Glucocorticoid receptor expression in target cells: ligand regulation, target gene responses and immunocytochemical localization

by
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A Thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

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June, 1995

To my parents, Xudong, Edmond and Eric

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ABSTRACT

Three aspects of the expression of the glucocorticoid receptor (GR) in rat tissues were examined. First, by using a radioimmunoassay to quantitate GR levels, a tissue-specific regulation of GR expression by glucocorticoids was found. While GR levels were decreased by glucocorticoids in cardiac atria of adrenalectomized rats, liver, cardiac ventricles, pituitary, hypothalamus and hippocampus, they were increased in the cardiac atria of the intact rats and spleen. GR levels in the thymus showed a biphasic time response to glucocorticoids, with an initial increase of GR level followed by a decrease. Second, the relevance of the tissue-specific regulation of GR was investigated in the cardiac tissues, using atrial natriuretic factor (ANF) as a model target gene to GR. A correlation between the GR levels and the ANF responses to glucocorticoids was shown, suggesting that the stimulatory effect of glucocorticoids on ANF synthesis was mediated by the cardiac GR and the magnitude of a target gene response to glucocorticoids can be regulated by modulating the GR level. Finally, by using immunocytochemistry, a cell-specific expression of GR was observed. GR was localized in the cardiac cells, the Leydig cells of the testis and the granular convoluted tubular cells (GCT) of the submaxillary gland. In addition, ANF was localized in the same cardiac cells which contained GR, supporting the possibility that glucocorticoid regulation of ANF synthesis was mediated by the cardiac GR. GR was colocalized with ANF, α 2u globulin and epidermal growth factor in the same GCT cells, suggesting roles of glucocorticoids in regulating these molecules therein. Adrenocorticotropic hormone (ACTH), a pro-opiomelanocortin (POMC) related peptide, was localized in the same Leydig cells which contained GR, suggesting that the presence of GR in the POMC containing cells does not always assure a response of the POMC gene to glucocorticoids and other tissue-specific factors may be involved in conferring POMC responsiveness to glucocorticoids, as glucocorticoids are known to inhibit POMC gene transcription in the anterior lobe of the pituitary but they do not regulate the same gene in the testis.

RÉSUMÉ

Trois aspects de l'expression du récepteur pour glucocorticoïde (GR) ont été étudiés. Premièrement, une méthode radioimmunologique a été utilisée pour quantifier le niveau de protéines du récepteur chez le rat. Les résultats démontrent que la régulation de l'expression du récepteur par le glucocorticoïde est spécifique à chaque tissu. Les niveaux de GR ont diminué suite à l'administration de glucocorticoïdes dans les oreillettes des rats adrenalectomisés, le foie, les ventricules, l'hipophyse, l'hypothalamus et l'hippocampe, et ont diminué dans les oreillettes des rats intacts et dans la rate. Les niveaux de GR dans le thymus ont répondu de manière diphasique en fonction du temps en réponse aux glucocorticoïdes, en présentant initialement une augmentation suivie d'une baisse des niveaux de GR. Deuxièmement, la signification de la régulation spécifique des GR a été étudiée dans le tissu cardiaque en utilisant le facteur natriurétique des oreillettes (ANF) comme gène cible modèle du GR. Une corrélation entre les niveaux de GR et la réponse du ANF aux glucocorticoïdes a été démontrée, suggérant que les GR cardiaques participent à l'effet stimulant des glucocorticoïdes sur la synthèse de ANF et que l'amplitude de la réponse du gène cible aux glucocorticoïdes peut être modifiée en fonction des niveaux de GR. Finalement, à l'aide d'immunocytochimie, l'expression du récepteur spécifique à la cellule a été démontré. Le GR a été localisé dans les cellules cardiaques, les cellules de Leydig des testicules et les cellules granuleuses (GCT) de la glande sous-maxillaire. De plus, le ANF a été localisé dans les mêmes cellules cardiaques contenant des GR, ce qui appui la possibilité que les GR cardiaques interviennent dans la régulation de la synthèse du ANF par les glucocorticoïdes. Les GR étaient colocalisés avec l'ANF, l' α 2u globuline et le facteur de croissance épidermal dans les GCT, suggérant que les glucocorticoïdes interviennent dans la régulation de ces molécules à l'intérieur de ces dites cellules. En plus des GR, l'hormone adrénocorticotropique, un peptide relié au proopiomelanocortine (POMC), a également été localisé dans les cellules de Leydig, ce qui suggère que la présence des GR dans les cellules contenant le POMC n'assure pas toujours une réponse du gène POMC aux glucocorticoïdes et que d'autres facteurs spécifiques aux tissus pourraient moduler la réponse du POMC aux glucocorticoïdes, puisqu'il est admis que les glucocorticoïdes inhibent la transcription du gène POMC dans le lobe antérieur de l'hipophyse et non pas dans le tissu testiculaire.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Dennis G. Osmond, the chairman of Anatomy Department and my supervisor, for giving me the opportunity of conducting my research in this department, for his enthusiasm, support and continuous encouragement throughout the course of my studies.

I am grateful to Dr. J. J. M. Bergeron, my supervisor, for his constructive criticism in writing this thesis.

I thank Dr. Tony Antakly for giving me the chance to carry out my research project and allowing me to do my experiments in his lab.

I thank Ms. Dolores Raquidan for her help in sacrificing the animals, teaching me the technique of radioimmunoassay, for her assistance in performing the affinity labelling and immunoprecipitation experiment, and most of all, for her friendship.

Dajan O'Donnell, Aria Shiffman, Rita Hannah and Andre Laferriere are thanked for their friendship, moral support, and valuable discussions.

I thank Mr. Geoff Turner for reading the manuscript.

I am grateful to Isabelle Dussault and Julie Paquette for translating the abstract into French.

I wish to extend my thanks to all the staff members and students in the Department of Anatomy, in particular, Ms. Karen Halse and Ms. Audrey Innes for their friendship and constant secretarial help.

Finally, I thank my husband, Xudong Wang. Without his love, constant encouragement and useful discussion of the experiment results, this work would not have been completed.

ABBREVIATIONS

ABC, avidin biotin complex

ACTH, adrenocorticotropic hormone

ADX, adrenalectomy

ANF, atrial natriuretic factor

ATP, adenosine triphosphate

C, cytosine

cAMP, cyclic adenosine monophosphate

CAT, Chloramphenicol acetyltransferase

CBG, corticosteroid-binding globulin

cDNA, cloned DNA

cGMP, cyclic guanosine monophosphate

CRF, corticotrophin releasing factor

DAB, 3,3'-diaminobenzidine

Dex, dexamethasone

DOC, deoxycholate

DNA, deoxyribonucleic acid

EDTA, ethylenediaminetetraacetic acid

EGF, epidermal growth factor

g, gram

G, guanine

GCT cells, granular convoluted tubular cells

GR, glucocorticoid receptor

GRE, glucocorticoid responsive element

h., hour

hGR, human glucocorticoid receptor

HPLC, High performance liquid chromatography

Hsp90, 90 Kd heat shock protein

HTC, hepatoma tissue culture cells

IC, immunocytochemistry

Kda, kilo dalton

L, liter

LH, luteinizing hormone

M, molar

mg, milligram

min., minutes

ml, milliliter

mM, millimolar

MMTV, mouse mammary tumour virus

MMTV LTR, MMTV long terminal repeat

mRNA, messenger ribonucleic acid

MSH, melanocyte stimulating hormone

NF1, nuclear factor 1

NP40, nonidet P-40

NRS, normal rabbit serum

PBS, phosphate buffered saline

Ph, $-(\log_{10}[H^+])$

POMC, pro-opiomelanocortin

RIA, radioimmunoassay

SD, standard deviation

SDS, sodium dodecyl sulfate

Tris, 2-(hydroxymethyl) 2-amino-1,3 propanediol

μl, microliter (10^{-6} L)

μM, micromolar (10^{-6} M)

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CHAPTER I. INTRODUCTION AND LITERATURE REVIEW

A. Adrenal glands and steroid hormones

Adrenal glands are paired structures that lie above the kidneys. Each adrenal gland consists of an outer cortex and an inner medulla. The adrenal cortex consists of three distinct zones. A small outer layer, the *zona glomerulosa*, is the site of mineralocorticoid synthesis. The wider, middle zone is the *zona fasciculata* that is primarily responsible for glucocorticoid secretion. The inner *zona reticularis* secretes adrenal sex hormones (androgen and oestrogen). Glucocorticoids, mineralocorticoids and sex hormones are steroid hormones. The primary effects of mineralocorticoids are on the cortical collecting tubule of the kidneys. They act to promote tubular sodium reabsorption and potassium excretion (Marver, 1981). The principal mineralocorticoid hormone is aldosterone and its secretion is primarily controlled by the renin-angiotensin system. Other regulators include sodium and potassium levels, ACTH, and neural components of the adrenergic and dopaminergic systems. The normal adrenal cortex does not secrete physiologically effective amounts of testosterone or oestrogenic substances (Migeon, 1972). The adrenal medulla consists of hormone-producing cells called chromaffin cells. These cells synthesize and secrete catecholamines (epinephrine, norepinephrine and dopamine), which are small compounds with structures that combine a catechol nucleus and an amine side chain. The proportions of epinephrine and norepinephrine found in the adrenal medulla vary with the species. In humans, the adrenal contains 15-20% norepinephrine and in rats, it contains 15% norepinephrine. Catecholamines are important neurotransmitters that mediate central nervous system and autonomic nerve functions and are important regulators of the cardiovascular system. The adrenal medulla, a highly specialized part of the sympathetic nervous system, functions under stress or whenever marked deviations from normal homeostasis occur, in contrast to the rest of the sympathetic nervous system, which is involved in the minute-to-minute fine regulation of most physiologic processes. Secretion of the adrenal medullary hormones is mediated by

the release of acetylcholine from the terminals of preganglionic fibers. The resulting depolarization of the axonal membrane triggers an influx of calcium ion. The contents of the storage vesicles are released by exocytosis. The catecholamines exert their physiologic effects by binding to receptor molecules on the surfaces of target cells. The actions of catecholamines are terminated quickly. Compared to hormones with a more prolonged action they have a relatively low affinity for their receptor and rapidly dissociate from it. The free hormone is removed by several mechanisms. These include reuptake by the sympathetic nerve ending, metabolism by the enzymes catechol-O-methyltransferase and monoamine oxidase, conjugation with sulfate ion, and direct excretion by the kidney. In addition to the catecholamines, the chromaffin cells of the adrenal medulla synthesize and secrete opiate-like peptides including met- and leu-enkephalin. Adrenalectomized individuals, having otherwise intact sympathetic nervous systems and receiving adrenocortical steroid replacement therapy, suffer no clinically significant disability.

Glucocorticoid hormones are important regulators of differentiation, development and growth (Ballard, 1979; Cidlowski and Cidlowski, 1982; Chang and Roth, 1979). There are species differences in the abundance, half-life, and circadian rhythms of these steroids. In humans, the most abundant glucocorticoid is cortisol, with a half-life of 1-2 h and peak levels in the early morning (Follenius, 1982). In rats, the most abundant glucocorticoid is corticosterone, which has a half-life of 15-30 min and peak levels in the early evening (Marotta et al., 1975). Although this general pattern is consistent, there is considerable intra- and interindividual variability, and the circadian rhythm may be altered by changes in sleep pattern, light-dark exposure, and feeding times. The rhythm is also changed by (1) physical stresses such as major illness, surgery, trauma, or starvation; (2) psychologic stress, including severe anxiety, endogenous depression, and the manic phase of manic-depressive psychosis; (3) central nervous system and pituitary disorders; (4) Cushing's syndrome (a condition caused by excessive glucocorticoid secretion); (5)

liver disease and other conditions that affect cortisol metabolism; and (6) chronic renal failure.

