells (2). In our assay system, the ANF inhibitory concentration range was 10^{-12}-10^{-8}M and there was no significant difference between either the presence or absence of CSI at 1 μg/ml as shown in Fig. 3-29. Interestingly, α-MSH specifically stimulates aldosterone production of isolated zona glomerulosa cells at a much lower concentration than is required for stimulation of corticosterone production in zona fasciculata cells despite the fact that α-MSH can potentiate the effects of ACTH on corticosteroid production in both zona fasciculata and glomerulosa cells (176,425). In our experiment, 0.5 nM α-MSH markedly increased aldosterone production by dispersed rat zona glomerulosa cells but only slightly increased B production even at 10^3 nM. CSI at 1 μg/ml can inhibit α-MSH stimulated aldosterone synthesis (Fig. 3-30), but CSI has no affect on α-MSH stimulated B production by zona glomerulosa cells (Fig. 3-31).

3.5.2. α-MSH receptor studies

From the results presented so far, we assumed that α-MSH action on aldosterone and corticosterone production by zona glomerulosa cells are different in nature. At that time there was no report demonstrating α-MSH binding to a receptor other than

Figure 3-29. The effect of CSI on the ANF inhibition of aldosterone production in the dispersed zona glomerulosa cell bioassay. Aldosterone log-dose-response to ANF in the absence (open circles) or presence(close circles) of 1 μg/ml CSI using rat adrenal zona glomerulosa cells at concentration of 200,000 cells/ml. The results are the mean of five separate experiments, each measured in duplicate. The vertical lines represent the standard deviations of the mean.

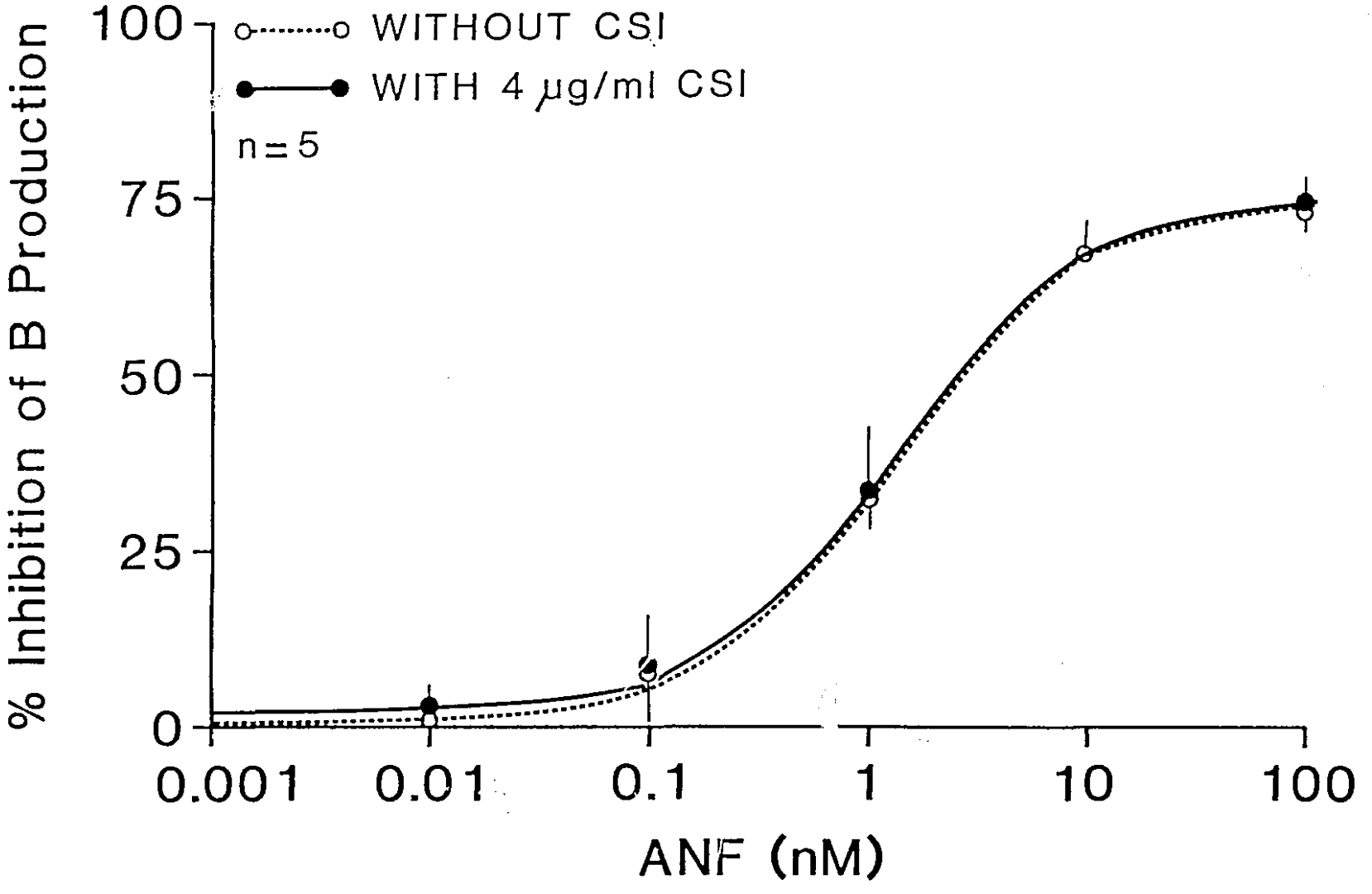

100
75
50
25
0
% Inhibition of B Production
WITHOUT CSI
WITH 4 µg/ml CSI
n=5
0.001
0.01
0.1
1
10
100
ANF (nM)

Figure 3-30. The effect of CSI on the α-MSH stimulated-aldosterone production in the dispersed zona glomerulosa cell bioassay. Aldosterone log-dose-response to α-MSH in the absence (open circles) or presence(close circles) of 1 μg/ml CSI using rat adrenal zona glomerulosa cells at concentration of 200,000 cells/ml. The results are the mean of four separate experiments, each measured in duplicate. The vertical lines represent the standard deviations of the mean.

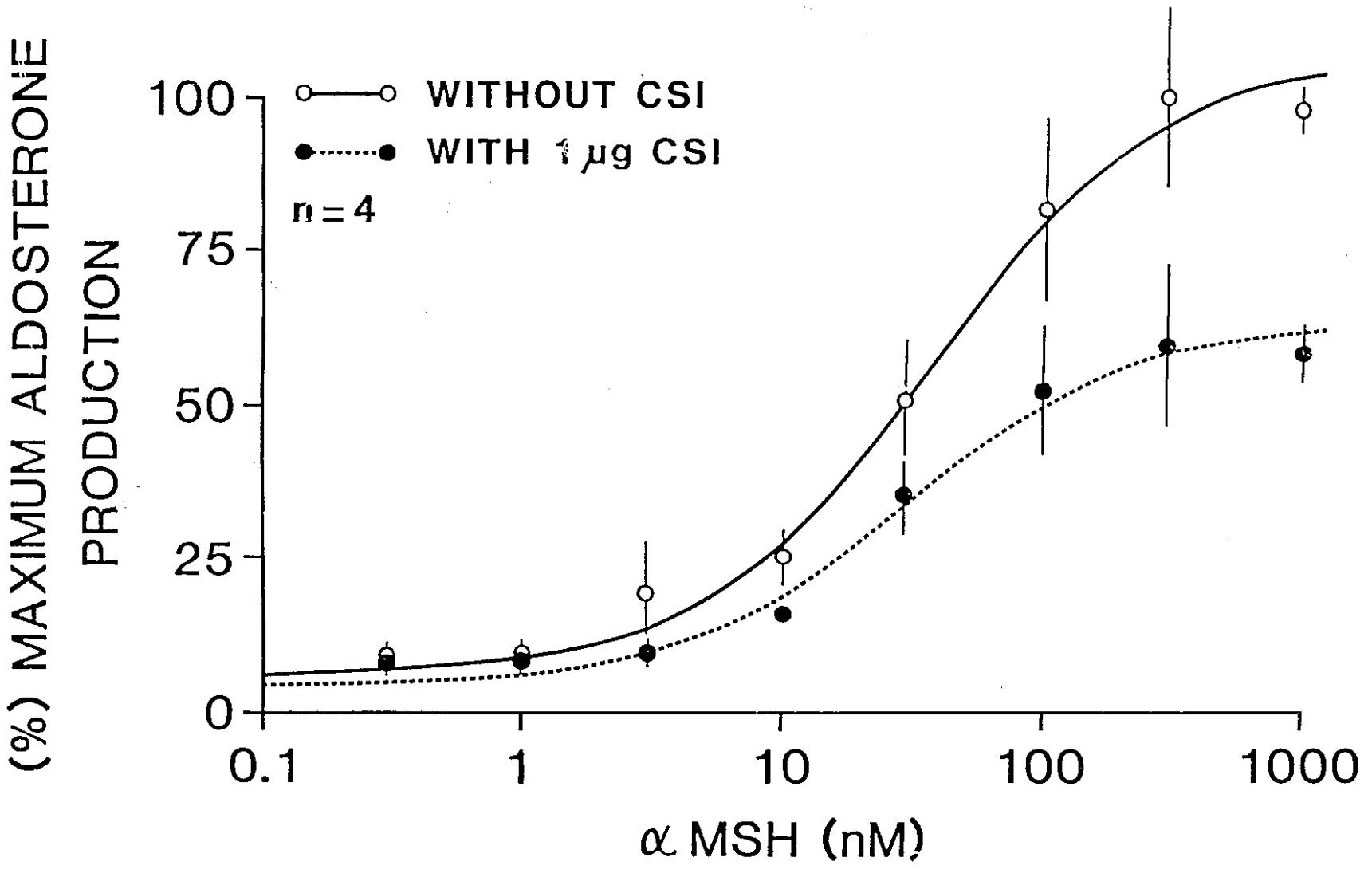
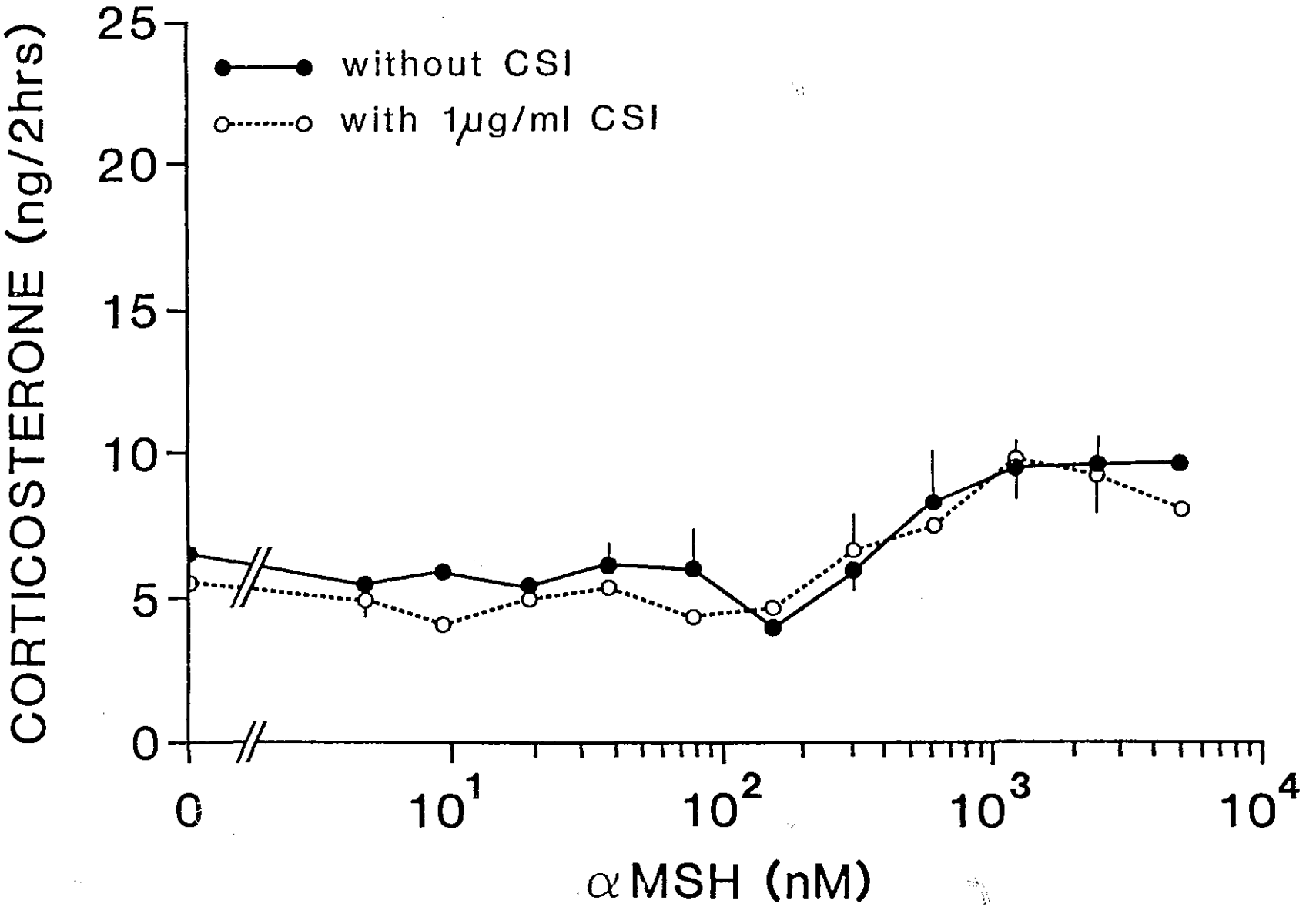

(%) MAXIMUM ALDOSTERONE PRODUCTION
100
75
50
25
0
WITHOUT CSI
WITH 1 µg CSI
n = 4
0.1
1
10
100
1000
α MSH (nM)

Figure 3-31. The effect of CSI on the α-MSH stimulated-corticosterone production in the dispersed zona glomerulosa cell bioassay. Corticosterone log-dose-response to α-MSH in the absence (open circles) or presence(close circles) of 1 μg/ml CSI using rat adrenal zona glomerulosa cells at concentration of 200,000 cells/ml. The results are the mean of four separate experiments, each measured in duplicate. The vertical lines represent the standard deviations of the mean.