The secretion of glucocorticoids is stimulated by adrenocorticotropic hormone (ACTH) which is synthesized by the pituitary gland and positively regulated by corticotrophin releasing factor (CRF) manufactured in the hypothalamus. The hypothalamus is predominantly under the control of higher centers of the central nervous system and responds to other types of stimuli (especially "stressful" ones such as trauma, burns, hypoglycaemia, exercise, infection, chemical intoxications, haemorrhage, pain, psychologic stress) as well. Glucocorticoids interact with specific receptors in the brain and pituitary gland to inhibit further release of both CRH and ACTH (Jingami et al., 1985; Munck et al., 1984; Keller-Wood and Dallman, 1984). The inhibitory action of glucocorticoids is exerted at multiple target sites, of which only two have been unequivocally identified to date: neurons in the hypothalamic paraventricular nucleus that produce CRF, and the corticotroph cells in the adenohypophysis (Dallman et al., 1987; Beyer et al., 1988). There are two distinct inhibitory mechanisms, fast and delayed feedback inhibition. The fast feedback inhibition of ACTH is rate-dependent; ie, it depends on the rate of increase of the glucocorticoid but not the dose administered. This phase is rapid, and basal and stimulated ACTH secretion both diminish within minutes after the plasma glucocorticoid level increases. This fast feedback phase is transient and lasts less than 10 minutes, suggesting that this effect is not mediated via cytosolic glucocorticoid receptors (see below) but rather via actions on cell membrane. The delayed feedback inhibition after the initial rate-dependent effects of glucocorticoids further suppresses CRF and ACTH secretion by mechanisms that are both time- and dose-dependent. Thus, with continued glucocorticoid administration, ACTH levels continue to decrease and become unresponsive to stimulation. The ultimate effect of prolonged glucocorticoid administration is suppression of CRF and ACTH release and atrophy of the zonae fasciculata and reticularis as a consequence of ACTH deficiency (adrenal

androgen production is also regulated by ACTH). The suppressed hypothalamic-pituitary-adrenal axis fails to respond to stress and stimulation. Delayed feedback appears to act via the classic glucocorticoid receptor (see below), thus reducing synthesis of the messenger RNA for pro-opiomelanocortin (POMC), the precursor of ACTH. The carefully maintained balance of glucocorticoid secretion is interrupted during periods of stress when high circulating levels of glucocorticoids are needed for survival. Plasma ACTH and cortisol are secreted within minutes following the onset of stresses and these responses abolish circadian periodicity if the stress is prolonged. Stress responses originate in the central nervous system and increase hypothalamic CRF and thus pituitary ACTH secretion. Stress responsiveness of plasma ACTH and cortisol is abolished by prior high-dose glucocorticoid administration and in spontaneous glucocorticoid excess syndrome (as in Cushing's syndrome); conversely, the stress responsiveness of ACTH secretion is enhanced following adrenalectomy.

Glucocorticoids exist in plasma in both unbound and protein-bound forms. The major binding protein is corticosteroid-binding globulin (CBG) which binds glucocorticoids noncovalently with a high affinity. Glucocorticoids can also bind loosely to albumin. The distribution of glucocorticoids between unbound and protein-bound forms is dependent on the levels of CBG and albumin. The relative proportion of each form of steroids has a profound effect on the amount of glucocorticoids available to the target cells. CBG does not bind synthetic glucocorticoids except for prednisolone.

The metabolism of the steroids renders them inactive and increases their water solubility, as does their subsequent conjugation with glucuronide or sulfate groups. These inactive conjugated metabolites are more readily excreted by the kidneys. The liver is the major site of steroid catabolism and conjugation, and 90% of these metabolized steroids are excreted by the kidneys.

B. Receptors for glucocorticoids (GRs)

1. Mediation of glucocorticoid action by glucocorticoid receptors

During the past two decades, studies from several laboratories produced a generally accepted hypothesis for the primary events involved in steroid hormone action (for reviews, see Munck and Leung, 1977; Baxter and Rousseau, 1979; O'Malley and Schrader, 1979). Although certain specific molecular details remain unclear, the overall steps in the mechanism have been elucidated. Little attention has been given to the mechanism of steroid entry into target cells. However, it is generally accepted that steroid hormones readily cross the cell membrane by simple diffusion due to their relatively small size and their lipophilic nature (Plagemann and Erbe, 1976; Ballard, 1979). Early studies based on cell fractionation and radioautography led to the development of the two-step model of steroid hormone action (Jensen and DeSombre, 1972; Chan and O'Malley, 1976; Gorski and Gannon, 1976). The premise of this model is that free steroid binds to unoccupied receptor which resides in the cytoplasm. The first step in the model is "activation" or "transformation" of the steroid-receptor complex, resulting in a conformational change of the receptor, which enhances the affinity of the receptor for chromatin. The second step is the translocation of the activated receptor-steroid complex from the cytoplasm to the nucleus where it interacts with DNA elements, resulting in modulation of transcription of target genes. The transcribed mRNAs code for proteins, which are in turn responsible for eliciting the hormonal effects. Although GR is associated with microtubules during different stages of the cell cycle (Akner et al., 1995), microtubules may not be required for either the nuclear translocation or biological activity of GR (Szapary et al., 1994). The steps of glucocorticoid action is depicted in Fig. 1.

For the past few years, the classical two step mechanism of steroid hormone action has been repeatedly challenged and is the subject of a heated controversy. Ironically, this two step model was first suggested following subcellular fractionation and radioautographic studies by Jensen and co-workers (1968) on the oestrogen receptor in the rat uterus and appeared to hold for all steroid receptors. However,

recent immunocytochemical and enucleation studies on oestrogen and progesterone receptors have demonstrated that these receptors are exclusively in the nucleus even in the absence of steroids (King and Greene, 1984; King et al., 1985; Press et al., 1985; Perrot-Appolanat et al., 1985; Welshons et al., 1984). These studies on the oestrogen and progesterone receptors are at variance with rapidly accumulating evidence from various laboratories that the glucocorticoid receptor is largely cytoplasmic and translocates to the nucleus following steroid binding (Govindan, 1980; Antakly and Eisen, 1984; Antakly et al., 1989; Fuxe et al., 1985; LaFond et al., 1988; Wikstrom et al., 1987; Picard and Yamamoto, 1987). Part of the problem probably derives from the use of antibodies which may be characterized only partially with respect to their reactivity with structural determinants of the native receptors. However, it remains unclear whether the histochemistry reflects epitope exposure or cellular localization. Probably, steroid receptors display distinct characteristics with regard to their subcellular localization. The nature of the receptor activation is unclear. The analysis of the receptor structure would be helpful in understanding the process of activation (see below). The activation itself is probably a two-step process. GR exists as a monomer (Okret et al., 1985; Mendel and Orti, 1988) associated with a 90 Kda heat shock protein (Hsp90) dimer (Mendel and Orti, 1988; Denis et al., 1987; Gehring, 1993) in the absence of the hormone. The first step in the ligand-induced activation process is probably the dissociation of the GR monomer from the Hsp90 dimer (Denis et al., 1987; Rexin et al., 1988; Pratt et al., 1988; Yang and DeFranco, 1994). The second step is the dimerization of two identical GR entities as suggested by Wrange et al. (1989). Receptor activation is favoured by conditions such as increased temperature, physiological ionic strength, and cytosol dilution (Jensen and Ellison, 1968; Gorski et al., 1968; Higgins et al, 1973; Milgrom et al., 1973; Kalimi, et al., 1975; Burnstein et al., 1991a).

Glucocorticoid antagonists bind to the glucocorticoid receptors but do not elicit the nuclear events required to cause a glucocorticoid response. These steroids

compete with agonist steroids for the receptors and thus inhibit agonist responses. Other steroids have partial agonist activity when present alone; ie, they elicit a partial glucocorticoid response. However, in sufficient concentration, they compete with agonist steroids for receptor and thus competitively inhibit agonist responses; ie, these partial agonists may function as partial antagonists in the presence of more active glucocorticoids. Steroids such as progesterone, 11-deoxycortisol, deoxycorticosterone, testosterone, and 17β-oestradiol have antagonist or partial agonist-partial antagonist effects on glucocorticoid receptors; however, the physiologic role of these hormones in glucocorticoid action is probably negligible, because they circulate in low concentrations. The activity of an antagonist can be blunted in certain conditions as the action of a glucocorticoid antagonist, RU486, disappears upon activation of protein kinase A (Nordeen et al., 1995).

2. History of GR discovery

The earliest evidence for the presence of GR was provided by experiments with the radioactively labelled ligand. In the early 1960s, intracellular glucocorticoid-binding components were identified in rat liver using radioactive steroid, but their involvement in hormone action was debatable (reviewed by King and Mainwaring, 1974). A major breakthrough occurred when binding studies were conducted on isolated cells in which steroid concentrations at the cellular level were known with precision and kinetic experiments could be performed, and when synthetic glucocorticoids were used that did not interact significantly with nonreceptor components of the cell. In 1966, Hollander and Chiu (1966) demonstrated the binding of cortisol to mouse lymphosarcoma P1798 cells, an interaction that was very much reduced in steroid-resistant cells. Then, critical investigations on rat thymocytes provided what was perhaps the first clear demonstration of the glucocorticoid receptor (Munck and Brinck-Johnsen, 1968; Schaumburg and Bojesen, 1968). In these cells, steroids bound to a limited number of sites with an affinity consistent with the cellular response to glucocorticoids. These findings were

extended to glucocorticoid-sensitive cell lines in culture: human HeLa cells (Melnykovych and Bishop, 1969), mouse L929 fibroblasts (Hackney et al., 1970), P1798 lymphosarcoma (Kirkpatrick et al., 1971), rat hepatoma tissue culture (HTC) cells (Baxter and Tomkins, 1970), and S49 lymphoma cells (Baxter et al., 1971).

Because the putative steroid receptor molecules were present in only trace amounts (10^3-10^4 sites per cell or less than 0.01% of the cellular protein), it has been difficult to characterize them. The development of high-affinity synthetic analogues of the ligands overcame many of the difficulties of receptor isolation. GR purification has been most extensively performed on rat liver (Wrang et al., 1979; Wrange et al. 1984; Grandics et al., 1984; Govindan and Gronemeyer, 1984). Rat GR consists of a polypeptide of 795 amino acids (Miesfeld et al., 1986) and has a molecular mass of about 94 Kda, according to SDS-polyacrylamide gel electrophoresis (Wrange et al., 1984).

3. Molecular structure and functional properties of GR

GRs are basally phosphorylated in the absence of hormone and become hyperphosphorylated after hormone treatment of intact cells (Orti et al., 1992; Orti et al., 1993). Most of the phosphorylation sites are located in the regions of the N-terminal domain that are necessary for maximum transcriptional activity and reduce nonspecific binding to DNA (Bodwell et al., 1995). Earlier protein chemical studies on purified GR suggested that DNA binding and hormone binding properties, both present in a single molecule, could be dissociated by limited proteolysis (Wrange and Gustafsson, 1978; Okret et al., 1981; Carlstedt-Duke et al., 1982, 1987; Wrange et al., 1984). These studies provided the first line of evidence that steroid hormone receptors are structurally organized into different domains. Subsequent cloning of GR cDNAs and deduction of their amino acid sequences confirmed this prediction (Rusconi and Yamamoto, 1987; Giguère et al, 1986; Carlstedt-Duke et al., 1987; Hollenberg et al., 1987; Kumar et al., 1986). The identification of functional domains for hormone binding, DNA binding, and transactivation was further

facilitated by co-transfection assay (Giguère et al., 1986). The trans-vector provides for the efficient production of GR in cells that do not normally express the receptor gene. The cis-vector contains a chloramphenicol acetyltransferase (CAT) gene (a reporter gene whose product is easily monitored) coupled to the promoter of mouse mammary tumour virus (MMTV) whose expression is induced by glucocorticoids (Ringold, 1979). Addition of glucocorticoids or the agonist will activate the CAT gene. The level of CAT activity is directly proportional to the effectiveness of the hormone receptor complex in activating the expression of the CAT gene. By means of this assay, it is possible to investigate the effects of in vitro mutations on receptor activity.

All steroid receptors studied to date share three distinct functional domains: a N-terminal domain, a short DNA-binding domain, and a C-terminal hormone-binding domain (Fig. 2) (for reviews, see Evans, 1988; Beato, 1989). The central core DNA-binding sequence is rich in Cys, Lys, and Arg residues and is highly conserved among GR from different species (Hollenberg et al., 1985; Miesfeld et al., 1986; Danielsen et al., 1986) and among all the steroid receptors, the v-erb-A gene product, as well as the receptors for human retinoic acid and thyroid hormone (for review, see Evans, 1988). The homology in the ligand-binding domain is more graded and generally parallels the structural relatedness of the hormones themselves. The N-terminal domain is variable and this may contribute to important functional differences among receptors.

The C-terminal domain, located in the carboxyl one-third of the protein, is the most complex both structurally and functionally. On one hand, deletion or insertion of amino acids in this region results in loss of both the capacity of steroid binding and the ability of transactivating responsive genes (Giguére et al., 1986; Rusconi and Yamamoto, 1987; Hollenberg et al., 1987; Danielsen et al., 1987). On the other hand, mutational studies revealed that loss of a portion of the hormone-binding region of the GR results in a constitutively active molecule (Hollenberg et al., 1987;

Godowski et al., 1987). These results provide the first mechanistic insight into the process of activation: neither the steroid-binding domain nor the steroid hormone itself is needed for DNA binding or transcription enhancement. It is now accepted that the hormone-binding region normally prevents the receptor from binding DNA and thus prevents the receptor from functioning (Evans, 1988). The addition of hormone apparently relieves this inhibition (Becker et al., 1986). In the hormone-binding domain reside the amino acid residues thought to be responsible for the interaction with Hsp90 whose binding to the receptor prevents the receptor from exerting its transactivating effect on the responsive genes (Denis et al., 1988; Pratt et al., 1988). The binding of the hormone promotes the dissociation of the receptor from Hsp90 and allows the receptor to gain the translocation and transactivation functions. Recently, Dalman et al. (1991) identified the amino acid sequences (from 574 to 632) in the C-terminus of the mouse GR which are responsible for interacting with Hsp90. The C-terminal domain also contains regions involved in transcriptional transactivation (Giguère et al., 1986; Hollenberg and Evans, 1988; Webster et al., 1988; Dahlman-Wright et al., 1992) and regions important for nuclear localization (Picard and Yamamoto, 1987). Some of the amino acid residues involved in the binding of natural or synthetic ligands have been identified by mutational and chemical analysis (Carlstedt-Duke et al., 1988; Smith et al., 1988; Chen and Stallcup, 1994).

The hypothesis that the DNA-binding domain is included within the highly conserved central core of the receptor was first based on: (1) the clustering of basic residues likely to interact with DNA, (2) the presence of a Cys-rich motif which is common to DNA-binding proteins, (3) the high homology of this core among the steroid receptors. This hypothesis was tested by mutation of different parts of the DNA-binding domain (Hollenberg et al., 1987; Godowski et al., 1987; Giguere et al., 1986; Kumar et al., 1986). Mutants are able to bind hormone, but the DNA-binding capacity is lost. A direct confirmation of this function was made by "finger

swap" experiments in which the putative DNA-binding domain of the human oestrogen receptor was substituted with that of the human GR, resulting in a chimeric receptor with glucocorticoid response when exposed to oestrogen (Green and Chambon, 1987; Zilliacus et al., 1991). The DNA-binding domain may also be involved in transcriptional activation (Miesfeld et al., 1987; Schena and Yamamoto, 1988; Schena et al., 1989).

The structure of GR-DNA complex has been determined using X-ray crystallography (Luisi et al., 1991), NMR spectroscopy (Hard et al., 1990a, b) and has recently been refined using two- and three-dimensional NMR spectroscopy on an ^{15}N-labelled DNA-binding domain fragment (Baumann et al., 1993). GR DNA-binding domain consists of two subdomains (or motifs) that both have the general composition zinc-coordinating domain, two major alpha-helices (helix I and III) and extended regions. Each zinc atom is tetrahedrically coordinated to four cysteines and the two major helices are perpendicular to each other. The hydrophobic side chains of these helices and the extended regions form the protein core (Hard et al., 1990b; Luisi et al., 1991). The two subdomains of DNA binding domain differ from each other both structurally and functionally. The first subdomain is involved in DNA interaction, with some of the residues in the zinc region forming specific interactions with the phosphate groups of DNA and the recognition helix I positioned in the DNA major groove. Helix I contains three residues that have been shown to discriminate betwseen glucocorticoid responsive element and estrogen response element (Danielsen et al., 1989; Mader et al., 1989; Umesono and Evan, 1989; Zilliacus et al., 1991, 1992). The second subdomain provides the dimerization interface where most of the intersubunit interactions are made by residues in the loop between Cys-476 and Cys-482. The second zinc-coordinating domain also contains a short fragmemt of distorted helix (helix II). The amino-terminal zinc subdomain determines the target gene specificity (Green et al., 1988). When the N-terminal zinc finger is contributed by the glucocorticoid receptor, the chimeric receptor is able to activate a

glucocorticoid responsive element (see below) independent of whether the C-terminal zinc finger is derived from a glucocorticoid or an oestrogen receptor and vice versa (Green et al., 1988).

Despite the highly conserved nature of the DNA-binding domain among steroid receptors, a recent study has shown that a few amino acid differences between the androgen receptor and GR DNA-binding domains contribute to altered receptor DNA interactions; however, it is likely that non-receptor factors are involved in further modulation of receptor-selective DNA binding and transactivation functions (Rundlett and Miesfeld, 1995).

Located between the central DNA binding domain and the C-terminal hormone binding domain of the GR is a short sequence, rich in basic amino acids, which is partly responsible for signalling the nuclear translocation of the GR (Picard and Yamammoto, 1987). This sequence is homologous to those required for nuclear localization of SV40 T-antigen (Kalderon et al., 1984).

The N-terminal domain is hypervariable in both size and amino acid composition among all the steroid receptors. This region of the receptor has been demonstrated to have modulatory effect on transactivation (Danielson et al., 1987; Hollenberg et al., 1987; Giguère et al., 1986; Godowski et al., 1988; Hollenberg and Evans, 1988). Deletions in this region of GR reduce activity by 10- to 20-fold (Hollenberg et al., 1987; Danielson et al., 1987). Other evidence for a functional role of the N-terminal sequence came from the analysis of the nt^i (nuclear transfer increased) GR mutants (Yamamoto et al., 1976). nt^i GR appears to contain an altered N-terminus and, although the receptor can bind hormone, it is not biologically functional. It is this portion of the protein to which most of the receptor antibodies thus far have been raised (Carlstedt-Duke et al., 1982; Westphal et al., 1982; Antakly et al., 1990).

According to structure and function, the steroid hormone receptors can be divided into two groups: one including the glucocorticoid, progesterone, androgen,

and mineralocorticoid receptors and the other including the oestrogen, thyroid hormone, retinoic acid, and vitamin D3 receptors (the receptors for thyroid hormone, retinoic acid, and vitamin D3 are structurally and functionally related to steroid receptors and thus belong to the superfamily of steroid hormone receptors). Members of the first group can all act through the hormone responsive element of MMTV. The primary amino acid sequence of the DNA binding domain exhibits stricter conservation within the subgroups (Beato, 1989).

The transactivation observed within the short DNA-binding domain alone is only a small fraction of the total activity of the receptor, and additional transactivation domains are located in the C-terminal and also in the N-terminal regions. In the human GR, two transactivation domains have been identified: one at the N-terminal region independent of the hormone and the other within the C-terminal region completely dependent upon hormone binding (Hollenberg and Evans, 1988).

4. Glucocorticoid responsive element (GRE)

By gene transfer studies, DNA sequences responsible for interacting with GR have been found in many inducible genes. These DNA sequences have also been identified by direct binding studies using partially purified GR (Geisse et al., 1982; Payvar et al., 1983; Karin et al., 1984; Cato et al., 1984; Slater et al., 1985; Miksicek et al., 1986; Danesch et al., 1987; Scheidereit et al., 1983; DeFranco and Yamamoto, 1986). The location of these sequences within regulated genes is very variable, ranging from positions several kilobase pairs upstream of a promoter (Jantzen et al., 1987) to positions within the first few hundred base pairs upstream (Karin et al., 1984; Buetti and Diggelmann, 1986; Kühnel et al., 1986) or downstream of the cap site (Slater et al., 1985). Thus these sequences, termed glucocorticoid responsive elements (GREs), function in a position- and orientation-independent fashion and behave like transcriptional enhancers. However, unlike other enhancers, the activity of the GRE is dependent on the presence or absence of receptor ligand (Chandler et al., 1983; Kumar et al., 1987). The partially

palindromic 15-bp GRE (15-mer), GGTACAnnnTGTTCT, is sufficient to render a non-regulated promoter inducible by glucocorticoids. Thus binding of hormone-receptor complexes to the GRE site on DNA is responsible for transcriptional regulation. The available information from DNAase I and methylation protection studies suggest that a receptor dimer binds to the functional GRE, in agreement with the partially palindromic structure of the conserved 15-bp GRE. Recent studies have shown that the GRE is recognized as two hexamer half-sites, each half-site is recognized by a single subunit of a receptor dimer, probably in a cooperative fashion (Nordeen et al, 1990).