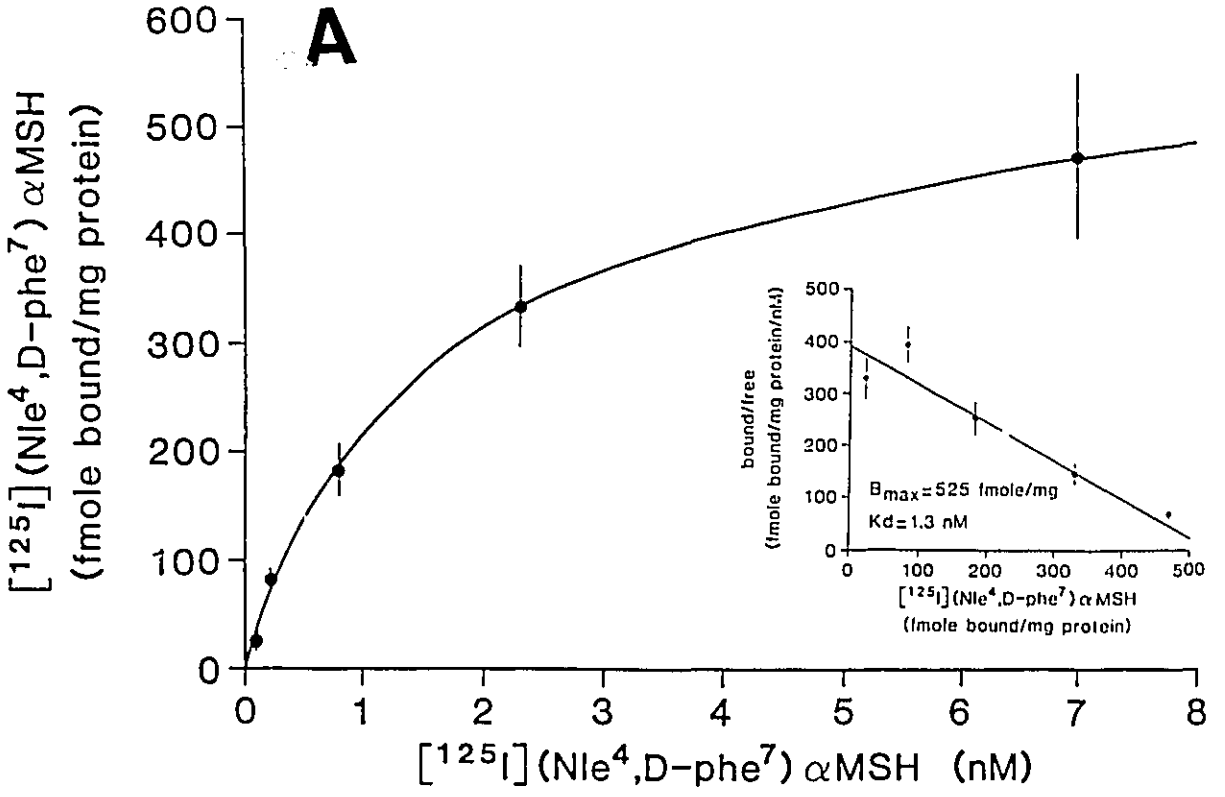

CORTICOSTERONE (ng/2hrs)
25
20
15
10
5
0
without CSI
with 1μg/ml CSI
0
10¹
10²
10³
10⁴
αMSH (nM)

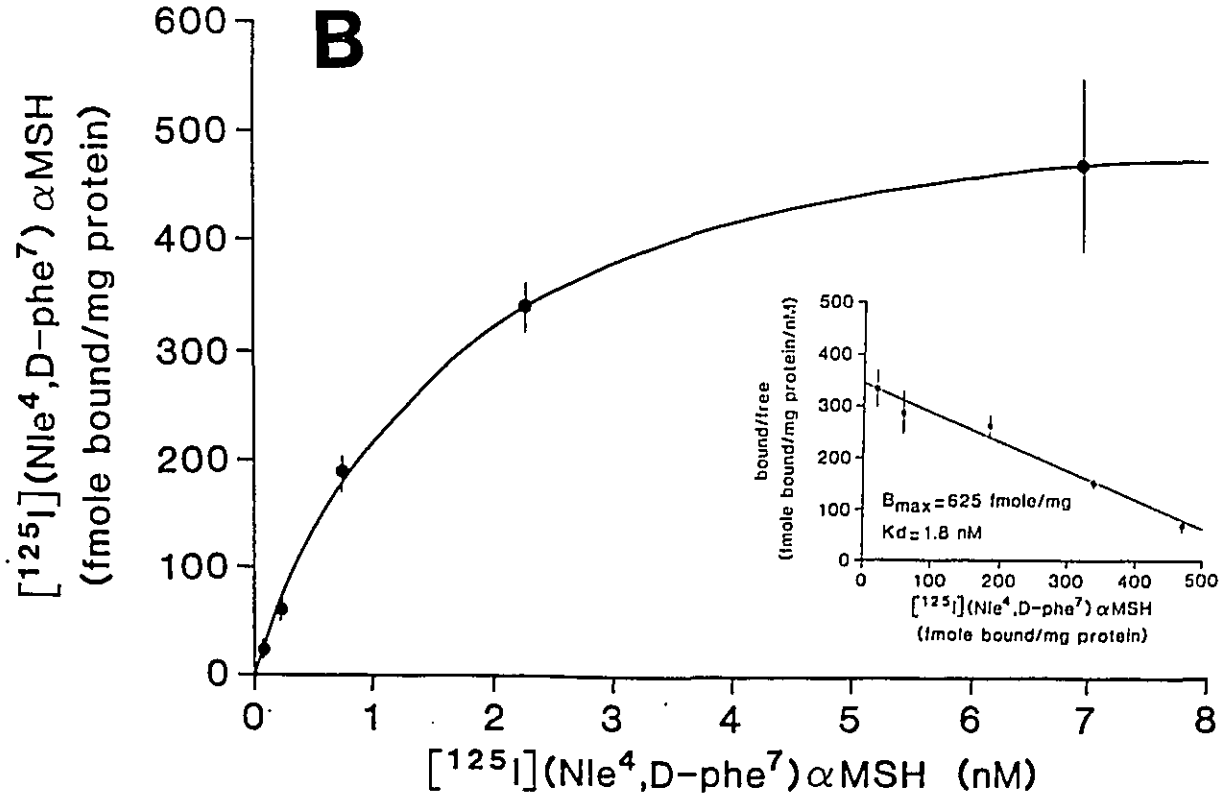
the ACTH receptor on the adrenal gland. In order to elucidate the mechanism of CSI inhibitory action on α-MSH-stimulated aldosterone production by zona glomerulosa cells, an α-MSH receptor binding study was initiated. Since α-MSH receptor studies on the membrane of the lacrimal gland had already been well documented, we did these binding studies first and they served as a control on future binding studies.

3.5.2.1. ***Specificity of binding***

Saturation binding properties of α-MSH sites were first studied in the membrane of the lacrimal gland, and then in the membrane of the zona glomerulosa cells of the adrenal gland. These results are shown in Fig. 3-32 A and B respectively. Binding was studied over a concentration range from 0 - 8 nM, with saturation achieved at approximately 7 nM. Nonspecific binding in adult rat lacrimal cell membrane preparation was lower (half) than that observed with adult rat adrenal glomerulosa cell membrane preparations, representing about 50% of the total binding at saturation. The α-MSH receptors on zona glomerulosa cells of the adrenal gland and lacrimal gland seem to have the same binding properties. The average apparent equilibrium dissociation constant (K_d), determined from Scatchard analysis, were 1.3 and 1.8 nM, and B_{max} were 525 and 625 fmole/mg protein in adult rat lacrimal cell membrane preparation and in adult rat adrenal glomerulosa cell membrane preparations respectively.

Figure 3-32. Specific binding of [^{125}I]iodo-[Nle^4,D-phe^7]α-MSH to membrane fractions of rat lacrimal and adrenal glands. The experiment was conducted as described in section 3.5.2.1. Specific binding of [^{125}I]iodo-Nle^4,D-phe^7]α-MSH to the membrane fractions of rat lacrimal (A) and zona glomerulosa cells of the rat adrenal gland (B) was plotted as a function of concentrations of free [^{125}I]iodo-Nle^4,D-phe^7]α-MSH. Kd was calculated from its corresponding Scatchard plot (inset). The results are the mean of three separate experiments, each measured in triplicate. The vertical lines represent the standard deviations of the mean.


A
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
[125I](Nle4,D-phe7) αMSH (nM)
[125I](Nle4,D-phe7) αMSH (fmole bound/mg protein)
bound/free (fmole bound/mg protein/nM)
Bmax=525 fmole/mg
Kd=1.3 nM
[125I](Nle4,D-phe7) αMSH (fmole bound/mg protein)


B
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
[125I](Nle4,D-phe7) αMSH (nM)
[125I](Nle4,D-phe7) αMSH (fmole bound/mg protein)
bound/free (fmole bound/mg protein/nM)
Bmax=625 fmole/mg
Kd=1.8 nM
[125I](Nle4,D-phe7) αMSH (fmole bound/mg protein)

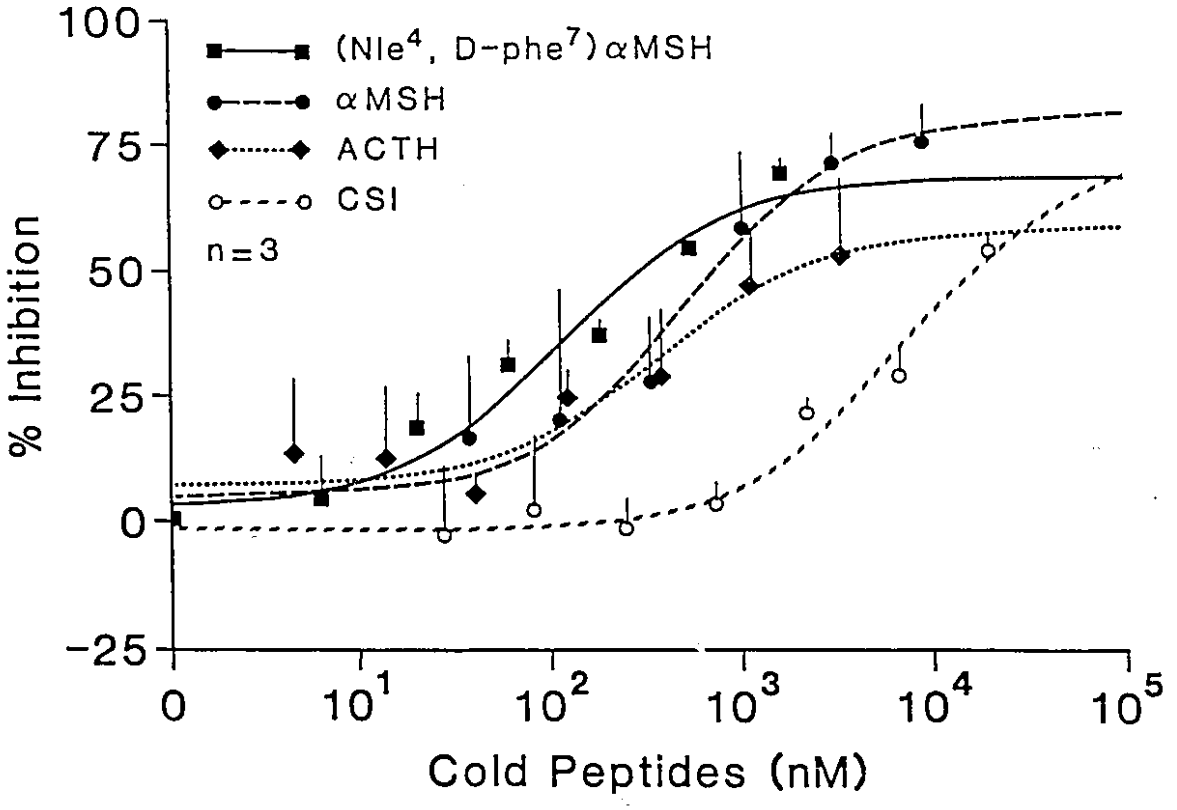
3.5.2.2. *Effects of CSI and other inhibitors on α-MSH binding*