5. Molecular mechanism of GR actions

a). GR and other transcriptional factors

GR can regulate gene transcription either positively or negatively depending on the particular genes and the content of the cells involved. The mechanism by which hormone receptors modulate transcriptional activity is largely unknown. A number of observations suggest that the presence of a GRE alone is not sufficient for hormone inducibility but that, in addition, other regulatory elements are required. Mutation of the nuclear factor 1 (NF1) binding site on the MMTV long terminal repeat (MMTV LTR) abolishes the ability of this promoter to respond to glucocorticoids (Buetti and Diggelmann, 1986; Miksicek et al., 1987), indicating the necessity of NF1 in the effect of glucocorticoids on MMTV. Exonuclease footprinting experiments suggest that glucocorticoid induction of transcription from the MMTV LTR promoter results from recruitment of transcriptional factors, presumably NF1 and TATA box binding factors, at the promoter region, indicating that receptor binding promotes the formation of a transcriptionally active complex (Cordingley et al., 1987). Subsequent experiments with artificial combinations of GREs and binding sites for transcriptional factors showed that not only NF1, but a whole battery of other well-characterized factors, are able to act in combination with the hormone receptor for the activation of an adjacent promoter (Schüle et al., 1988a;

Schüle et al., 1988b). In fact, two GREs are as effective as one GRE in combination with a binding site for NF1 (Schüle et al., 1988b), probably resulting from co-operative interaction between the bound receptors and other transactivation factors. The cooperative DNA-binding between two receptors is mediated by direct protein-protein interactions between the receptor proteins as suggested by Wright and Gustafsson (1991). Removal of sequences with a strong similarity to the CACCC element of the β-globin promoter (Myers et al., 1986), adjacent to the proximal GR binding site of the tryptophan oxygenase gene, abolishes glucocorticoid induction, implying the importance of this element for the activity of the GRE (Danesch et al., 1987). In addition, there is also dependence on the spacing between the GRE and the binding sites for other factors. The GRE can function independently of additional upstream factors when positioned immediately upstream of a TATA box but one GRE alone is not able to mediate transcription when inserted further upstream, 351-bp from the transcription initiation site; inducibility can be restored at the further upstream position by either two GREs or a single GRE in combination with a CCAAT box (Strähle et al., 1988). The effect of individual transcriptional factors varies considerably with the cell line used for the gene transfer study, probably reflecting the differences in the cellular content of the factors (Strähle et al., 1988). Induction of transcription from a promoter with two upstream glucocorticoid response elements is 10- to 20- fold greater than that from a similar promoter with only one response element. Using the mobility shift assay, a factor has been identified that enhances specific DNA binding of androgen and glucocorticoid receptors by 25- and 26-fold, respectively, through the formation of heteromeric complexes, suggesting that this receptor binding factor may influence the ability of these nuclear receptors to activate transcription (Kupfer et al., 1993).

Functional antagonism between GR and other factors has also been described. For example, GR and the transcription factor Jun/AP-1 can reciprocally repress one another's transcriptional activation through a mechanism likely involving protein-

protein interactions (Schüle et al., 1990). Calreticulin, a Ca^{2+}-binding protein in the lumen of the endoplasmic reticulum which is also found in the nucleus, interacts with the DNA-binding domain of GR and prevents the receptor from binding to its specific GRE (Burns et al., 1994). Pearce and Yamamoto (1993) described that GR and mineralocorticoid receptor had antagonistic effects toward each other. Taken together, a novel transcriptional control may be modulated by inactive "antagonists" competing for receptor binding sites on the hormone response element.

b). Role of chromatin structure

Chromatin structure may also be involved in modulating the hormonal function. Unlike the cooperative function of GR and NF1 by whole cell assay mentioned above, when GR and NF1 are added to DNA fragments containing the MMTV-LTR in vitro, they compete for binding to their respective cognate sequences (Brüggeneier et al., 1990), implying the involvement of chromatin structure in mediating the interaction between receptors and other transcriptional factors. The observation that following hormonal treatment DNAase I hypersensitive regions appear in the vicinity of the regulated promoters indicates that the hormones may act by altering the chromatin structure of target genes (Becker et al., 1984; Zaret and Yamamoto, 1984).

c). Negative regulation of target genes

Most of the systems studied thus far have concerned genes that are induced by steroid hormones. However, GR has also been shown to repress expression of a variety of genes, including the POMC gene (Drouin et al., 1987 and 1989), the prolactin gene (Sakai et al., 1988), the proliferin gene (Mordacq and Linzer, 1989), chorionic gonadotropin α subunit gene (Akerblom et al., 1988; Oro et al., 1988), and collagenase gene (Weiner et al., 1987). GR negatively regulates the bovine prolactin gene promoter by binding to a so-called "negative glucocorticoid responsive element (nGRE)" (Sakai et al., 1988). The nGREs differ substantially from the consensus sequence for positive GREs. How nGREs confer inhibitory effect on transcription

is unknown. A competition mechanism has been proposed to explain repression of the POMC gene by glucocorticoids. In this case, binding of the receptor to a site around -60 could prevent the CAAT box binding factor from interacting with its target sequence (Droiun et al., 1987 and 1989). However, recent work from Drouin's group has generated a new mechanism for the glucocorticoid repression of POMC gene (Drouin et al., 1993; Therrien and Drouin, 1993). The POMC nGRE binds three molecules of GR whereas the positive GRE binds a receptor homodimer. The nGRE complexes appeared to form by the initial binding of a GR homodimer on one side of the double helix followed by the cooperative binding of a third GR molecule on the opposite side of the double helix. It may be that nGRE-bound GR represses transcription by interfering with the action of other transcription factors bound upstream and/or downstream of the nGRE. The negative regulation of the α-fetoprotein gene by glucocorticoids was suggested to be due to the interference of AP-1 activity by the glucocorticoid receptor either by direct competition for DNA binding or via protein-protein interaction (Zhang et al., 1991). In the case of the α subunit gene, glucocorticoid repression seems to be mediated by a competition between the hormone receptor and the cAMP-mediator protein for binding to overlapping DNA regulatory sequences (Akerblom et al., 1988). In this system, inhibition by glucocorticoids is only detectable when constructions contain the cAMP responsive element in cells able to respond to cAMP. In other cells with constructions not containing the cAMP responsive element, the same receptor binding sites can act as positive modulators of adjacent promoters (Akerblom et al., 1988). Thus the nGRE may not be the sole determinant for negative regulation.

Recent studies showed that a 25-bp element conferred on linked promoters either positive or negative glucocorticoid regulation, depending upon physiological context (Diamond et al., 1990). In this system, c-Jun and c-Fos served as selectors of hormone responsiveness; a 25-bp sequence that resided upstream of the mouse proliferin gene was inactive in the absence of c-Jun, whereas it conferred a positive

glucocorticoid effect in the presence of c-Jun, and a negative glucocorticoid effect in the presence of c-Jun and relatively high levels of c-Fos; the transcriptional action of this sequence involved both DNA binding and protein-protein interactions by the receptor and the nonreceptor factors. Similarly, Nechushtan et al. (1987) has shown that phosphoenolpyruvate carboxykinase (PEPCK), a single copy gene, is stimulated by glucocorticoids in some tissues and repressed in others (Nechushtan et al., 1987; Short et al., 1986). The expression of the glycoprotein hormone alpha gene is regulated divergently by glucocorticoids in different cell types. Coexpression of the GR with an alpha-CAT reporter gene caused activation of alpha promoter activity in fibroblasts, but repression in JEG-3 choriocarcinoma cell, indicating that cell-specific factors dictate positive versus negative regulation of this promoter by GR. This supports a mechanism in which GR mediates repression in JEG-3 cells by receptor interference with transactivating potential of enhancer-binding protein or associated transcription factors (Chatterjee et al.,1991). Collectively, these studies indicate that whether glucocorticoids result in positive or negative regulation on responsive genes depends not only upon the DNA sequence on the target genes but also upon the physiological context involved.

C. Initial studies on glucocorticoid functions on target tissues

Glucocorticoids play an important role in the metabolism of target tissues. In a given tissue, glucocorticoids frequently affect only a small subset of the expressed genes. The phenotypic expression of glucocorticoid action varies according to the target tissues involved. For example, a major effect of glucocorticoids on liver is stimulation of gluconeogenesis (Cahill et al., 1971), involving induction of enzymes, such as tyrosine aminotransferase and tryptophan oxygenase (Melman, 1974; Sarkar and Griffith, 1976). However, glucocorticoid treatment results in lymphocyte lysis and cell death by the induction of a lysis gene product that alters the lymphocyte genome (Munck and Crabtree, 1981; Compton and Cidlowski, 1987; Compton et al., 1988; Miller et al., 1994; Peiffer et al., 1994). Glucocorticoids play an important

role in mood and behaviour. Patients with Addison's disease are often depressed or anxious, and these symptoms are ameliorated by proper hormonal replacement therapy (Tepperman and Tepperman, 1987). In the brain, both mineralocorticoid receptor and GR are present (Trapp et al., 1994). The balance of mineralocorticoid receptor and GR-mediated effects appears critical for the long-term control exerted by corticosteroids over specific aspects of neuronal activity, stress responsiveness, and behavioral adaptation (De Kloet et al., 1994). Mineralocorticoid receptors respond to low concentrations of the steroid, while higher concentrations are needed for additional activation of GR. Mineralocorticoid receptor activation appears relevant in hippocampal neurons for stability and sensitivity of the stress response system, and for behavioral reactivity and response selection. Additional transient GR activation suppresses excitability, facilitates recovery from the stress response, and promotes information storage. The mineralocorticoid receptor and the GR can form a heterodimeric complex with DNA-binding and transactivation properties different from those of the respective homodimers, suggesting an unrecognized mechanism for the transcriptional regulation of glucocorticoid-responsive genes in tissues such as the brain which coexpress these receptors (Trapp et al., 1994). The production of glucocorticoids is regulated by pituitary-adrenal axis and, in turn, the secretion of corticotrophin-releasing factor in hypothalamus and POMC in the pituitary is regulated by glucocorticoids (Roberts et al., 1982; Jingami et al., 1985).

Because glucocorticoids have a wide spectrum of action in all tissues studied (Munck and Leung, 1977), it was speculated that most tissues contained GR. Indeed, GR was found in all the tissues mentioned above (Ballard et al., 1974; Gustafsson et al., 1987). However whether there is variance in GR content among the various cell types within a given tissue remains to be examined. In this respect, submaxillary gland, testis, and heart provide interesting systems to study the cellular distribution of GR for the reasons described below.

1. Submaxillary gland

The submaxillary gland is a heterogenous tissue composed of two main cell types, acini and tubules. An enlarged portion of the tubules is composed of specialized epithelial cells, known as granular convoluted tubular cells (GCT). These cells secrete a great number of physiologically important polypeptides, including epidermal and nerve growth factors (Barka, 1980), and atrial natriuretic factor (Gutkowska and Nemer, 1989). α 2u globulin, a peptide with unknown function originally thought to be exclusively produced in the liver, was also found to be synthesized in the GCT cells of submaxillary gland (Antakly et al., 1982b). α 2u globulin is under developmental and multi-hormonal regulation. Two steroids, androgens and glucocorticoids, greatly influence the growth, differentiation, and secretory activity of the GCT cells (Walker, 1982; Walker et al., 1981; Gresik et al., 1981). Since androgen and glucocorticoid effects are mediated by their respective receptors, studies on the expression and cellular localization of androgen and glucocorticoid receptors in the submaxillary gland are important for understanding the mechanisms of action of these steroids. Androgen receptors have been well described in the rat and mouse submaxillary gland (Verhoeven, 1979; Nemoto et al., 1985; Ohara-Nemoto et al., 1988; Nemoto et al., 1986; Minetti et al., 1986; Kyakumoto et al., 1986 and 1987; Sato et al., 1986). Unfortunately, little information is available on the glucocorticoid receptor in the submaxillary gland. Because glucocorticoids can partially substitute or synergize with the effects of androgens on GCT cells (Sato et al., 1981), glucocorticoids were thought to act via androgen receptors in these cells; this hypothesis was reinforced by data showing binding of cortisol to androgen receptors in mouse submaxillary gland (Maruyama and Sato, 1986). However, glucocorticoid effects can not be all attributed to androgen-like activity, since reversal of tubular atrophy in adrenalectomized and castrated or hypophysectomized mouse requires the administration of corticosterone along with thyroid hormone or androgens (Chretien, 1977). Furthermore, adrenalectomy and glucocorticoid administration affect specific gene products in the

GCT cells (Gerald et al., 1986) in a different manner than androgens. Even if glucocorticoids acted on GCT cells by simply increasing the levels of androgen receptor, this action would necessitate the presence of glucocorticoid receptors. Thus it is interesting to examine whether GR is present in the GCT cells of the submaxillary gland.

2. Testis

Testis, the male reproductive organ, is composed of seminiferous tubules where sperm cells are generated and interstitial cells (Leydig cells) which produce androgen under the stimulation of luteinizing hormone (LH) secreted by the pituitary gland. The epithelium of the seminiferous tubules consists of spermatogenic cells and Sertoli cells. Glucocorticoids, when administered in vivo and in vitro, are known to inhibit normal steroidogenic function of the Leydig cells (Monder et al., 1994). Treatment with glucocorticoids resulted in decreased androgen production and LH receptor content (Saez et al., 1977; Bambino and Hsueh, 1981). These inhibitory effects of glucocorticoids on normal Leydig cell function corroborate well with earlier binding studies of GR in testicular cells. Although, specific binding sites for ^{3}H-dexamethasone in the testis were first demonstrated in cytosols of whole testis of juvenile rats (Ballard et al., 1974), an abundance of such binding sites was subsequently detected in the cytosolic and nuclear fractions of enriched interstitial cell populations as compared to the rest of the testis (Evain et al., 1976). However, the precise localization of GR in the different cell types of the testis remains to be determined.

POMC, the precursor to ACTH and other related peptides, was originally identified in the corticotropic cells of the pituitary. Recent studies have shown that the Leydig cells in many species also contain POMC related peptides such as ACTH, endorphin, and γ-melanocyte stimulating hormone (γ-MSH) (Sharp and Pekary, 1981; Tsong et al., 1982a and 1982b; Shaha et al., 1984). POMC transcripts were subsequently identified in rat and mouse testis by blot hybridization analysis (Chen

et al., 1984; Pintar et al., 1984; Lacaze-Masmonteil et al., 1987) and precisely located in the mouse Leydig cells by in situ hybridization (Gizang-Ginsberg and Wolgemuth, 1985). However, unlike in the anterior lobe of the pituitary, the POMC gene in testis is not regulated by glucocorticoids (Tremblay et al., 1988). The non-responsiveness of the POMC gene to glucocorticoids in the intermediate lobe of the pituitary is due to the lack of functional GR in these cells (Antakly et al., 1985). Whether this mechanism applies to the testicular system remains to be determined. Identification of GR and POMC related peptides in the same Leydig cells will answer this question.

3. Heart

Several observations suggest that glucocorticoid hormones might have an effect on the heart: patients with Addison's disease (primary adrenocortical insufficiency) and adrenalectomized animals have atrophic changes of the heart (Sayers and Travis, 1970); the contractile force of the heart is impaired in adrenalectomized animals (Brown and Remington, 1955; Lefer and Sutfin, 1964); glucocorticoids effectively restore normal circulatory parameters in animals that are in adrenal crisis (Swingle et al., 1957) and potentiate the circulatory and myocardial effects of catecholamines (Baxter and Forsham, 1972). These observations do not prove unequivocally a direct effect of glucocorticoids on heart since they could also be the consequence of a more general influence of glucocorticoids on the circulation. Recent studies showed that glucocorticoids regulated atrial natriuretic factor synthesis both in vivo and in vitro (Nemer et al., 1988; Sheilds et al., 1988; Weidmann et al., 1988), implying that glucocorticoids have a direct effect on the heart tissue. This effect is presumably mediated by GR.

Beato and Feigelson (1972) have failed to demonstrate GR in the rat heart. However, Ballard et al. (1974) have established the presence of high affinity binding sites of [^{3}H] dexamethasone in rat and dog heart cytosol. In addition, Gregory et al. (1976), Boer and Oddos (1979), and Turner (1986) have shown that the GR number

in the heart is significantly increased after bilateral adrenalectomy. All these lines of information indicate the presence of GR in the heart tissue.

The clarification of the cellular GR distribution in the heart is important to understand the glucocorticoid action on the cardiac tissue. Radioautographic studies have demonstrated an uptake of [^{3}H]-dexamethasone by cardiac nuclei in vivo in the mouse (Coutard et al., 1978). However, further studies are needed to localize GR in the heart tissue, particularly by using specific antibodies to the GR.

D. Initial studies on GR regulation

1. Cellular GR concentration and target gene responses

A prerequisite for glucocorticoid response is the presence of intracellular GR, since the hormonal response is mediated by the GR protein (Grove et al., 1980). For instance, the cytolytic response of T lymphocytes to glucocorticoids is dependent on the presence of functional GR (Harmon and Thompson, 1981). Absence of GR in normal rat tissue is associated with resistance to the hormone (Antakly and Eisen, 1984) and the induced expression of the functional GR established the biological response of the cells to the hormone (Antakly et al., 1985). However, the hormonal sensitivity in GR containing cells is not always guaranteed. The cellular response to glucocorticoids is influenced by several factors such as the degree of cellular differentiation (Ballard, 1979), receptor activity (Northrop et al., 1986), receptor modifying factors (Housley et al., 1984), and receptor concentration (Bourgeois and Newby, 1979; Gehring et al., 1984). Cellular GR levels have been shown to vary as a result of endocrine manipulations (Svec and Rudis, 1981; Cidlowski and Cidlowski, 1981; Sapolsky et al., 1984; Isohashi et al., 1979), neural influence (Mcginnis and De Vellis, 1981; Antakly et al., 1985; Antakly et al., 1987), during different stages in the cell cycle (Crabtree et al., 1980; Cidlowski and Cidlowski, 1982; Distelhorst et al.,1984) and during aging (Chang and Roth, 1979).

One of the essential questions is whether there is a relationship between the cellular sensitivity to glucocorticoid hormones and the concentration of GR.

Conflicting data are found in the literature, although most of them support a relationship.

Several lines of evidence indicate that the intracellular GR concentration is an important factor in determining the extent of the biological response of cells to glucocorticoids. Earlier work demonstrated a correlation between nuclear GR binding and cellular response implying the importance of GR level in the extent of cellular response to glucocorticoids (Bloom et al., 1980). GR levels correlate with response in vivo to glucocorticoid therapy in certain leukemias (Lippman et al., 1978; Bloomfield et al., 1981a; Ho et al., 1981; Costlow et al., 1982; Bell et al., 1983; Moalli and Rosen, 1994) and lymphoma (Bloomfield et al., 1980; Bloomfield et al., 1981b). A direct relationship between GR concentration and glucocorticoid sensitivity in mouse T lymphoid cells was observed by two laboratories (Gehring et al., 1984; Bourgeois and Newby, 1979). Furthermore, Danielsen and Stallcup (1984) isolated a cell variant that became less sensitive to glucocorticoids by decreasing its GR number. More recently and more directly, it has been shown that the magnitude of transcriptional regulation by glucocorticoids in cells that have been transfected with the GR gene is proportional to the level of GR expression achieved (Miesfeld et al., 1986; Giguère et al., 1987; Vanderbilt et al., 1987; Hirst et al., 1990). Moreover, no evidence of saturability was observed even when receptor level was increased 50-fold over the control level (Hirst et al., 1990).

On the other hand, other studies have failed to demonstrate a relationship between GR concentration and glucocorticoid response. Manipulations of cells with different substances such as butyrate (Littlefield et al., 1980), mitogens (Smith et al., 1977) and hydroxyurea (Littlefield et al., 1986), cause changes in cellular GR concentration. However, the changes in cellular GR concentrations under these conditions have not been reflected in parallel changes in cellular response. In these cases, it cannot be excluded that these substances also affect other mechanisms involved in the cellular response cascade.

2. Regulation of GR expression

Since GR is the limiting factor in the response cascade and small changes in the receptor concentration will be reflected in parallel changes in the cellular sensitivity to glucocorticoid hormone, it is of particular importance to understand how cellular GR levels are regulated. However, little is known about the factors which regulate GR expression. In general, hormone receptors can be regulated by their own ligands and by other regulatory molecules.

Throughout this report, up-regulation of receptors is referred to as the phenomenon of increase of receptor concentration and down-regulation of receptors is referred to as the phenomenon of decrease of receptor concentration.

a). GR levels and adrenalectomy (ADX)

The adrenals are the organs where glucocorticoids are produced. One way to know the influence of the hormones on the receptor level is to examine whether the receptor level is changed after removal of the hormones by means of adrenalectomy. As early as 1974, McEwen et al. (1974) showed that the GR level in the hippocampus was increased by 50% after male Sprague-Dawley rats were adrenalectomized for 3 or 7 days. Two years later, the same group reported that the GR level was also increased in the pituitary, hypothalamus, septum, amygdala and cerebral cortex after rats were adrenalectomized (Olpe and McEwen, 1976). Gregory et al. (1976) found that the number of cardiac and hepatic GR increased 12-48 hours after adrenalectomy. Boer and Oddos (1979) reached the same conclusion by measuring the receptor levels in the heart and the liver after rats were adrenalectomized. The GR level was also found to be increased in rat skeletal muscle (Mayer and Rosen, 1978) after removal of the adrenal glands. Recently, Turner (1986) showed that the GR content was increased by adrenalectomy in various tissues examined which included liver, heart, kidney, hippocampus, cerebral cortex, amygdala-entorhinal cortex, hypothalamus and pituitary. All these studies were carried out by ligand binding assay. More recently, Kalinyak et al. (1987) demonstrated that, by RNA blot

hybridization, the GR mRNA level was increased in brain and kidney but not in liver and lung after rats were adrenalectomized. Thus regarding the liver, the GR protein level (the number of ligand binding sites) was increased (Turner, 1986; Gregory et al., 1976; Boer and Oddos, 1979) but the GR mRNA level was unchanged by adrenalectomy (Kalinyak et al., 1987). This might be because the GR mRNA level was not changed but the translation efficiency of the GR mRNA or the half-life of the GR protein was increased. Alternatively, more receptor might become capable of binding to the ligand after adrenalectomy. While Olpe and McEwen (1976) observed an increase of GR number in hypothalamus of adrenalectomized male rats in a binding study, Peiffer et al. (1991) showed that the GR mRNA levels were unchanged in the hypothalamus after the female rats were adrenalectomized for 2 weeks. The discrepancy may be due to sex differences in rat brain glucocorticoid binding (Turner and Weaver, 1985) and/or due to a mechanism of GR regulation at the post-transcriptional level.