CSI, α-MSH, ACTH were tested in competition studies with [^{125}I]-[Nle^4, D-Phe^7]-α-MSH, a fully biologically active α-MSH analogue. The effect of nonradioactive [Nle^4, D-Phe^7]-α-MSH, α-MSH, $ACTH_{1-24}$ and CSI to in various concentrations on the binding of [^{125}I]iodo-[Nle^4,D-Phe^7]α-MSH to membranes derived from zona glomerulosa cells of the adrenal gland and lacrimal gland is shown in Fig 3-33. In the zona glomerulosa of the adrenal gland, CSI was able to displace 14 nM of ^{125}I-[Nle^4, D-Phe^7]-α-MSH in the concentration range 0.5 μM to 30 μM being about 57.6 times less potent than [Nle^4, D-Phe^7]-α-MSH, 14.5 times less potent than native α-MSH, and 12.9 times less potent than $ACTH_{1-24}$. In the lacrimal gland, similar results were observed. The lower panel of Fig. 3-33 shows that [[Nle^4, D-Phe^7]-α-MSH was the most effective peptide, having an IC_{50} of 77.4 nM, approximately 2.5 times more potent than α-MSH (IC_{50} of 200 nM), 6.2 times more potent than ACTH (IC of 480 nM). CSI showed the lowest receptor affinity (IC of 6000 nM). Peptides, such as ANF and Angio II, were totally without effect (data not shown here).

3.5.3. CSI receptor binding studies

Specific binding for CSI to the membrane fractions of these tissues could be demonstrated for CSI only after polylysine saturation of the non-specific sites (Fig. 3-34). These data are similar to the results of a CSI receptor binding assay which was

Figure 3-33. The inhibitory effects of α-MSH, ACTH and CSI on α-MSH receptor binding. The percentage inhibition of 14 nM ^{125}I-Nle^{4}, Phe^{7}-α-MSH on the membrane fractions of zona glomerulosa (upper panel) and rat lacrimal gland (lower panel) with increasing concentrations of Nle^{4}, Phe^{7}-α-MSH, α-MSH, ACTH and CSI. The results are the mean of three separate experiments, each measured in quadruplicate. The vertical lines represent the standard deviations of the mean.


100
75
50
25
0
-25
% Inhibition
(Nle4, D-phe7) αMSH
αMSH
ACTH
CSI
n=3
0
10^1
10^2
10^3
10^4
10^5
Cold Peptides (nM)

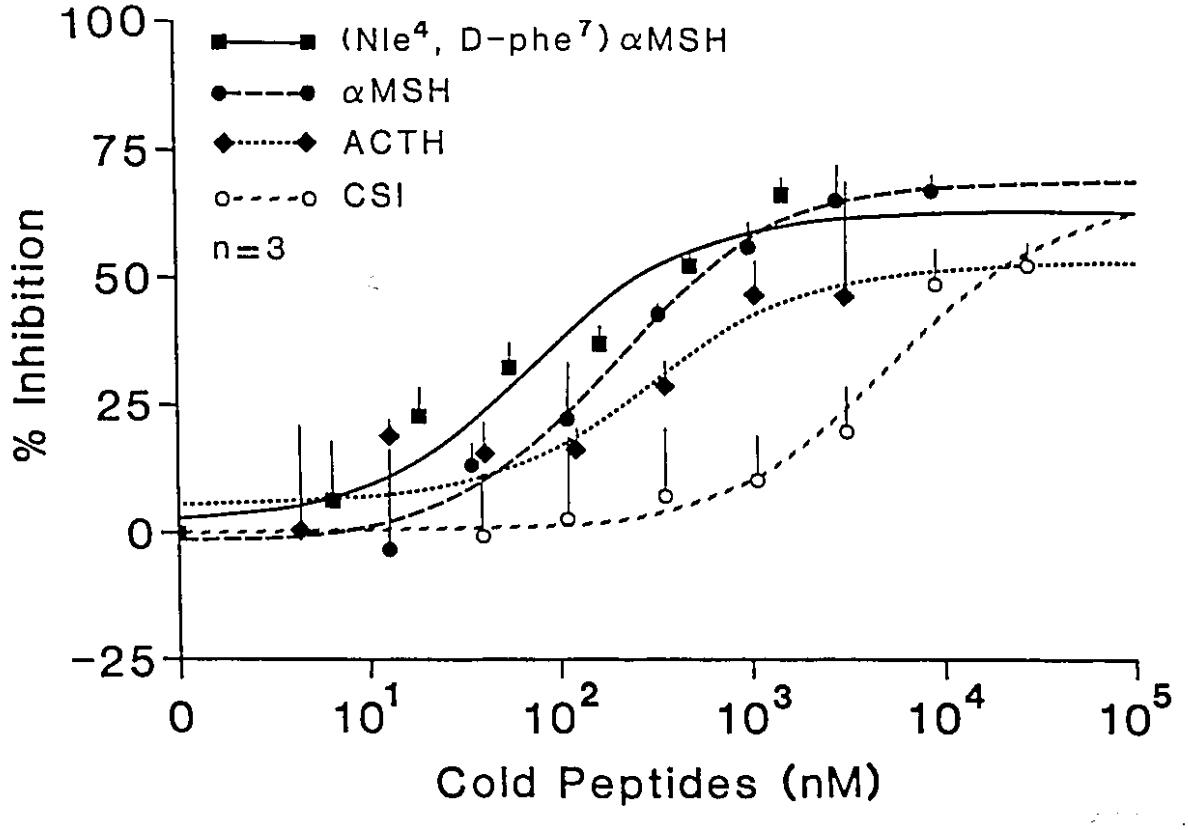
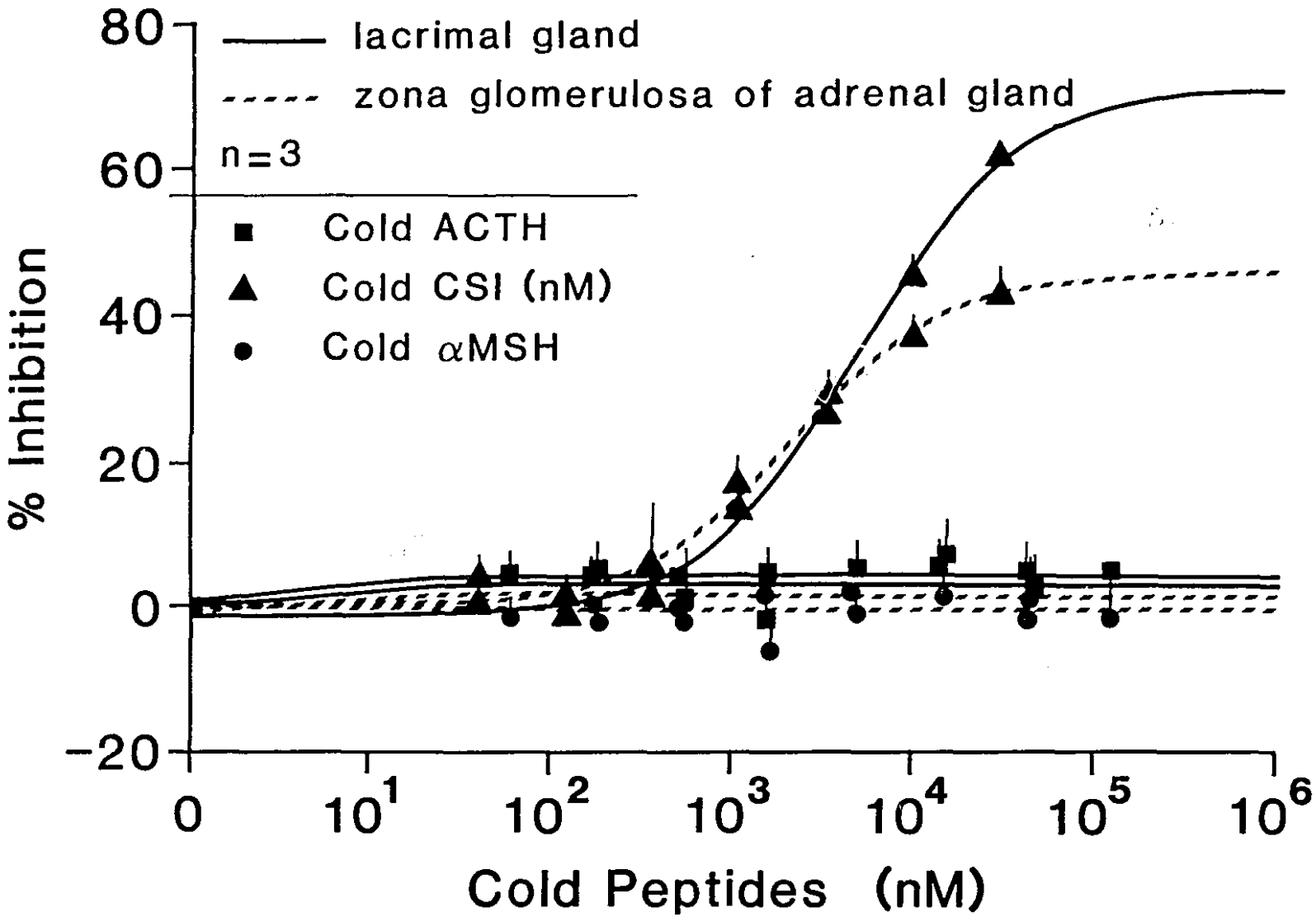

100
75
50
25
0
-25
% Inhibition
(Nle4, D-phe7) αMSH
αMSH
ACTH
CSI
n=3
0
10^1
10^2
10^3
10^4
10^5
Cold Peptides (nM)

Figure 3-34. The effects of CSI, α-MSH and ACTH in the CSI receptor binding assay. Membrane fractions of rat lacrimal gland and zona glomerulosa cells of the rat adrenal gland were incubated with [^{125}I]-CSI with increasing concentrations of CSI, α-MSH and ACTH as indicated. Binding was determined as described in Methods. The results are the mean of three separate experiments, each measured in quadruplicate. The vertical lines represent the standard deviations of the means.

CSI Receptor Binding



done in rat adrenal cells (426). In both membranes prepared from rat lacrimal gland and zona glomerulosa of the adrenal gland, CSI binding was only displaced by a large excess of unlabelled CSI, but not by 100,000-fold excess of unlabelled hACTH and α-MSH. It is obvious from these data that the CSI was able to specifically bind to some membrane components other than the α-MSH receptor in these two glands.

CHAPTER 4 DISCUSSION

4.1. Isolation and characterization of corticostatic peptides from guinea pig and rabbit bone marrow

As mentioned in the first chapter, corticostatic peptides were first isolated from the rabbit (8) and then from human tissues (300). At this point it was interesting to know whether these peptides could also be found in other species such as rat, guinea pig, mouse or bovine and whether all corticostatic peptides isolated belonged to the same family. As part of this project we describe here our data which dealt with the isolation of corticostatic peptides from guinea pig and rabbit bone marrow.

4.1.1. RP-HPLC

The peptide purification procedure used in this study was a slightly modified version of the method described by Bennett et al (286,287). This method is simple, rapid and ensures the recovery of unmodified peptides with high yield. In the initial protocol, the use of the acidic extraction medium (1M HCl, 5% formic acid, 1% TFA and 1% NaCl, pH 0) at 4°C was found to be the most efficient way of inhibiting peptidase activity during the first stage of isolation. At this pH (close to 0) and the temperature (4°C), most proteolytic enzymes are not active.