The literature review on the GR regulation by adrenalectomy is listed in Table 1.

b). GR levels and glucocorticoid treatment

In AtT-20 mouse pituitary tumour cells, exposure to dexamethason (Dex) for 48-96 hours resulted in a 75% reduction of total receptor content per cell (Svec and Rudis, 1981). Similar findings were reported nearly simultaneously using the HeLa S_3 cells (Cidlowski and Cidlowski, 1981). Over the past few years, the same phenomenon of glucocorticoid induced GR down-regulation has been reported in HTC cells (Danielsen and Stallcup, 1984), W7 mouse thymoma cells (Danielsen and Stallcup, 1984), cultured normal human T-cells (Lacroix et al., 1984), and cultured human skin fibroblasts (Oikarinen et al., 1987; Berkovitz et al., 1988). Down-regulation of the GR by glucocorticoids was also observed in intact rat tissues such as skeletal muscle, hippocampus, and amygdala (Mayer and Rosen, 1978; Tornello et al., 1982; Sapolsky et al., 1984). However, there are different results regarding

the GR regulation in hypothalamus and pituitary. While Sapolsky et al. (1984) found that stress or exogenous corticosterone down-regulated the GR level in the hippocampus and amygdala but not in the hypothalamus or pituitary of adult rats, Yang et al. (1990) showed that after ACTH treatment (glucocorticoids are elevated by ACTH) of sheep fetus, there was a decrease of GR number in both the pituitary and hypothalamus. The obvious discrepancy among these results is probably due to species and/or age differences. Glucocorticoid down-regulation of GR was also demonstrated in the lymphocytes of normal human volunteers (Schlechte et al., 1982). All these studies were conducted by ligand binding assay.

With the cloning of the GR gene, it is possible to determine the receptor mRNA levels under different endocrinologic conditions. Kalinyak et al. (1987) showed that Dex treatment resulted in a consistent decrease of 40-60% in the accumulation of GR mRNA in all the tissues studied which included lung, spleen, brain, liver, kidney, and adrenal glands. In addition, no correlation between the initial GR concentration and the magnitude of GR regulation was observed. In fetal rat lungs of 16-21 days gestation as well as adult lungs betamethasone treatment resulted in a significant decrease of glucocorticoid receptor mRNA to 50-65% of the control level (Bronnegard et al., 1991). Peiffer et al. (1991) reported that the GR mRNA was decreased by Dex treatment of adrenalectomized rats in amygdala, hippocampus and hypothalamus. The decrease of GR mRNA was also demonstrated in human IM-9 lymphocytes and rat pancreatic acinar AR 42J cells (Rosewicz et al., 1988), rat hepatoma culture cells (Okret et al., 1986; Dong et al., 1988), and in NIH 3T3 cells (Hoeck et al., 1989). Down-regulation of GR occurred not only in natural GR-containing cells (Hela S_3 cells) but also in cells with GR introduced by DNA transfection (Burnstein et al., 1991b; Alksnis et al., 1991). Bronnegard observed that in normal human fibroblasts the GR mRNA was down-regulated by 60% while in fibroblast from familial glucocorticoid resistance patient the mRNA was down-regulated by only 40% after Dex treatment for 12 hours (Bronnegard et al., 1991).

Thus the GR regulation by Dex may be, in part, subject to the control of the physiological conditions in the cells. The literature review on the GR regulation by glucocorticoid treatment is listed in Table 2.

The mechanism of GR down-regulation is currently unclear. Treatment of HeLa S_3 cells with Dex for 24 or 48 hours resulted in more profound down-regulation of GR protein than of GR mRNA (Silva et al., 1994), suggesting posttranscriptional mechanisms. In COS-1 monkey kidney cells transiently transfected with a GR expression vector, in which GR transcription is under the control of the Rous sarcoma virus promoter (Burnstein et al., 1990), and in stably transfected Chinese hamster ovary cells expressing the GR under the control of either the Rous sarcoma virus (Burnstein et al., 1991a) or human metallothionein-IIa (Alksnis et al., 1991) promoter, there was a decrease in GR mRNA after steroid treatment, again suggesting posttranscriptional regulation of GR expression. Several laboratories have sought to understand the mechanism of GR regulation by investigating the effect of glucocorticoids on the rate of GR turnover in cells. Svec and Rudis (1981) found that GR in AtT-20 cells are depleted more rapidly when cells are incubated with both cycloheximide and Dex than when cells are incubated with cycloheximide alone, suggesting that the half-life of the GR is shorter in the presence of hormones than in the absence of hormones. The effect of glucocorticoid hormones on the intracellular receptor half-life has been extensively investigated by Samuels and coworkers using the technique of dense amino acid (heavy isotopes, i.e., ^{2}H, ^{3}C, ^{15}N) labelling (Raaka and Samuels, 1983; McIntyre and Samuels, 1985). Their results indicate that the GR half-life in GH_1 pituitary tumour cells is significantly shorter when the cells are cultured in the presence of glucocorticoid hormone (GR half-life was 9.5 hours) than when the cells are cultured in the absence of the hormone (GR half-life was 19 hours). The increased turnover rate of GR protein by glucocorticoids was also demonstrated in HTC cells (Dong et al., 1988) and NIH-3T3 cells (Hoeck et al., 1989). The mechanism for ligand-induced increase in the turnover rate of GR protein

is not known, although it is possible that the activated GR which was dissociated from Hsp90 (Denis et al., 1988; Picard et al., 1988) or translocated to the nucleus (Wikström et al., 1987) is more susceptible to proteolytic degradation. In contrast, Distelhorst and Howard (1989) showed that the GR half-life (9 hours) in S49 mouse lymphoma cells, by kinetic pulse-chase labelling study, was not altered by glucocorticoids. The discrepancy between the results from different laboratories may be related to differences in the methods used to measure receptor half-life or may represent a difference in receptor regulation in different types of cells.

In some cases, decreased GR expression has been attributed to a decreased rate of transcription of the GR gene. The observation that GR mRNA was decreased after glucocorticoid treatment in a variety of tissues and cells suggests that regulation of GR activity may occur at the level of GR transcription and/or mRNA stability (Kalinyak et al., 1987; Gustafsson et al., 1987; Hoeck et al., 1989; Vig et al., 1994). The decrease of GR mRNA is not due to an alteration of GR mRNA stability but rather to a decreased transcription rate. Rosewicz et al. (1988) showed that the GR mRNA half-life of approximately 120 min in human lymphoma IM-9 cells and 240 min in rat pancreatic acinar AR42J cells was not affected by Dex treatment but the rate of GR gene transcription was decreased. Similarly, Dong et al. (1988) demonstrated that the half-life of GR mRNA in the HTC cells was approximately 4.5 hours and was unaffected by Dex. Furthermore, the glucocorticoid-caused down-regulation of the GR transcription observed in the presence of the inhibitor of protein synthesis, cycloheximide, indicates that this response might not be dependent on the synthesis of a putative second transactivating factor but directly mediated by the GR protein itself (Okret et al., 1986; Vig et al., 1994). The identification of GR binding sequences within the 3'-nontranslated part of the rat GR cDNA clone suggests a direct interaction of GR with its mRNA and/or even its gene in the regulation of the receptor expression (Okret et al., 1986). More recently, Burnstein et al. (1990, 1991c) showed that certain sequences of human GR cDNA bound GR, and these

sequences were necessary and sufficient for glucocorticoid-induced down-regulation of the receptor. The analysis of the genomic structure of hGR helps better understand the mechanisms of the glucocorticoid down-regulation of GR (Encio and Detera-Wadleigh, 1991; Leclerc et al., 1991). Careful examination of the hGR promoter led to the recognition of the down-regulatory sequences between the region -250 and -750 5' to the transcription start site (Govindan et al., 1991; Leclerc et al., 1991). Despite the fact that the hGR promoter region is extremely rich in G+C sequences, the region identified which contains the responsive elements for ligand-induced down-regulation does not contain any such steroid response elements shown to be essential for the transactivation or trans-repression (Beato et al., 1989) of other glucocorticoid-responsive genes.

In contrast to the largely documented down-regulation of GR by glucocorticoids, several groups recently reported that GR protein and mRNA were increased by glucocorticoid treatment in a leukemic cell line (Eisen et al., 1988; Antakly et al., 1989; Ashraf et al., 1991). Gomi et al. (1990) found that glucocorticoid induced GR mRNA in glucocorticoid-sensitive myeloma cells and correlated this induction with glucocorticoid-evolved cell lysis. Dex treatment was reported to increase GR mRNA in cerebral cortex cells but decrease GR mRNA in hypothalamus cell (Pepin et al., 1990). Administration of Dex to rats significantly reduced GR mRNA levels in hippocampal subfields CA1-2 and CA-3, but had no effect on GR mRNA levels in the dentate gyrus (Herman et al., 1989). Thus different tissues or cells might respond differently in terms of GR regulation. In addition, it appears to be developmentally regulated; there was no negative regulation in several fetal or neonatal tissues in which GR was shown to have functional activity and is negatively regulated in the adult animal (Kalinyak et al., 1989). While down-regulation represents negative feedback mechanism, up-regulation may be important for cells to obtain the maximal response to the hormones.

c). GR levels and other steroid hormones

Other steroid hormones may also regulate GR levels. For example, GR mRNA was down-regulated by oestrogen in pituitary cells (Peiffer and Barden, 1987). A recent report showed that GR was up-regulated by oestrogen in brain regions such as the septum and the hypothalamus (Ferrini and De Nicola, 1991). Thus oestrogen has different regulatory effects on GR expression in different tissues or cells. In primary cultures of mouse mammary epithelial cells, the level of GR is maintained by glucocorticoids whereas progestin antagonizes the glucocorticoid effect (Schneider et al., 1988).

d). GR levels and other factors

In addition to steroid hormones, GR can be regulated by several other factors. For instance, alterations in cell energy-metabolism may affect receptor number (Wheeler et al., 1981). Concanavalin A treatment of C_6 rat glioma cells in primary culture leads to a 90% reduction in receptor activity, indicating that cell surface modulators may affect receptor levels (Cidlowski and Munck, 1976). Age-associated decreases in glucocorticoid responsiveness and GR number by rat adipocytes, splenic leukocytes and WI-38 fibroblasts have been described (Roth, 1974; Roth, 1976; Roth and Livingston, 1976; Rosner and Cristofalo, 1981; Cristofalo and Rosner, 1979). Kidney of immature rats contain higher GR level than that of mature rats (Sharma and Timiras, 1988). However, when Ono et al. (1988) examined GR in cultured human pubic skin fibroblasts of young and aged men, they did not find age-related differences in either receptor number or the affinity of the receptor to the ligand. Similarly, Kalimi (1982) and Kalimi and Banerji (1982) did not find significant age-related changes in glucocorticoid responsiveness and GR levels in rat splenic leukocytes. Receptor number may depend on the phase of the cell cycle as cells in the S phase have a 1.5- to 3-fold increase in receptor number compared with those in the G_1 phase (Cidlowski and Cidlowski, 1982). In addition, receptor function may be subject to cell cycle control as many cells are unresponsive to glucocorticoid hormone action during the G_2 phase of mammalian cell cycle (Hsu and DeFranco,

1995). Neural factors may alter GR level as the expression of GR in the intermediate lobe of the pituitary is under the control of dopamine (Antakly et al., 1985; Antakly et al. 1987). Changes in GR number in brain or pituitary cytosols have been reported to be induced by vasopressin (Veldhuis and de Kloet, 1982a), ACTH (Veldhuis and de Kloet, 1982b), and insulin (Tornello et al. 1981). In AtT-20 pituitary tumour cells, treatment with CRF resulted in a decrease in GR binding and GR mRNA level and this down-regulation of GR was mediated via a cAMP dependent mechanism (Sheppard et al., 1991). In the primary cultures of mouse mammary epithelial cells, GR number was controlled by cortisol as well as prolactin (Schneider and Shyamala, 1985). Yang (1992) showed that, in the newborn period compared to the fetal life of lambs, GR mRNA levels were higher in hypothalamus and pituitary but lower in adrenal gland, indicating a developmental and tissue-specific change in GR content.