Furthermore, the formic acid and high salt concentration helped to free peptides from the tissue due to the ability of formic acid to penetrate the cell membrane. Since the salts are strong precipitating agents for proteins, most of the large proteins will be precipitated and removed by centrifugation. Tominaga et al. have used an acidic extraction medium (1 N acetic acid, 0.1 N HCl) with 5 minutes boiling as part of their extraction procedure (427). The recovery using the boiling method was relatively poor (427). This is probably due to the fact that boiling peptides in acid will chemically alter the structure (decarboxylation, formation of double bonds etc.) and destroy the molecule. Corticostatic peptides are a group of highly positively charged cationic peptides which will bind to some negatively charged cellular components under low salt and relatively mild acidic conditions. Therefore, corticostatic peptides are coprecipitated with other proteins after denaturation at high temperature before being totally extracted. This might be the main reason why the level of CSI in different rabbit tissues as reported by Tominaga et al.(427) were 10 times less than that obtained by us.

At the next step of purification, the peptides in the supernatant are simultaneously concentrated, desalted and deproteinized by adsorption onto ODS-silica cartridges. Small peptides can be concentrated on ODS-silica cartridges based on their ability to penetrate the pores of the ODS-silica, whereas

proteins are usually too big and salts are too polar to be retained. Peptides bound to the ODS-silica cartridges can be recovered by eluting with 80% acetonitrile. The eluates from the ODS-silica cartridge, usually in 80% acetonitrile containing 0.1% TFA, can be stored safely below -20°C until used. At this low pH, in a non-polar solvent and in the frozen condition, most of the peptidases are not active. After extraction and deproteinization, peptides can now be readily separated by RP-HPLC. Before the eluates are injected onto RP-HPLC columns, the acetonitrile has to be evaporated under vacuum or diluted with aqueous buffer until the final concentration of acetonitrile reaches 1%.

The separation of peptides on RP-HPLC is widely practised (428,429). This is primarily due to its versatility and ability to resolve peptides exhibiting very minor differences in structure. There are other advantages of using reversed-phase such as efficiency, speed and high reproducibility. RP-HPLC utilizes hydrophobic interaction to retain peptides, and the interactions of peptides with ODS-silica packing material are enhanced by using a pH below the pKa of carboxyl groups. An "ion-pairing" reagent is usually included in the solvent to increase the resolution. It is because these agents (i.e. TFA or HFBA) can minimize the polarity of free amino groups. The relative elution position of a particular peptide depends to a large extent on the number of positive charges and hydrophobic residues. By performing RP-HPLC purification in the present studies using TFA

and HFBA as ion-pairing reagents, we have isolated and identified a variety of corticostatic peptides from guinea pig and rabbit bone marrow and various tissues. Peptide solubility dictates the choice for both type and concentration of ion-pairing agent. TFA is usually the first choice because it is an excellent solubilizing agent and it does not interfere with optical absorption in the ultraviolet range and therefore allows detection of peptide bonds below 230nm. The counter-ion HFBA, is a stronger hydrophobic ion-pairing reagent than TFA and maximizes the retention of basic peptides. This is the reason why corticostatic peptides were eluted at a relatively lower acetonitrile concentration in TFA system and at a higher acetonitrile concentration in the HFBA system. These properties greatly facilitated the purification of corticostatins. Since the solvent systems are completely volatile, the peptide eluates can be directly used in radioimmunoassay, bioassay and amino acid analysis following evaporation of the solvents.

4.1.2. Adrenal cell bioassay

Historically, the bioassay has been a great asset in the isolation of biologically active peptides and proteins. In vitro bioassays are most convenient for generating concentration-response curves for peptides. Several parameters related to the protein-protein interaction can be generated from such curves. The most commonly used is the EC_{50} (i.e. the concentration of peptide that produces half-maximal effect), which defines the

potency of the peptide in a given system. Another parameter is the maximal effect elicited by the peptide, which is directly related to its intrinsic activity. In structure-activity studies, these parameters are compared for several related peptides.

The bioassay system used here was essentially the same as the one for the ACTH assay devised by Sayers (430,431). Adrenal capsular strippings containing predominantly zona glomerulosa cells were used to generate a cell suspension for studying aldosterone responsiveness, and decapsulated adrenal glands were used to obtain predominantly zona fasciculata/reticularis cell suspension for studying the corticosterone response. In order to achieve maximum production of aldosterone and corticosterone, potassium and calcium were supplemented respectively in corresponding medium (for a detailed discussion of K^+, and Ca^{++} see sections 1.3.2.2. and 1.3.1.2.). A complete separation of zona glomerulosa cells from the inner zona cells is almost impossible, however, a good preparation of glomerulosa cells is always accompanied by a higher responsiveness to Angio II-stimulation and lower responsiveness to ACTH-stimulation. These adrenal cell bioassays are used by us predominantly for determining corticostatic activity of unknown peptides, comparing the potency among different corticostatic peptides and their analogues, and demonstrating the interaction between CSI and other related hormones.

The EC_{50} of ACTH in the present bioassay system was

approximately 150 pg/ml consistent with the results obtained by most laboratories (431). The maximal corticosterone response to ACTH stimulation varied from one assay to another. This is due to the variations of the bioassay conditions as mentioned before such as the temperature of incubation, the gas phase condition, and the method of handling the cells and especially the conditions of the animals. Most of the results obtained from our bioassay have been normalized as % of maximal corticosterone production.

4.1.3. GPCS1,2,3 and CSV and VI

Using bone marrow as the starting material, three guinea pig peptides (GPCS1, GPCS2, GPCS3), and two rabbit peptides (CSV, CSV) with corticostatic activity have been isolated by RP-HPLC using the rat adrenal cell bioassay system to monitor the column fraction (Fig. 3-1, 3-2, 3-6, and 3-7). Although the total amount of corticostatic peptides in lung and in the bone marrow are comparable, concentrations of these peptides are much higher in the bone marrow than lung. Therefore the cationic ion-exchange Sep-pak purification step used previously described was not necessary especially when trying to purify anti-ACTH components in the current studies (288).

During the course of isolation of the first eluates of guinea pig corticostatin as shown in Fig 3-1A, a Vydac reversed-phase column and a shallow gradient have been used in the

subsequent step in order to provide a better resolution. Surprisingly, what we considered to be a homogeneous peak split into two adjacent linked peaks (Fig.3-1C). Amino acid analysis showed that there was only one amino acid difference between the front of the first peak (GPCS1) and the tail of the second peak (GPCS2) (Table 3-1). Therefore, the front and tail of these two peaks were rechromatographed separately until GPCS1 and GPCS2 reached homogeneity. Sequence analysis confirmed the amino acid analysis results showing that GPCS2 differed from GPCS1 at residue 21, a leucine instead of isoleucine in GPCS1 (This is one of the best examples demonstrating how powerful RP-HPLC can be to resolve peptides with such a minor difference in structure). GPCS1 and GPCS2, had typically conserved six cysteines in their backbone as found in rabbit and human corticostatic peptides but unlike other members of the corticostatin family, they did not have basic amino acids at their C-terminal end, instead they had the basic amino acids at their N-terminal end.

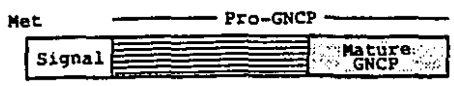
GPCS1 was also recently isolated by Selsted and Harwig (432) as a single guinea pig peritoneal neutrophil peptide with antimicrobial activity. GPCS1 and GPCS2 were then isolated from the same source by Yamashita and Saito (433) and cloned by Nagaoka et al. (434). Analysis of these clones indicated that both GPCS1 and GPCS2 were synthesized as pre-pro precursor proteins comprising 93 amino acid residues, which were composed of a signal sequences (N-terminal 19 residues), propeptide

sequences (43 residues) and mature GPCS1and GPCS2 sequences (31 residues) (Fig.4-1). The deduced amino acid sequences showed that there were only two amino acid differences between GPCS1 and GPCS2, one in the pro-peptide region and one in the mature peptide region. According to these results, GPCS1 and GPCS2 mRNA was only detected in bone marrow but not in mature neutrophils. These observations suggest that the mature neutrophils, despite their abundant content of GPCS1 and GPCS2, lose the capacity to synthesize these peptides. It is of interest, that GPCS1 and GPCS2 are only biosynthesized in bone marrow cells. The anti-ACTH activity of GPCS1 and GPCS2 are considerably lower than that of CSI (Fig.3-5), the most active rabbit corticostatin, but comparable to other rabbit corticostatins.

Interestingly, GPCS3 does not structurally belong to the corticostatin/defence family of peptides despite its cysteine-rich and arginine-rich content. GPCS3 has a very unique structure, it is a 13 amino acid peptide and is present as an anti-parallel dimer linked by disulphide bonds as shown in Table 3-2. The evidence that it is a dimer came from both ion spray mass spectroscopy and size-exclusion HPLC (Fig.3-3). Enzymatic digestion (Fig.3-4) suggested an anti-parallel configuration. This was the first corticostatic peptide found so far which did not have a six cysteine backbone structure. This challenges the concept that there is a requirement of a highly conserved cysteine backbone structure for biological activity. It is

Figure 4-1. Nucleotide sequences of GNCP cDNA clones and the deduced amino acid sequences of prepro-GNCPs. (Adapted from Nagaoka I, Someya A, Iwabuchi K, and Yamashita T 1991 . Characterization of cDNA clones encoding guinea pig Neutrophil Cationic Peptides. FEBS 280:287)

```
                                                                       -78
                                                                        CGG CAC GAG AGG

          A        -50A   C                                                            -1
GAC AAA AGC CTG TTG TGG TTT CAC CTC TGC CTG CCC AGC TTC AGT CCA GAA AGG TGA CTC CCA GCC

1                                                               50
ATG AGG ACC GTC CCT CTC TTT GCT GCC TGT CTT CTG CTG ACC CTG ATG GCC CAG GCT GAG CCT CTC
Met Arg Thr Val Pro Leu Phe Ala Ala Cys Leu Leu Leu Thr Leu Met Ala Gln Ala Glu Pro Leu
-62                                             -50                        ↑

                                        100
CCA AGA GCA GCT GAC CAC TCT GAC ACT AAG ATG AAA GGA GAC AGA GAA GAC CAT GTT GCT GTC ATT
Pro Arg Ala Ala Asp His Ser Asp Thr Lys Met Lys Gly Asp Arg Glu Asp His Val Ala Val Ile
-40                                     -30                                     -20

                    150                 C
TCT TTT TGG GAG GAA GAA AGC ACC AGT CTT GAA GAT GCA GGT GCA GGT GCA GGC CGG GCG TGT ATT
Ser Phe Trp Glu Glu Glu Ser Thr Ser Leu Glu Asp Ala Gly Ala Gly Ala Gly Arg Arg Cys Ile
                                -10     Gln                         -1↑ 1

200                                                             C 250
TGC ACA ACA AGA ACC TGC CGT TTT CCA TAT CGC AGG CTG GGA ACC TGC ATC TTC CAG AAT CGA GTC
Cys Thr Thr Arg Thr Cys Arg Phe Pro Tyr Arg Arg Leu Gly Thr Cys Ile Phe Gln Asn Arg Val
                     10                                      20 Leu

                                            300
TAC ACA TTC TGC TGC TAA GCT TCC AGA ATA AAA AAC AAT TCT ATT TTG CTT TGA GGC CTC TAA GAG
Tyr Thr Phe Cys Cys ***
             30

                        350
AAT TGC TGC TTT CCT GTA GCT ATG TCT TCC ATT GTT TTC TTT CTG TTA AAT AAA TTG CTG TGG AAT

399
TGC
```



possible that GPCS3 does not act through the same mechanism as other corticostatins. GPCS3 also has the ability to release histamine (435). Other biological roles for GPCS3 have not as yet been determined.

CSV and CSVI were much more difficult to purify compared to other members of the family. These two corticostatic peptides had not been observed in the past due to their relatively low abundancy. The elution time of CSV was also only slightly earlier than CSI in the first step of RP-HPLC purification. In order to seach for minor corticostatic components such as GPCS3-like peptides, we increased the amount of starting tissues and monitored all minor components with both amino acid analysis and by the anti-ACTH rat adrenal cell bioassay. In this way CSV and CSVI were purified to homogeneity and their structures were determined by sequence analysis.

Structurally, CSV and CSVI are identical to NP-4 and NP-5 (290) respectively (Table 3-4). Very recently the cDNA structures for CSI(NP-3a), CSV(NP-4) and CSVI(NP-5) were published by Michaelson et al (436). Their cDNA sequences indicate that the peptides are synthesized as 94-95 amino acid prepro-CSs which are similar to the previously characterized preproCSIII and CSIV, consistent with their lysosomal localization. CSIII and CSIV mRNAs were found in bone marrow and spleen, organs which contained immature polymorphonuclear leukocytes. CSIII and CSIV mRNA were detected in lung macrophages but not in macrophages

from other organs, nor in monocytes, the putative macrophage precursors. In macrophages, the expression of CSIII and CSIV appears to be a marker of lung-specific differentiation. It will be very interesting to know how the synthesis of these peptides is regulated. These studies are currently underway by other investigators in our laboratory.