Finally, a number of reports described roles of cAMP in controlling the cellular GR level. cAMP increases glucocorticoid binding capacity in cultured skin fibroblasts (Oikarinen et al., 1984; Gruol et al., 1986). Oikarinen et al. (1984) implied that it was the ratio of cAMP to cGMP which determined the receptor concentration and thus the receptor activity during cell proliferation. This increase in glucocorticoid binding required functional cAMP-dependent protein kinase activity, since cells containing reduced kinase activity showed impaired response to cAMP. More recently, it has been found that cAMP increases GR mRNA and protein levels in rat hepatoma cells and lymphoma cell lines, suggesting that cAMP regulates glucocorticoid binding at the level of increased GR expression (Dong et al., 1989; Gruol et al., 1989). Furthermore, Dong et al. (1989) showed that the cAMP increased GR level correlated well with the increase in inducibility of two glucocorticoid regulated genes, the endogenous tyrosine aminotransferase and the stably integrated MMTV, implying the limiting nature of the GR protein in determining the biological response. In contrast to what was described above, Sheppard et al. (1991) showed that cAMP decreased both the GR binding and the

level of GR mRNA in AtT-20 pituitary tumour cell line. This may suggest that cell-specific factors are involved in the cAMP-mediated regulation of GR expression.

E. Atrial natriuretic factor (ANF) and its relationship with glucocorticoids

1. Structural and functional properties of ANF

ANF is a potent diuretic, natriuretic, and vasorelaxant hormone which was initially discovered in mammalian atria (Currie et al., 1983; deBold et al., 1981). ANF is released by the atria in response to an increase in atrial stretch. Kidneys, vessels and adrenal are the major ANF target tissues. The ANF inhibitory effect on the renin-angiotensin-aldosterone system potentiates its natriuretic and vasodilatory actions (reviewed by Dussuale, 1994). By decreasing the venous return to the heart, ANF exerts an indirect negative feedback on its own synthesis. The cDNA for prepro-ANF predicts a 151- and 152-amino acid protein in man and rat, respectively, including an N-terminal signal sequence (Zivin et al., 1984; Yamanaka et al., 1984; Maaki et al., 1984; Oikawa et al., 1984; Seidman et al., 1984a; Nakayama et al., 1984). Rat atria store mostly a single 126-amino acid prohormone, ANF-126, which is derived from the prepro-ANF by removing the N-terminal signal sequence and the two C-terminal arginine residues predicted by the cDNA sequence (Kangawa et al., 1984; Tanaka et al., 1984; Flynn et al., 1985; Miyata et al., 1985; Glembotski et al., 1985; Vuolteenaho et al., 1985; Gibson et al., 1987). Immunocytochemical studies have shown that ANF is stored in cardiac atrial cells within electron dense secretory granules, similar to those found in other endocrine cells (Cantin et al., 1984; Metz et al., 1984; Zisfein et al., 1986). The principle circulating form of ANF is a 28-amino acid peptide which is derived from the C-terminus of pro-ANF and has been identified as ANF-(99-126) (Schwartz et al., 1985; Thibault et al., 1985). The posttranslational processing of ANF-(1-126) to ANF-(99-126) was suggested to occur within or in close association with the cardiac myocytes (Shields et al., 1988). ANF-(99-126) acts on a variety of target tissues through binding to specific membrane receptors (De Léan et al., 1984; Lowe et al., 1989).

While ANF is synthesized predominantly in the cardiac atria (deBold et al., 1981), small amounts are also produced in several other tissues including the cardiac ventricles and submaxillary gland (deBold et al., 1981; Nemer et al., 1986; Gutkowska and Nemer, 1989). Ventricular myocytes present an interesting contrast to atrial myocytes with regard to the dynamics of ANF secretion. Whereas atrial myocytes store large quantities of ANF in the electron-dense secretory granules, ventricular myocytes contain far fewer granules but secrete ANF constitutively at a rate about 10-fold greater than that of atrial cells (Bloch et al., 1986).

The rat ANF cDNA clone was used as a probe to isolate human (Nemer et al., 1984) and rat (Argentin et al., 1985) ANF genes. The ANF genes are composed of three exons and two introns, the second intron contains a potential glucocorticoid receptor binding site (Nemer et al., 1984; Argentin, et al., 1985). The presence of this GR binding site suggests that ANF gene is regulated by glucocorticoids, a hypothesis which has been recently confirmed by in vitro and in vivo experiments (see below).

2. ANF regulation by glucocorticoids

ANF expression is regulated by developmental (Claycomb, 1988), hemodynamic (Needleman and Greenwald, 1986; Yamaji et al., 1985; Espiner et al., 1986; Haller et al., 1987; Uehlinger et al., 1987; Nemer et al., 1988) and hormonal factors (Ladenson et al., 1988; Nemer et al., 1988). One of the hormonal factors is glucocorticoids which have been shown to stimulate ANF production both in vivo and in vitro. For example, glucocorticoid administration to ADX rats leads to a 2- to 3-fold increase in atrial ANF mRNA levels and a 4-fold increase in the left ventricular ANF mRNA levels (Nemer et al., 1988). The glucocorticoid stimulation of ANF mRNA is due to a direct action of the steroid at the level of the heart as confirmed in primary cardiocyte cultures (Nemer et al., 1988). Shields et al. (1988) showed that glucocorticoids stimulated ANF secretion in cultured atrial cells as well as cultured ventricular cells. Glucocorticoid-stimulated ANF secretion was also

observed in normal men receiving glucocorticoid treatment (Weidmann et al., 1988). Argentin et al. (1991) found that, by DNA-mediated gene transfer studies, glucocorticoids affected ANF gene transcription via a glucocorticoid response element located in the distal 5'-flanking sequences of the rat ANF gene between -697 and -1029 base pairs. These authors further revealed that, by in vitro DNase I footprinting experiments, there were two binding sites for purified glucocorticoid receptor within this region. Since glucocorticoid activation of the ANF promoter appears specific to cardiac cells, these authors suggested that there was an interaction between the glucocorticoid receptor binding sites and cardiac-specific regulatory elements of this promoter.

F. Objectives

The present study has been aimed to answer three questions.

1). In terms of GR regulation by glucocorticoids, there seems to be a controversy since up-regulation has been reported (Eisen et al., 1988; Antakly et al., 1989) despite the largely accepted concept that glucocorticoids down-regulate their own receptors (for reviews, see Svec, 1985; Gustafsson et al., 1987). Whether different tissues exhibit different responses still remains an open question although Kalinyak et al. (1987) have shown that GR mRNA level is increased in certain tissues but is not altered in other tissues by ADX (a tissue-specific response). Thus a systematic study on several rat tissues has been undertaken to examine this issue.

2). Whether induction or reduction of GR results in parallel changes in target gene response has been a challenging question. To examine this issue, cardiac ANF has been chosen as a model target gene of the GR. The cardiac tissue contains GR and synthesizes ANF, thus providing a model system for studying the relationship between changes in GR levels and alteration in the responses of ANF to glucocorticoids. The induction of cardiac ANF has been well documented by both in vivo and in vitro experiments (Nemer et al., 1988; Shields et al., 1988; Weidmann et al., 1988). Recently, GR binding sites have been identified on the ANF gene

(Argentin et al., 1991).

3). The general presence of GR has been documented in mammalian tissues (Ballard et al., 1974). However, whether the expression of GR is cell-specific, and if this is the case, what is the biological significance of this cell-specific expression of GR are two important questions. To examine these issues, heart, testes and submaxillary glands provide interesting examples to study the cellular distribution of GR and the relationship between GR and other substances by immunocytochemical methods for the reasons below.

a). The heart: ANF, the main secretory protein in the heart, is regulated by glucocorticoids. For this reason, ANF and GR have been speculated to be present in the same cardiac cells. However, to date, no immunocytochemical information has been available in this regard. Thus the aim of the present study is to investigate whether GR and ANF are co-expressed in the same cardiac cells.

b). The testes: Our previous studies showed that while the anterior pituitary POMC gene was inhibited by glucocorticoids, testicular POMC was not subject to glucocorticoid regulation (Tremblay et al., 1988). Whether the non-responsiveness of testicular POMC to glucocorticoids is due to the lack of GR in the POMC-containing cells, as in case of the intermediate lobe of the pituitary, remains to be answered. The present investigation is aimed to examine this issue by immunocytochemical localization of GR and ACTH (a POMC derived peptide) in the testis.

c). The submaxillary glands: Glucocorticoids and androgens influence the development and differentiation of the GCT cells in the submaxillary gland (Walker, 1982; Walker et al., 1981). However, glucocorticoid effects have long been thought to be elicited through the action of androgen receptor. Our hypothesis is that glucocorticoid effects are mediated by the GR. To prove this hypothesis, the present study has been undertaken to examine the presence and cellular distribution of GR in the submaxillary glands. At the same time, the presence of ANF, α 2u globulin and

EGF have also been investigated to gain information on the relationship between GR and these molecules.

CHAPTER II. MATERIALS AND METHODS

A. Intact rats

Sprague-Dawley rats weighing 250-300 g were purchased from Charles River Laboratory (St. Constant, Quebec). The rats, housed three or four per cage, were kept under a 12 h light-dark cycle and maintained on standard Purina chow food and tap water ad libitum.

B. Adrenalectomy

Some rats were bilaterally adrenalectomized to deplete the endogenous glucocorticoids. ADX was performed through the dorsal approach under ether anaesthesia. In an initial experiment, GR levels were measured by radioimmunoassay (RIA) (see below) in several tissues of normal rats and of rats adrenalectomized for 1 week, 2, and 3 weeks. Since optimal GR regulation was found in the rats adrenalectomized for 2 weeks (see results), adrenalectomy for 2 weeks was chosen for further study. Adrenalectomized rats were kept and maintained under the similar conditions as those for normal rats except that ADX rats were given 0.9% sodium chloride instead of tap water since diet salt is necessary for the survival of the adrenalectomized rats.

C. Glucocorticoid treatment

A synthetic glucocorticoid, Dex, was used. Dex binds to GR with a high affinity and is specific to GR (Roberts-Thomson et al., 1991; Funder, 1992). First, a dose response analysis of Dex on GR levels was done. Briefly, intact rats were injected intraperitoneally with Dex of various doses ranging from 1 to 8 mg/kg for 4 days. GR levels were measured by radioimmunoassay (see below) in the liver cytosols. A Dex dose of 4 mg/kg resulted in a reduction of GR level by 53.2% and this dose was chosen for further study. Second, a time response analysis of Dex on GR levels was done. Intact rats were injected intraperitoneally with Dex (4 mg/kg) for different time lengths ranging from 2 to 24 hours. The GR levels were measured by radioimmunoassay in the liver cytosols.

In further studies, both intact and adrenalectomized rats were injected intraperitoneally with Dex (4 mg/kg body weight in phosphate-buffered saline [PBS]) daily for 1 day, 4, or 8 days. The injections were given at a constant time of the day (9:00-10:00 h) to avoid variations. It was known that stress (histamine, cold exposure, immobilization and ether) increased serum corticosterone and decreased GR levels in experimental rats (Sapolsky et al., 1984). In order to know whether the action of injection applied in the present study was such a stress inducer to alter GR levels in rat tissues, intact rats were either injected with the same volume of PBS as that used for Dex injection for 1 day, 4 or 8 days or were uninjected before being sacrificed. The method for PBS injection was exactly the same as that for Dex injection. GR concentration was measured by RIA in liver, spleen, cardiac ventricles and atria. Since no significant difference (by analysis of variance) was detected among GR levels while rats were uninjected or injected with PBS (see results), uninjected rats were used as controls for Dex injection in the experiments thereafter.