4.1.4. The structure of corticostatic peptides and their anti-ACTH activity

As mentioned above, corticostatins and defensins constitute a family of structurally related cysteine-rich peptides. These low molecular weight, cationic polypeptides have been isolated from human, rabbit, rat and guinea pig neutrophils and the cDNA encoding some of the members also have been cloned (434,437-439). All corticostatins and defensins, except GPCS3, contain 31-34 amino acids, including a consensus sequence of 11-12 residues in which cysteine and arginine align in a highly conserved fashion. The most striking feature of these structures is the high conservation of the position of the six cysteines residues within these peptides (except for GPCS3). The cationic nature of these peptides is highly variable. They range from a net-positive charge of +9 for CSI to +2 for HP3 (297). Some members of the family are closely related and constitute structural subsets. For instance, HP1, HP2 and HP3 differ by only one amino acid residue (297). Similarly, CSIII and CSIV differ only at residue 13 (8),

and R-4 and R-5 only at residue 7 (302). The primary structures of the corticostatin/defensin family from human, rabbit, rat and guinea pig are summarised in table 4-1.

Table 4-1. The comparison of the primary sequences of members of the corticostatin/defensin family and their anti-ACTH activity

Peptide	Sequence	$I.D._{50}$(nM)
Rabbit		
CSI(NP-3a)	GICACRRR FCPNSERFSGYCRVNGARYVRCCSRR	25
CSII(NP-3b)	GRCVCRKQLLCSYRERRIQDCKIRQVRFPFCCPR	120
CSIII(NP-1)	VVCACRRA LCLPRERRAGFCRIRGRIHPLCCRR	375
CSIV(NP-2)	VVCACRRA LCLPLERRAGFCRIRGRIHPLCCRR	500
CSV(NP-4)	VSCTCRRF SCGFQERASQSCTVNQVRHTLCCRR	650
CSVI(NP-5)	VFCTCRQF LCGSGERASGSCTINGVRHTLCCRR	6000
Human		
HP-1	ACYCRIP ACIAGERRYGTCIYQGRLWAFCC	not active
HP-2	CYCRIP ACIAGERRYGTCIYQGRLWAFCC	not active
HP-3	DCYCRIP ACIAGERRYGTCIYQGRLWAFCC	not active
HP-4	VCSCRLV FCRRTELRVGNCLIGGVSFTYCCTRV	475
Rat		
R-1	ACYCRIG ACVSGERLTGACGLNGRIYRLCC	not active
R-2	VTCYCRTS SCRFGERLSGACRLNGRIYRLCC	not active
R-3	CYCRTS SCRFGERLSGACRLNGRIYRLCC	not active
R-4	VTCYCRRT RCGFRERLSGACGYRGRIYRLCCR	50
R-5	VTCYCRST RCGFRERLSGACGYRGRIYRLCCR	1500*
Guinea Pig		
GPCS1(GNCP-1)	RRCICTTR TCRFPYRRLGTCIFQNRVYTFCC	250
GPCS2(GNCP-2)	RRCICTTR TCRFPYRRLGTCLFQNRVYTFCC	250
GPCS3	RRPRCFCRLHCRC CRCHLRCFCRPRR	2000

* Amount is value predicted from the data in reference No.20.

The corticostatic peptides but not the defensins have been found to induce L-type Ca^{2+} channels in guinea pig jejunal villus enterocytes (440). Therefore there must be some correlations in structural requirements for anti-ACTH and L-type Ca^{2+} channel agonist activities as all the corticostatic peptides examined to

date are active in inducing Ca^{2+} channels.

The fact that all members of the corticostatin/defensin family are basic suggests that the cationic nature of these peptides accounts for the displacement of the binding of the Lys-Lys-Arg-Arg- the "address region" of ACTH to its receptor (441). While basic charge is most probably important, it is not the sole factor controlling biological activity. This is because CSI, CSII, CSIII, CSIV, CSV, GPCS1, and GPCS2 vary considerably in potency and yet they have all similar overall positive charges. Comparison of the structures of corticostatic and non-corticostatic peptides is highly informative. For example, R-1 and R-4 share 72% sequence homology. R-1 which is a non-corticostatic peptide lacks a carboxyl-terminal extension and also lacks two arginine residues within the 5 to 10 residues of the sequence which is present within R-4, the second most potent peptide (ED_{50} of 50 nM). If the structures of corticostatic peptides are compared with non-corticostatic peptides, we also find that corticostatic peptides share one common feature and that is basic amino acids at their terminals either at C- or at N-terminals as is the case for GPCS1 and GPCS2. The non-corticostatic peptides have no carboxyl-terminal extension. This hypothesis for the requirement of terminal basic amino acids for corticostatic activity was tested experimentally.

Important information on the structural requirements of the peptides can be obtained by designing chemical modifications of

the peptide and evaluating the conformation and biological activity of the synthetic products. Shortening at the amino and carboxyl ends is often used to study modified peptides. Removal of one or both of the carboxyl-terminal arginines generated peptides whose corticostatic activity was lower compared to CSI itself (Fig.3-11 and 3-12). On the other hand, removal of amino-terminal arginine from the mixture of GPCS1 and GPCS2, either singly or both of them was without effect on the biological activity (Fig.3-9 and 3-10). The conclusion from this study can be that the presence of the pair of arginines at the carboxyl-terminal of CSI and amino-terminal of mixture of GPCS1 and GPCS2 are not critical for corticostatic potency. These data make it difficult to assign a minimum structural requirement necessary for biological activity of the corticostatic peptides.

If we rearrange the order of table 4-1 as shown in table 4-2 according their corticostatic potency except HP-4, it will not be too difficult to draw some meaningful conclusions.

Table 4-2. The comparison of the primary sequences of members of the corticostatin/defensin family and their anti-ACTH activity

Peptide	Sequence	I.D.$_{50}$(nM)
GPCS3	RRPRCFCRLHCRC CRCHLRCFCRPRR 1 6 10 15 20 25 30	2000
CSI(NP-3a)*	GICACRRR FCPNSERFSGYCRVNGARYVRCCSRR	25
R-4	VTCYCRRT RCGFRERLSGACGYRGRIYRLCCR	50
CSI(1-33)	GICACRRR FCPNSERFSGYCRVNGARYVRCCSR	85
CSI(1-32)	GICACRRR FCPNSERFSGYCRVNGARYVRCCS	125
CSII(NP-3b)*	GRCVCRKQLLCSYRERRIQDCKIRQVRFPFCCPR	120
GPCS1(GNCP-1)*	RRCICTTR TCRFPYRRLGTCIFQNRVYTFCC	250
GPCS2(GNCP-2)*	RRCICTTR TCRFPYRRLGTCLFQNRVYTFCC	250
GPCS1(2-31)	RCICTTR TCRFPYRRLGTCIFQNRVYTFCC	250
GPCS2(3-31)	CICTTR TCRFPYRRLGTCLFQNRVYTFCC	250
CSIII(NP-1)*	VVCACRRA LCLPRERRAGFCRIRGRIHPLCCRR	375
CSIV(NP-2) *	VVCACRRA LCLPLERRAGFCRIRGRIHPLCCRR	500
CSV(NP-4)*	VSCTCRRF SCGFQERASQSCTVNQVRHTLCCRR	650
R-5	VTCYCRST RCGFRERLSGACGYRGRIYRLCCR	1500**
HP-4	VCSCRLV FCRRTELRVGNCLIGGVSFTYCCTRV	475
CSVI(NP-5)*	VFCTCRQF LCGSGERASGSCTINGVRHTLCCRR	6000
HP-1	ACYCRIP ACIAGERRYGTCIYQGRLWAFCC	not active
HP-2	CYCRIP ACIAGERRYGTCIYQGRLWAFCC	not active
HP-3	DCYCRIP ACIAGERRYGTCIYQGRLWAFCC	not active
R-1	ACYCRIG ACVSGERLTGACGLNGRIYRLCC	not active
R-2	VTCYCRTS SCRFGERLSGACRLNGRIYRLCC	not active
R-3	CYCRTS SCRFGERLSGACRLNGRIYRLCC	not active

* The designations for these peptides were done at the time of isolation as defensins (290,433).
** Value is estimated from the data in reference No.302.

Because GPCS3 is the smallest corticostatic peptide which structurally resembles the N-terminal part of the corticostatic peptides, the corticostatic element must reside in the first half of these peptides. The number of basic residues in the region between the second and third cysteine, especially the second and

third or third residues from the second cysteine are most important to their anti-ACTH activities. (e.g. in the case of CSI, the most important residues are No.7 and No.8 arginines). CSI and R-4, the two most potent corticostatic peptides, have three basic residues in this region. Position 8 or 9 is the most significant for corticostatic activity followed by position 7. This conclusion was based on these facts that CSI(1-33) and R-4 both have three arginines in these regions and CSI(1-33) with arginine at position 8 almost as potent as R-4 with arginine at position 9, and also that GPCS1 and GPCS2 only have one arginine in this region at position 8, but they are more potent than CSIII and CSIV with two arginines in this region but at position 6 and 7. Furthermore, R-5 is missing one arginine at position 7 and lost most of corticostatic activity. Fourth, C-terminal or N-terminal basic residues are not critical to their anti-ACTH activities (Fig. 3-10 and 3-12), but two C-terminal arginines strongly potentiate these activities (Fig. 3-12).

The human peptide HP-4, should be a weak corticostatic peptide with an ID_{50} at around 1500 nM according to its structure. The reason that it has a potent anti-ACTH activity is difficult to explain. Perhaps two arginines at 11 and 12 compensate for the missing arginine at positions 7-9.

The hypothesis that requirements for basic residues at postions between the second and third cysteine needs to be further tested experimentally. If it is true, we are now in the

position to design some more potent agonists of corticostatin by increasing positive charges in the region between the second and third cysteines.

4.2. Distribution and localization of CSI in rabbit tissues

During the course of isolation of the peptides, it was noted that lungs, bone marrow and spleen contained much greater amounts of corticostatins than other tissues. One important question that needed to be addressed was to what cell type in these tissues these corticostatic peptides are localized and to what extent. We decided to start with rabbit CSI as a model because it is the most potent anti-ACTH peptide isolated, and because the synthetic peptide is available to prepare antibodies. It was also the most extensively studied of the corticostatic peptides.

4.2.1. *RIA*

Ideally, the labelled ligand should be totally free from corresponding unlabelled peptide and only one ^{125}I linked to one specific tyrosine to reach a specific activity of slightly less than the theoretical 2125 Ci/mmol. Practically, we did not find it necessary to use a highly purified ligand for RIA unless a receptor study was also involved. The method used in this study, as described in the Materials and Methods sections, matches all the requirements for the current purposes. This is because, first, both monoiodo and diiodo or other iodinated forms of CSI can be recognized by our polycolonal antisera and most of the

iodinated peptide was in the monoiodo-form. In addition, over 70% of the peptide would be iodinated under our conditions, and the free iodine was separated almost completely from the peptide pool by reversed-phase cartridge separation. We applied this protocol to other RIAs, and so far we have not encountered any major problems.

4.2.2. *Immunocytochemistry*

Immunocytochemical studies have provided a powerful tool in supplementing and extending the data obtained by RIA and other techniques. Its strength is that it combines a high degree of specificity with the resolution of the light and electron microscope. Therefore it can be more or less regarded as "biochemistry within the tissue section," and even more than that if the amount of immunoreactivity can be quantified. At present, a wide variety of compounds can be localized by immunocytochemistry (442). The most common procedures used in peptide immunocytochemistry have also been used in our CSI immunocytochemical study.

Immunoperoxidase staining is invariably the best stains when and if appropriate antibodies are available, because of their versatility, sensitivity, and specificity. Unlike fluorescent labels, which require the use of a special microscope, enzyme labels are visible by conventional light microscopy, provided that appropriate chromogenic substrates are used. Horseradish peroxidase has been used in the present study because among the

several different enzymes that are used for conjugation with antibodies, it produces the best results. In the first place, It is very efficient because, compared with immunofluorescence and indirect methods, the dilution of the first antiserum can be very high. Second, the background staining appears to be minimal.

The antisera against the synthetic rabbit CSI peptide proved to be highly specific and was able to distinguish some of the isoforms of CSI. The specificity of the antibody in localizing CSI in rabbit tissues was demonstrated by the single band obtained from a Western blot for spleen, intestine, adrenal and lung (Fig3-13A). It has very low cross-reactivity with other rabbit corticostatic peptides (Fig.3-13B). Cross-reactivity with the corticostatic peptides from other species such as rat and human was not detectable (Fig.3-13B). However the titres of the antisera are relatively low from all 5 guinea pigs used to obtain antibodies. We had to use 1:2400 and 1:100 dilution for radio-immunoassay and immunocytochemistry studies respectively. Despite its low titre, the background for both assays were reasonably low. This is possibly due to the high specificity of the antisera. Similar data on the specificity of a synthetic rabbit CSI antisera also prepared in guinea pigs was recently published by Tominaga et al (427).

In our study, the immunoreactivity of CSI was focal and was seen in immunocell rich tissues such as spleen and lung, as well as some other tissues such as zona reticularis and fasciculata of

the adult adrenal, in villus cells of the small intestine, and in anterior pituitary cells. The intensity and distribution of CSI varied among the tissues. For example, CSI staining was strong in the spleen and lung but weak in the adrenal. The immunolocalization of CSI in tissues in general is consistent with the immunoreactivity of CSI in these tissues measured by RIA. For instance, large amounts of CSI have been measured in the placenta by RIA where a strong CSI staining was also detected.

4.2.3. CSI in the immune system

It is known that bone marrow and peritoneal neutrophils contain a large amount of CSI (299). Immunocytochemistry studies show that the predominant CSI-immunostained cells in lungs and spleen were large macrophages (Fig.3-25). At present the possibility cannot be excluded that the presence of CSI in macrophages may be due to the release of this peptide from the neutrophil granule in these tissues and its uptake by adjacent macrophages. Several CSI immunoreactivity peaks were eluted from spleen but on further chromatography only CSI could be identified (Fig.3-14). The difference between this data and the data reported by Tominaga et al. (427) may be due to the method used in extraction of the tissues. As we discussed in section 4.1.1.1. artifact formation may be due to the boiling method used by Tominaga et al (427).

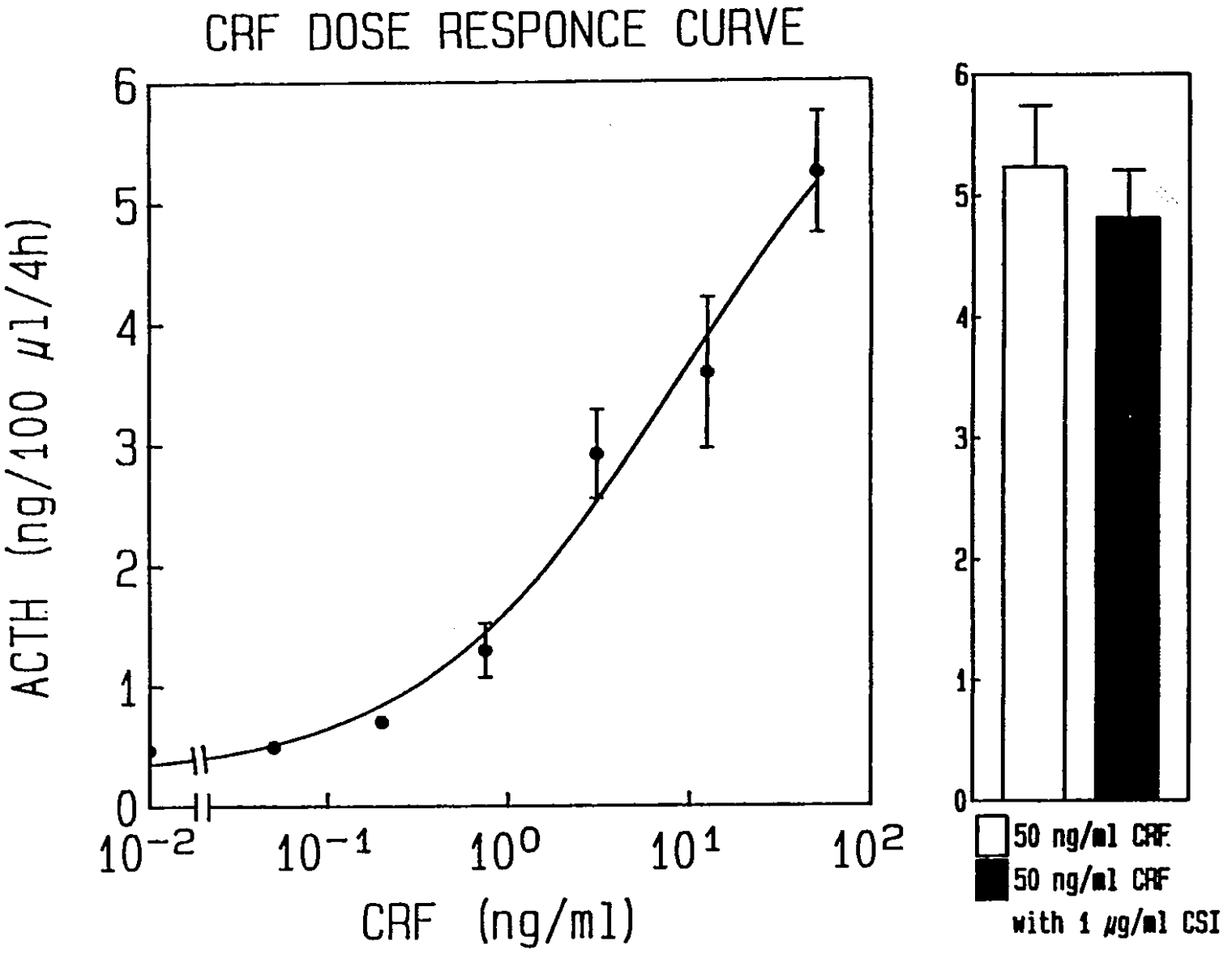
Both RIA and immunocytochemistry studies demonstrated that liver, with its rich blood supply, and kidney contain

undetectable amounts of CSI. This raises a very interesting question. As we now know, biosynthesis of these peptides is turned off after monocyte differentiation into macrophages, because the mRNA of some corticostatic peptides could not be detected in these mature cells (24). Corticostatic peptides must therefore be synthesized during stem cell differentiation. There is a possibility that corticostatic peptides are only synthesized in subsets of leukocytes during maturation. Corticostatin-positive cells migrate to the spleen and lung, but corticostatin-negative ones migrate to liver and kidney. Therefore, corticostatic peptides might be involved in an unknown but very important maturation and sorting mechanism.

4.2.4. CSI in the central nervous system.

Our RIA results on the CNS show that CSI was unevenly distributed in rabbit brain. In the normal female rabbit, the pituitary contained the highest amount of immunoreactive CSI and this was followed by the hypothalamus (Fig.3-16) which may imply that CSI is possibly involved in the hypothalamus-pituitary-adrenal axis. However, CSI had no effect on CRF stimulated ACTH release in rat pituitary cell culture (Fig.4-2). So far whether CSI has any effect on other neuropeptides in this area of the brain is unknown. The CSI-immunostaining can also be detected in the anterior pituitary with one third of the cell population being positive (Fig.3-27). The type of the cells stained has not

Figure 4-2. The effect of CSI on the CRF stimulated-ACTH production in rat pituitary cell culture. The left panel shows the CRF dose response curve and the right panel shows CRF alone (open bar)and CRF plus CSI (solid bar) in the rat pituitary cell culture. The results are the mean of four separate experiments, each measured in duplicate. The vertical lines represent the standard deviations.


CRF DOSE RESPONCE CURVE
ACTH (ng/100 µl/4h)
0
1
2
3
4
5
6
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
CRF (ng/ml)
50 ng/ml CRF
50 ng/ml CRF
with 1 µg/ml CSI

as yet been determined. Similar results were also reported by Tominaga et al. (427). However, there is a striking difference in the amounts of CSI found in the normal female rabbit pituitary in our study (ca. 1100 ng/mg DNA) and that reported by Tominaga et al (427) which was 5.6±0.27 ng/mg wet tissue weight. This difference may due to the extraction method they used as we discussed in section 4.1.1.1.. Similar differences were noted for other tissues in our study compared to their results.

In the brain, CSI staining is only present in the blood vessels of the hypothalamus which are associated with macrophages. We do not have any evidence to suggest that the uneven distribution of CSI in the brain is due to some uneven distribution of the blood vessel in the brain. The presence of CSI in the CNS needs to be further evaluated.

4.2.5. CSI in the adrenal grand

We isolated and characterized the corticostatic peptides based on their biological activities in vitro particularly by their specific inhibitory activity on the ACTH-induced adrenocortical steroidogenesis (8). The results from immunocytochemical studies on the adrenal gland are quite different from the results obtained in other tissues. First CSI immunostaining was found directly in or on endocrine cells of zona reticularis and fasciculata and not in the immune cells such as macrophages (Fig.3-27). This is in contrast to the report by Tominaga et al. (427) who reported CSI immunostaining in

adrenal medullary cells. Second, unlike lung and spleen, CSI positive stainings in the adrenal was not abolished with preabsorption but surprisingly was slightly enhanced by using CSI preabsorbed antiserum (data not shown). Like other tissues, there was no immunostaining on the adrenal gland if preimmune-serum is used. Our interpretation of these results are as follows: the staining of adrenal and other tissues are somehow different in nature, and the antibody and antigen (CSI) complex in the preabsorbed antiserum can still bind to the adrenal possibly to some receptors such as the ACTH receptor but cannot bind to other tissues. This might imply that the antiserum used here and the adrenal CSI binding proteins bind to a different part of the CSI molecule. Recent experiments support the hypothesis that the antiserum was unable to abolish the anti-ACTH activity of CSI at 1:100 dilution in the rat adrenal cell bioassay in vitro (data not shown here). To date it is not known whether the adrenal is able to produce endogenous CSI, these immunocytochemistry results suggest that adrenal is one of the important target tissues of CSI.

4.2.6. CSI in the small intestine

We have recently found that corticostatic peptides can cause volume reduction in guinea pig villus enterocytes by activating L-type Ca^{2+} channels (440). In this study, immunoreactivity of CSI was observed in the villus cell of rabbit small intestine (Fig.3-26). This further supports the hypothesis (21) that

corticostatic peptides might also be involved in the calcium channel regulation.

As mentioned in the introduction, cryptdin cDNA encodes the precursor of a corticostatin/defensin-related peptide that accumulates in high levels in mouse intestinal crypt epithelium during postnatal development. The physiological role of cryptdin in the small bowel remains to be determined. Cryptdin may inhibit bacterial translocation, modulate intestinal hormone synthesis, influence hormonal sensitivity of the intestinal epithelium, or exhibit a multiplicity of related activities. In our study, there was no CSI staining in the Paneth cells which are the richest source of cryptdin. This suggests that CSI and cryptdin are not produced by same type of cell in the small intestine. It is therefore possible that there are unique Paneth cell peptides in the rabbit small intestine as has been shown for the mouse. It will be extremely interesting to know how these very similar genes are regulated and by what factors.

4.3. CSI and parturition

As discussed in the first chapter, parturition in mammals is under the control of neural as well as endocrine and possiblly immune factors as well. The rise in fetal glucocorticoids acts as a trigger for the onset of parturition in some species (331,337,443). We have to keep in mind that the fetus is endowed with several unique and transient endocrine organs, and also that

many factors, such as neuropeptides, can be synthesized in many tissues of the fetus or embryo.

A systematic rise in fetal ACTH and other POMC-derived peptides during pregnancy has been well documented in sheep (444). This increase is accompanied by the maturation of the fetal HPA axis. However, the final rise in fetal glucocorticoids which acts as a trigger for the onset of parturition is not preceeded by a significant increase of ACTH. The question as to how the responsiveness of the adrenal gland to ACTH increases during the onset of parturition has not yet been answered. Is there a factor other than ACTH that might be involved in the process. One clue that suggests that the immune system is actually involved in parturition came from the observation that infection during pregnancy is commonly associated with the premature onset of labor. The infection can be localized in uterine or extrauterine maternal tissues or in the extraembryonic fetal membranes (445). We noticed that rabbit plasma CSI levels are much higher in rabbits with surgical infection than normal ones, and also the presence of large amounts of corticostatins in the fetal lung (8) at a time when HPA axis undergoes activation.

4.3.1. The level of CSI in the fetal and maternal plasma and tissues

The amounts of immunoreactive CSI present in various tissues of the brain (Fig.3-16 and 3-17) at 24,27, and 30 days of gestation indicates that more of the peptide was present in the

rabbit maternal brain than in the fetal brain during gestation. Surprisingly, the levels of CSI in the pituitary of the normal female rabbit brain were much higher than that in the mother and fetus. The significance of these changes of CSI level is not clear. Fig.3-16 indicates that the concentration of CSI in the fetal pituitary remains relatively constant between 24-30 days of gestation at a time when there is a four fold increase in plasma ACTH (Fig.3-20). In contrast, maternal ACTH remains constant throughout this period but there is a two fold decrease in CSI levels in the pituitary on day 30 of gestation. Both the lung and the placenta are by far the richest source of CSI. The increase in pulmonary CSI occurs between 27 and 30 days in the mother and between days 24 and 27 days in the fetus. Moreover, in the mother, the increase in plasma CSI precedes the increase in lung CSI while in the fetus, the decrease in plasma CSI occurs at a time when the lung CSI levels are the highest. Therefore, one may conclude that the lung is not the only source for the circulating levels of CSI. It is possible that the placenta contributes to the circulating levels of CSI but the relative amounts secreted by the placenta into the fetal circulation may decrease after day 27 of gestation and a greater proportion may be secreted into the maternal circulation. As mentioned in the Introduction, the placenta can elaborate POMC peptides and CRF, and whether CSI is synthesized in the placenta remains to be determined. It is possible that CSI could interact with other

peptides formed in the placenta to modulate physiologic processes during pregnancy.

The comparison studies of fetal and maternal tissue by RIA shows that the amount of CSI in the fetal adrenal were much higher than that in maternal and normal female (Fig.3-18). However CSI immunostaining was undetectable in the fetal adrenal by immunocytochemistry. The reasons for this is unknown.

Fig.3-18 clearly shows that fetal adrenal levels of CSI appear to decrease two-fold during gestational days 24 to 30. The circulating level of CSI in the fetus decreases two fold as well (Fig.3-20A). The maternal plasma CSI levels increase from 14.9 to 44.7 ng/ml (Fig.3-20A). It has been shown for the rabbit that the fetal adrenal begins to differentiate into distinct zonas at about 20 days of gestation and by day 22 they elaborate measurable amounts of F and B (446). Therefore, it is very attractive to hypothesize that CSI plays an important role in modifying the response of adrenal cells to ACTH such that the rise in fetal F and B levels between 24-30 days of gestation (446) may be a consequence of the relative changes in ACTH and CSI in the fetal rabbit. During this period, the concentration of CSI in fetal plasma decreases at a time when there is a four fold increase in fetal plasma ACTH (Fig.3-20). In contrast, maternal ACTH remains relatively constant during the last 7 days of gestation but there is a increase in levels of CSI. The significance of this increased level of CSI in the maternal

rabbit and decreased levels of CSI in the fetus is still unknown. Considering that CSI also has potent histamine-releasing activity (detail in section 4.5.2) and L-type Ca^{2+} channel activation activities, the hypothesis that CSI plays some important role in the initiation of parturition becomes very attractive.

4.3.2. Effects of CSI on length of gestation

Results obtained from in vitro studies have greatly enriched our understanding of the possible physiological roles of these corticostatins. In order to study their effects on the endocrine system and to test our hypothesis that CSI might be involved in parturition, we did a series of in vivo studies designed especially to evaluate the possible effect of CSI on the length of gestation.

The half life of CSI in the blood circulation is very short, it has been estimated to be from 1 to 10 min as determined in studies where ^{125}I-CSI was injected into the tail vein of the rat and blood levels of the labeled peptide were determined. According to results obtained from in vitro studies we have to maintain CSI at a concentration 500-fold higher than ACTH to be able to see a significant depression of glucocorticoid production. To maintain a pharmacological dose of CSI in the adult animal was too costly. Therefore, we injected CSI or its antisera directly into fetuses. The control group was injected either with saline or preimmune sera. There was no acceleration of delivery of the newborn either by injection of CSI or CSI

antibody (Table 3-5). However the plasma levels of ACTH and cortisol both increased while the levels of CSI and corticosterone were unchanged in the plasma of the newborn after administration of CSI indicating that CSI had caused a rebound in ACTH concentration (Fig.3-21). Furthermore, the CSI level in the fetal adrenals within the CSI treated group were significantly higher than those within control groups treated with saline or preimmune sera (Fig.3-22). These results are not sufficient to conclude that CSI caused a direct desensitization of the adrenal gland which was overcome greater secretion of ACTH but it does support the hypothesis that CSI is involved in adrenal gland regulation. Many more experiments are needed to be able to make definitive conclusions from such studies.

There was no acceleration of delivery of the newborn and there was no significant difference in plasma levels of ACTH, CSI, B and F following injection of the CSI antisera (Fig.3-23). These results were consistent with the view that CSI and antibody complex are still biologically active. It is still too early to reach the conclusion that CSI has an important role to play in the onset of the parturition.

In future studies a more potent synthetic corticostatin should be used and administered by an implanted drug delivery pump to achieve more precise control of the levels of corticostatic peptides. The blocking of corticostatins in the parturition process will be very hard to do because we have to

titrate accurately the amount of antiserum to inject. Another approach to this problem is to use transgenic techniques to knockout the corticostatin gene by double replacement strategies.

4.4. The effects of CSI on the zona glomerulosa of the rat adrenal gland

As we discussed in the Introduction, the zona glomerulosa of the rat adrenal is mainly under angiotensin II control which results in the production of aldosterone. Other factors such as ANF, αMSH or ACTH also play an important role in aldosterone production. There is some controversy about the populations of ACTH and αMSH receptors on the zona glomerulosa and on the zona fasciculata (447). The results from our laboratory were mostly obtained from studies on zona fasciculata and these results suggest that CSI was unable to inhibit αMSH-stimulated corticosterone production in the isolated rat adrenal cell bioassay (288) but it inhibits ACTH-stimulated corticosterone production by competing with the Ala-Arg-Arg-Lys-Lys motif of the ACTH molecule when it binds to its receptor (288).

4.4.1. CSI and angiotensin II, ANF and αMSH

Steroid production by collagenase-dispersed rat adrenal glomerulosa cells was highly responsive to angiotensin II and ACTH. CSI had no effect on the increase of aldosterone and B production elicited by angio II (Fig.3-28) but did inhibit the ACTH-stimulated increase in aldosterone and B production in the

same way that it does on the zona fasciculata cells (8).

ANF can directly inhibit basal aldosterone secretion and antagonize the stimulatory effects of ACTH and angio II on the secretion of aldosterone by rat adrenal glomerulosa cells (242). Since the biological activity of ANF is an integral part of the homeostatic mechanisms regulating sodium retention this suggests that ANF may be responsible for the attenuated effects of ACTH and Angio II on the adrenal cortex during sodium loading. It is not surprising that CSI has no effect on ANF inhibition of formation of both basal and stimulated aldosterone production (Fig.3-29).

αMSH potentiates the effect of ACTH in adrenocortical steroidogenesis by decreasing the EC_{50} of ACTH on zona fasciculata B, and on zona glomerulosa aldosterone production. αMSH at 0.5 nM markedly increased aldosterone production in rat zona glomerulosa cells and slightly increases B production at 10^3 nM. CSI can inhibit α-MSH stimulated aldosterone synthesis (Fig.3-30). In the dispersed rat adrenal cell system, αMSH only increases B production at much higher concentrations (10 μM) and this action is not inhibited by CSI although the stimulatory activity of $ACTH_{1-18}$ amide was inhibited (Fig.3-31). The question we wanted to answer is whether that CSI interacts with the αMSH receptor in zona glomerulosa cells and inhibits the formation of aldosterone by this mechanism.

4.4.2. Radioligand binding assay

CHAPTER 4 DISCUSSION

Radioligand binding assays are useful in providing biochemical data concerning peptide-receptor interactions that precede the biological response. Labelling peptides as agonists or as antagonists would be a preferable approach for binding studies, because agonists or antagonists afford significant advantages in metabolic stability. In the present study, a potent agonist for αMSH, [Nle^4,D-phe^7]αMSH (448), has been used for binding studies.

Many investigators have used crude or semi-purified membrane preparations from various tissues to identify and characterize peptide receptor interactions. The membrane preparation used for the αMSH and CSI studies was first described by Leiba et al (423). The purpose of the two centrifugation steps in our experiments was to remove any soluble interfering substances such as guanine nucleotides which may interfere with the radioligand-binding assay.

Radioligand binding studies of peptide receptors pose several problems. First, peptides have a propensity to absorb non-specifically to many materials commonly used in binding assays, including glass and plastic polymers. Several methods of circumventing this problem have been used, including presoaking filters in BSA or other large inexpensive proteins. We have used filters soaked in 1mg/ml BSA in binding assays to reduce nonspecific binding of αMSH. Tracer degradation can be another major problem in peptide assay, especially in membrane

preparations in which protease activity can be quite high. Use of specific inhibitors of suspect peptidases are required to minimize this problem. In the present study, DTT, leupeptin, PABA and trypsin inhibitor were used to protect membrane function.

4.4.3. αMSH receptor study in the cell membranes of the lacrimal gland and the zona glomerulosa of the adrenal gland

In general, the αMSH receptor binding results (Fig 3-32 A) in our system are consistent with results previously reported by Leiba et al.(423) except that we did not find several magnitudes difference between [Nle^4, D-Phe^7]-αMSH, and αMSH in the rat lacrimal gland. A similar displacement pattern was also found in the zona glomerulosa cell membrane preparation. This may be due to the fact that the amount of iodinated [Nle^4, D-Phe^7]-αMSH used in these studies was higher (14 nM) than that used by Lieba et al (423) which was 2 nM. At the higher concentration maximum aldosterone production was obtained. We were, therefore, unable to detect the higher affinity binding site for this analogue. Because the main purpose of the current study was to find out whether the αMSH receptor in zona glomerulosa has the same property as the one in the lacrimal gland and whether CSI can specifically displace the radioligand from binding to the receptor. A relatively higher concentration was used in order to obtain more reliable and reproducible results at the cost of sacrificing assay sensitivity. Scatchard analysis (Fig 3-33) confirmed these results and suggested that there was only one

class of αMSH receptor in these two glands. In both systems, CSI was able to displace αMSH at a concentration above 1μM (Fig 3-32). In contrast to the αMSH receptor binding study, none of these peptides were able to displace iodinated CSI from binding to its membrane binding protein (Fig 3-34). Similar results also have been obtained using a membrane preparation of adrenal cells (426). These results are quite different from Tominaga et al. (449) who found ACTH was able to displace iodinated CSI from its membrane binding protein using adrenal cell preparation. We were unable to obtain a higher specific binding site for CSI in both zona glomerulosa of adrenal glands and lacrimal gland. Attempts to obtain Scatchard analysis of the results have never been successful. Based on these observations, the following conclusions were made. The αMSH receptor in the zona glomerulosa may share some properties with the one in lacrimal gland. CSI inhibits αMSH-stimulated aldosterone production in the dispersed zona glomerulosa cells by competing for αMSH binding sites. CSI binds to some other membrane components which are not displaceable with ACTH and αMSH.

As mentioned in the Introduction, there is a close structure relationship between ACTH and αMSH, their receptors also belong to the same subfamily of receptors coupled to G proteins (107). They cause an activation of adenylate cyclase and have an absolute requirement for extracellular Ca^{2+} to facilitate ligand binding (450,451). The melanophore receptors may be triggered not

only by ACTH-(4-10) but also by the C-terminal part of αMSH-(11-13) (452). CSI inhibits corticosterone production by competing with ACTH binding to its adrenal receptor. However CSI has been found to be a weak agonist in the αMSH bioassay using Anolis lizard skin (302). This is also consistent with the data showing no antagonistic effect of CSI on αMSH stimulated corticosterone production in the rat adrenal bioassay (288).

4.5. General discussion

4.5.1. Corticostatic peptides and their cDNAs

The cDNA coding for the three known human defensins has been reported as the first cDNA structure of any member of the CS/defensins family (437,438,453,454). The clones encoding HP-1 and HP-3, and probably HP-2, have been characterized. Nucleotide sequence analysis provides evidence that the defensins are synthesized as 94-amino acid precursor proteins which are processed to form the mature peptides. The clones encoding HP-1 and HP-3 differ by just two nucleotides; one in the coding region which accounts for the different N-terminal amino acids present in the mature peptides. HP-2 may be formed by degradation or processing of HP-1 and/or HP-3, because it lacks this N-terminal amino acid and is otherwise identical to the other two human defensins. Daher et al found that defensin mRNA were detected in unfractionated bone marrow but not in normal peripheral blood leukocytes, which contained 61% neutrophils (438). Their

hybridization results suggested that defensin RNA synthesis may occur primarily in neutrophil precursor cells in the bone marrow (438). Defensin transcripts was also detected in the peripheral blood leukocytes of some leukaemia patients (438). But there is no evidence for defensin expression in human macrophages (438). These results define important aspects of the mechanism of synthesis and the tissue-specific expression of a major group of neutrophil granule proteins.

Very recently, the cDNA clones encoding the HP-4 precursor (human corticostatin) have been isolated from a human bone marrow cDNA library (455). The nucleotide sequence shares about 72% identity with the cDNA encoding defensin HP-1, but differs from it, and from other cDNAs of this family characterised to date, by an extra 83 base segment. This extra segment is not adjacent to an intron, and is apparently the result of a recent duplication within the coding region corresponding to most of the mature HP-4 peptide. The HP-4 cDNA is typical of other corticostatin/defensin cDNAs which have been studied. However based on its distinct amino acid sequence in comparison with HP-1,2,and 3, there is a suggestion that the divergence of HP-4 and HP-1 genes commenced more recently than the divergence of the HP-1/HP-4 lineage from the rabbit and guinea pig corticostatin/defensin genes so far characterized.

The cDNAs encoding the two rabbit corticostatins, CSIII(MCP-1,NP-3) and CSIV(MCP-2,NP-4), and the tissue-specific expression

of the CSIII and CSIV genes were described by Ganz et al (437). The two genes are highly homologous throughout and are closely linked, reflecting the homology between the two corticostatins. Such duplications thus appear to be very recent manifestations of the evolutionary process within each species. Very recently, Michaelson et al (436) isolated and sequenced three rabbit corticostatin cDNAs that encode for preproprotein precursors for the mature corticostatin/defensins CSI(NP-3a), CSV(NP-4), and CSVI(NP-5). As for the previously characterized preprodefensins, they lack consensus sequences for N-linked glycosylation, suggesting that defensins are targeted to lysosome-like granules by a mechanism not dependent on the mannose-6-phosphate receptor (436).

As discussed previously, the cDNA clones encoding guinea pig neutrophil cationic peptides (GPCS1 and GPCS2) have been isolated from a bone marrow cell cDNA library (434). Analysis of all seven known procorticostatins/defensins revealed a structure wherein an anionic propiece neutralizes the cationic nature of the mature peptide. Because the defensins apparently require cationic epitopes for cell membrane permeabilization and cytotoxicity, charge neutralization of mature peptides by their anionic propieces may prevent autotoxicity during defensin synthesis and processing.

Cryptdin mRNA is one of many low molecular weight mRNAs that accumulate in the mouse small intestine during postnatal

development and code for a 6-kD cysteine-rich polypeptide (314). CS/defensins isolated from all sources contain a cysteine- and arginine-rich consensus sequence that confers a beta plated sheet structure on these related proteins (456). Previously the identification of intestinal cryptdin, CS/defensins had been found only in cells of myeloid origin (8,297,300,433,439 and 457). Expression of this CS/defensin-related gene in intestinal epithelium, and its presence in adult testis and brain, provides an opportunity to define broader physiologic functions for cryptdin and perhaps other CS/defensins in cells of none myeloid origin.

4.5.2. Biological activities of corticostatic peptides

Antibacterial activity of the defensins exhibit a remarkably broad array of properties in vitro (457). At micromolar concentrations, many defensins kill a wide variety of gram-positive and gram-negative bacteria and fungi. Some defensins are also effective against enveloped viruses, including herpes simplex and vesicular stomatitis. The work of Lehrer and colleagues shows that defensins permeabilize both the inner and outer membranes of Escherichia coil, and that inner-membrane permeabilization is coincident with cell death (458). A membrane potential is apparently required for defensin action, since cells are killed only when metabolically active and they are protected by membrane-depolarizing agents such as carbonylcyanide M-

chlorophenylhydrazone (CCCP). The high-resolution crystal structure of the defensin HP-3 which was determined by Hill et al. (459) reveals a dimeric ß sheet that has an architecture very different from other lytic peptides. HP-3 crystallizes as a dimer. The two molecules in the asymmetric unit are in close contact and are related to each other by a local twofold rotation axis. Three ß sheets of the monomer are extended across this interface to form a six-stranded sheet in the dimer. The dimeric assembly suggests mechanisms by which defensins might bind to and permeabilize the lipid bilayer.

The corticostatic and defensin activities seem to be completely dissociated since there is no positive correlation between them. In the rabbit, CSI is the most potent ACTH antagonist and in contrast it is the least potent cytotoxic agent. Similarly, CSVI is a weak corticostatic peptide but does not kill fumigatus hyphae at concentrations up to 100 µg/ml (460). It is also true in the human that the corticostatic peptide HP4 has no cytotoxic activity and HP1, a human cytotoxic peptide which shares great homology in structure with corticostatins, has no detectable corticostatic activity in the dose range used (300). The only exception is rat corticostatin R4 which has both potent corticostatic activity (IC_{50}=50 nM) and cytotoxic activity (IC_{50}=2 µM) (302). GPCS1 was isolated from guinea pig neutrophils and found to be antimicrobial (433) and anti-ACTH (461).

CHAPTER 4 DISCUSSION

The corticostatic peptides, but not the defensins, have been found to stimulate L-type Ca^{2+} channels in guinea pig jejunal villus enterocytes. Recently It has been found that corticostatic peptides are also active in the release of histamines from rat peritoneal mast cells (435). The most active corticostatic peptide CSI from the rabbit was the most potent in releasing histamine followed by mixture of GPCS1&GPCS2 and GPCS3. HP-4 was less potent in histamine release while the defensin HP-1, which has no corticostatic activity, was the least potent. CSI was capable of releasing histamine at concentrations as low as 0.006 nmoles/ml and at 1.26 nmoles/ml 67% of the histamine was released. These peptides did not affect cell viability. It appears that there is some correlation with corticostatic activity and histamine release. GPCS3 is an antiparallel heterodimer with a charge of +9 and 1/80th the corticostatic activity of CSI but with a fairly good ability to release histamine (71% at 2.5 nmoles/ml). Mast cells have been located in the adrenal (462). Histamine, as well as serotonin, is capable of stimulating steroidogenesis (463,464). Thus the corticostatic peptides may control adrenal formation of steroids by yet another mechanism involving mast cell histamine and serotonin release.

4.5.3. The role of corticostatic peptides in the immune-endocrine system

The interactions of the immune-endocrine system has been

well documented for some time (465). The importance of normal adrenal function to the immune reaction was also noticed in human endocrinology. In patients with Cushing's disease, infections are a major cause of death (466). Hyposecretion of glucocorticoids also increases susceptibility to infection (467). As mentioned in the Introduction, glucocorticoids exert a negative control over the immune system (193,194) and macrophage derived-IL-1 and other cytokines act as potent CRF releasing factors in the hypothalamus and pituitary (215,216,219). This immune and endocrine bi-directional regulation can explain the old observation that glucocorticoids cause monocytopemia (468), and lymphocytopenia (469). However, it is still difficult to explain how glucocorticoids stimulate neutrophil egress from bone marrow and cause neutrophilic leukocytosis (470).

It is of great interest that there are large amounts of corticostatic peptides present in the macrophages and the neutrophils of spleen and lung tissues. As discussed previously, in some rabbits infected during surgery, blood circulation of CSI levels were as high as 300 to 500 ng/ml (data not shown here), whereas the level of CSI is only about 40 ng/ml in the normal situation. Tominaga et al also reported that the concentration of CS-1-LI increased more than 20-fold during inflammation (427). These anti-ACTH peptides can be secreted in response to phorbol ester stimulation (471) and so it is likely that they enter the circulation during inflammation and cause an inhibition of

adrenal steroidogenesis. Moreover, the positive CSI immunostaining is not only present in immunocells but also in anterior pituitary cells and zona glomerulosa and fasiculata cells suggest that corticostatic peptides may directly counter act the ACTH action on the pituitary and adrenal cells by paracrine control mechanism.

The negative control of the HPA axis by the immune system has been investigated for some time (464), and evidence has now accumulated to suggest that some products of the immune system are capable of inhibiting the HPA axis at the level of the adrenal (472). These inhibitory factors, as mentioned in the Introduction, including murine macrophage factors, TGFß, and corticostatic peptides may also be involved in the negative control. However, their effects may be negated by positive mediators such as IL-1. This is a rich area for future research.

Summary

GPCS1, GPCS2 and GPCS3 were purified and characterized from guinea pig bone marrow cells. GPCS1 and GPCS2 were structuraly identical to GNCP-1 and GNCP-2 which were recently isolated from guinea pig peritoneal neutrophils and had antimicrobial activity. GPCS3 was a novel 13-amino acid peptide with a homodimer structure. This was the first corticostatic peptide found which did not have the six cysteine cocensus sequence characteristic of corticostatin/defensin family of peptides. GPCS3 also has the ability to release histamine from rat peritoneal mast cells . During the course of further study CSV and CSVI were purified and sequenced from rabbit bone marrow cells and both had corticostatic activity. Their structures were identical to NP-4 and NP-5 which were discovered as defensins. They were less potent than CSI with an $I.D._{50}$ 650 nM and 6000 nM for CSV and CSVI, respectively. Sturcture studies of the corticostatic peptides indicated that two C-terminal arginines of CSI were not crucial to its anti-ACTH activity, but they were strong potentiators; removal of the two N-terminal arginines of GPCS1 and GPCS2 did not change their anti-ACTH activity.

Using the specific CSI antibody, RIA and immunocytochemistry we were able to demonstrate that CSI was unevenly distributed in the rabbit. It was prominent in the phagocytes (macrophages and neutrophils) rich tissues (e.g. lung and spleen). CSI

immunostaining was also found in the cytoplasm of the anterior pituitary gland with one third of the cell population staining positive. It was found in the zona reticularis and fasciculata cells of adrenal gland, and in the surface epithelium of the small intestine. Immunoreactive CSI was undetectable in kidney, liver, heart and ovary.

In vivo studies showed that CSI was developmentally regulated in both mother and fetus of the rabbit during days 24, 27 and 30 of gestation. Large amont were measured during gestation in a number of fetal and maternal tissues such as pituitary, adrenal, lung, spleen and placenta, and in plasma and changes in concentration with advancing gestation indicated a physiological role for corticostatic peptides during pregnancy.

The mechanism studies of CSI action on rat zona glomerulosa cells indicated that CSI had no efffect on the action of angiotensin II or ANF. However CSI inhibited αMSH-stimulated aldosterone production in the dispersed zona glomerulosa cells by competing for αMSH binding sites, but not the MSH-stimulated corticosterone production in the zona reticularis and fasciculata.

Conclusions

1. Corticostatins belong to a corticostatin/defensin peptide family of peptides with a highly conserved consensus sequence, rich in cysteine and arginine.
2. 14 corticostatic peptides have been identified so far from rabbit, human, rat, and guinea pig. All of them contain either C-terminal or N-terminal arginine(s) and a arginine-rich region between the second and third cysteines.
3. The loss of N-terminal basic residues does not alter their anti-ACTH activities, but the loss of C-terminal arginines reduces these activities.
4. The number and position of basic residues between the second and third cysteines are critical to their anti-ACTH activities.
5. Corticostatic peptides also have histamine-releasing and calcium channel activation activities and can inhibit αMSH-stimulated aldosterone production in rat adrenal glomerulosa cells. These activities are highly specific and operate in a concentration dependent manner. The detailed mechanisms for histamine-releasing and calcium channel activation are unknown, but the anti-ACTH and anti-αMSH activities of corticostatin I act through competition for the receptor binding sites.
6. Corticostatin I-immuno staining was found predominantly in

the macrophages and neutrophils and to a less extent in the zona reticularis and fasiculata cells of the rat adrenal, in the surface epithelium of the small intestine and some parts of CNS such as the pituitary.

7. Corticostatins/defensins are also growth and differentiation associated peptides which can only be synthesized in some committed progenitor cells.
8. Corticostatin I seems to be involved in the regulation of HPA during parturition, but its exact role is unknown.
9. cDNAs of some number of this family have been cloned and characterized. It will be very interesting to know how these genes are being regulated.

Claims to original research

1. Two new rabbit corticostatic peptides, CSV and CSVI, were purified and identified from rabbit bone marrow cells. CSV had an ID_{50} of 650 nM and CSVI and ID_{50} of 6000 nM in the rat adrenal cell bioassay.

2. The finding of corticostatic peptides in the guinea pig bone marrow extracts and sequential purification and characterization of three guinea pig corticostatic peptides GPCS1, GPCS2 and GPCS3.

3. Unlike GPCS1 and GPSC2, GPCS3 was a novel peptide. It is a 13 member homodimer with corticostatic activity. GPCS3 was the first corticostatic peptide found that did not have the cysteine cocensus sequence of the corticostatin/defensin family of peptides.

4. Experimentally demonstrating that two C-terminal arginines of CSI were not crucial for its anti-ACTH activity and that two N-terminal arginines of GPCS1 and GPCS2 were not important to their biologic action.

5. Quantitative CSI radioimmunoassays of HPLC-fractions from various tissue extracts demonstrated that CSI is distributed

both in the peripheral and CNS system.

6. Cellular localization of CSI by immunocytochemistry studies demonstrated that it was mainly localized to macrophages in spleen, lung and placenta. The CSI immunostaining was also found in the cells of anterior pituitary gland, zona reticularis and fasiculata cells of the adrenal gland and the epithelial cells of the upper half of the villi of the small intestine.

7. In vivo studies showed that the amounts of CSI present in tissues was developmentally regulated in the fetus. The change of CSI levels both in fetal and maternal tissues during the last six days of pregnancy suggested a possible involvement of corticostatic peptides in rabbit parturition.

8. CSI inhibited the α-MSH-stimulated aldosterone production in the zona glomerulosa by competing for α-MSH binding sites, but not the α-MSH-stimulated corticosterone production in the zona reticularis and fasciculata. CSI had no effect on the action of angiotensin II or ANF in the zona glomerulosa of the rat adrenal gland.

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