D. Grouping of rats

To investigate the effect of glucocorticoids on the GR and ANF levels in experimental rats, the rats were divided into several treatment groups in a random manner: 1). intact/no medication (control), 2). intact/Dex 1 day, 3). intact/Dex 4 days, 4). intact/Dex 8 days, 5). ADX/no medication (control for ADX rats), 6). ADX/Dex 1 day, 7). ADX/Dex 4 days, 8). ADX/Dex 8 days.

E. Tissue handling

At the time indicated above, the rats were killed by decapitation between 10:00-12:00 h. Several tissues were excised; these were liver (left lobe), spleen, thymus, heart, pituitary, brain, the right testis and the right submaxillary gland. Immediately, the heart was further dissected into the left and the right atria and ventricles, the pituitary was separated into the anterior and neurointermediate lobes, and the hypothalamus and the hippocampus were dissected from the brain. All the tissues were quickly rinsed free of blood with PBS. Small pieces of heart, testis and

submaxillary gland tissues were cut and immediately fixed in Bouin's solution (mixture of 375 ml of a saturated aqueous solution of picric acid, 125 ml of 40% formaldehyde, and 25 ml of glacial acetic acid) for immunocytochemical (IC) study (see below). The rest of the heart (after the pieces were cut for IC), and other tissues were quickly frozen in liquid nitrogen before being stored in a -80^{0}C freezer for later determination of the ANF levels and GR levels for the heart and the GR levels for all the other tissues. Testis and submaxillary gland were not processed for RIA study.

When the rats were killed, it was observed by the naked eye that the thymuses shrank after Dex treatment. Thus thymuses were weighed quickly before they were processed for RIA study.

F. Preparation of RIA samples

In order to measure GR levels in rat tissues, GR had to be extracted from the tissues by homogenization. Tissues were thawed in ice-cold homogenate buffer which contained Tris HCl (pH 7.2) 25 mM, NaCl 10 mM, EDTA 1 mM, $MgCl_2$ 2 mM, dithiothreitol 1 mM, glycerol 5% and leupeptin 10 nM. Homogenization was performed in a 4^{0}C cold room using a motorized glass-on-teflon homogenizer (2300 rpm). A consistent volume of homogenate buffer was used for all the samples, i.e., the ratio of tissue : buffer was 1 gram : 3 ml. After 6 strokes of homogenizing, 0.4% Nonidet P-40 (NP40) (Sigma) and 0.06% deoxycholate (DOC) (Sigma) were added and 6 more strokes were done. Homogenates were centrifuged for 5 min in an Eppendorf microcentrifuge (12,000 g). The "pellets" referred to as "particulate fractions" were stored at -80^{0}C for the RIA experiment. The supernatants were centrifuged in a Beckman TL 100.2 desktop ultracentrifuge (128,000 g, 10 min) and the second supernatant referred to as "cytosol" was aliquoted and stored at -80^{0}C until GR measurement by RIA. The pellet resulting from the second centrifugation was added to the "particulate fraction" pool. Total protein in the "cytosol" was assessed using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

G. "Cytosol", "particulate fraction", and "particulate extract"

As described above, "cytosol" was defined as the supernatant after the second centrifugation of the tissue homogenate and the "particulate fraction" was defined as the pellet after the first centrifugation of the tissue homogenate. GR concentration was measured in all the "cytosols".

In order to determine the amount of GR which was unextracted and left in the "particulate fractions", GR was extracted and quantitated in the "particulate fractions" of liver and spleen as two examples. A procedure similar to tissue homogenization (see above) was applied for GR extraction from the "particulate fraction". Instead of the homogenate buffer used for tissue homogenizing, a high salt buffer containing 0.01 M phosphate (pH7.4) and 0.4 M NaCl was used to homogenize the "particulate fraction". Homogenization was achieved using a motorized glass-on-teflon potter (6 strokes, 2300 rpm). The homogenate was spun in an Eppendorf microcentrifuge (12,000 g, 5 min) and the supernatant was then spun in a Beckman TL 100.2 desktop ultracentrifuge (128,000 g, 10 min). The second supernatant, referred to as "particulate extract", was assayed for total protein using Bio-Rad protein assay kit and GR level by RIA. Fig. 3 shows the procedure for RIA sample preparation.

H. Preparation of the antiserum to a synthetic peptide (14-mer) of the GR

A 14 amino acid peptide (14-mer) corresponding to amino acids 171-184 of the human GR (Hollenberg et al., 1985) was prepared. The amino acid composition of this peptide is Lys, Gly, Gln, Thr, Gly, Thr, Asn, Gly, Gly, Asn, Val, Lys, Leu, Tyr (from the N-terminus to the C-terminus). An antibody was generated against this 14-mer peptide. Briefly, the peptide was coupled at its NH_2 terminus to bovine serum albumin (BSA) at a molar ratio of peptide : carrier of 14.3 : 1, according to an earlier procedure (Parker and Hodges, 1985). The conjugate (250 μg), mixed with Freund's complete adjuvant (Difco, Detroit, MI), was injected subcutaneously into male New England white rabbits. Subsequent booster injections at 3-week intervals were administered using Freund's incomplete adjuvant. Two rabbits were used for antisera production and these rabbits were named rabbit B and C. The

binding properties of the antisera from these two rabbits were tested by RIA. The antiserum from rabbit B after the second booster injection yielded the best binding and this antiserum was referred to as $14B_2$ that was used in this study.

The antibody titer, which represents the concentration and the affinity of an antibody, was monitored by incubating progressive dilutions of the $14B_2$ with a fixed amount of ^{125}I- labelled antigen (^{125}I-14-mer) in test tubes. To separate the antibody bound and free 14-mers, a goat anti-rabbit serum was added to the incubation followed by centrifugation. The amount of the bound 14-mer was measured by counting the radioactivity in the precipitate. The titer of the $14B_2$, which was determined as the antiserum dilution that bound 50% of the added antigen, was 1:15,000.

I. Immunoblotting of the GR with 14B2

HTC cells (kindly provided by Dr. K.R. Yamamoto) were used. These cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (GIBCO). Following their dissociation from monolayers, the HTC cells were collected by centrifugation, washed, resuspended in homogenate buffer, and finally homogenized by hand using a ground-glass homogenizer. Homogenates were centrifuged at 4 ^{0}C and 100,000 x g for 20 min in a Beckman TL-100 ultracentrifuge. The supernatant was used for immunoblotting.

An immunoblotting procedure (Gametchu and Harrison, 1984) was used to verify the specificity of the $14B_2$ antibody. Briefly, tissue homogenates of HTC cells were electrophoresed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels. The proteins separated on the gels were electrophoretically transferred on nitrocellulose paper and incubated with $14B_2$ or preimmune serum. After washing to remove the unbound antibodies, the nitrocellulose paper was incubated with ^{125}I-protein A (NEN, Boston, MA; specific activity 8.8 μCi/mg) and radioactive bands were revealed by autoradiography.

J. Affinity labelling and immunoprecipitation

Pituitary cells maintained in primary culture were used (Antakly et al., 1987). Thirty to 50 male Sprague Dawley rats were used per culture. The rats were decapitated, the pituitary glands were immediately excised, and the neurointermediate lobes were removed from the anterior lobes. After enzymatic digestion, the anterior lobes were cultured in 35-mm regular Falcon dishes in Dulbecco's Modified Eagle's medium containing 10% horse serum and 2.5% fetal bovine serum (Gibco, Grand Island, NY). The cells were washed in serum-free medium and incubated in PBS in the presence of [^{3}H]dexamethasone mesylate (NEN, 49 Ci/mmol) which binds covalently to the GR (Simons et al., 1987). Following affinity labelling, the cell cytosols were extracted and reacted with 5-10 μl of either the $14B_2$ antiserum or the preimmune serum. After an overnight incubation at 4 ^{0}C, the rabbit antibodies were immunoprecipitated using protein A sepharose (Pharmacia Uppsala, Sweden) and washed several times in PBS containing 0.1% NP-40. The precipitates were dissolved in SDS sample buffer [glycerol 1 ml; SDS 0.2 g; 0.25 mM Tris (pH 7.4) 0.6 ml; 0.3 ml B-mercaptoethanol; 0.15 g dithiothreitol; 10% bromophenol blue 0.2 ml], electrophoresed, fixed, treated with Entensify (DuPont-NEN, Boston, MA), and dried for fluorography.

K. Radioiodination of the 14-mer

Since the 14-mer peptide contains a tyrosine residue, it could be labelled with ^{125}I using the chloramine T method (Greenwood et al., 1963). Essentially, 2.5 μg of reverse-phase High Pressure Liquid Chromatography (HPLC) pure 14-mer dissolved in 5 μl of 0.1 M phosphate buffer (pH 7.4) was mixed with 12.5 μl (0.25 mCi) of Na^{125}I (New England Nuclear, Boston, MA). The iodination reaction was initiated by addition of 5 μl of 0.1 mg/ml chloramine T. After 30 min at 4^{0}C the reaction was stopped by the addition of 12.5 μl of 0.2 mg/ml sodium metabisulfate (Fisher), and neutralized by 250 μl of 10 mg/ml potassium iodide. The reaction mixture was immediately passed through Sep-Pak C_{18} cartridge (Waters, Milford, MA). The Sep-Pak was washed successively with 5 x 3 ml fractions of increasing concentrations of

acetonitrile dissolved in 0.1% trifluoroacetic acid in water. The radioactivity was monitored in 10 μl aliquots of these fractions. The iodinated 14-mer was eluted by acetonitrile as a single peak on a Sep-Pak column. The average specific activity of the iodinated 14-mer was 135 Ci/mmole (n=16). The ^{125}I-14-mer was stored at 4^{0}C. In some experiments, the purity of the Sep-Pak cartridge eluted tracer was examined by HPLC (Dionex, Canada). Briefly, the tracer was passed within 65 min through a Zorbax RP300-C18 reverse-phase column maintained at 60 ^{0}C and a gradient of acetonitrile from 5% to 20% in H_2O containing 0.01 M HCl. The eluted peak was detected by a FLO-ONE Radioactivity Detector (Radiomatic, A Canberra Company, Canada).

L. GR RIA procedure

RIA buffer containing 0.1% normal rabbit serum (NRS) in PBS was used to dilute tissue samples, antibodies and ^{125}I-14-mer (tracer). RIA was performed in polystyrene tubes to which were added 50 μl of either known amounts of 14-mer ranging from 0.6 to 1,500 fmoles/tube (standard curve) or the unknown samples, 100 μl of RIA buffer containing 20,000 cpm of ^{125}I-14-mer, 100 μl of $14B_2$ (the primary antibody) at the final dilution of 1/20,000. After one hour incubation at 22^{0}C, 150 μl of ice-cold goat anti-rabbit antiserum (the secondary antibody) (Biomega, Montreal) diluted at the final dilution of 1/200 was added and the precipitation reaction was allowed to occur for 18-22 hours at 4^{0}C. Then, the tubes were centrifuged at 4^{0}C (5,000 g, 30 min.) after 1 ml of ice-cold RIA buffer was added. A fine precipitate was then observed, and the supernatant was discarded by draining. The precipitate in the test tube was counted in an LKB-Wallac (Sweden) Compugamma counter and RIA data were calculated by the program supplied with the counter. The principle of RIA is depicted in Fig. 4. In addition to the tubes with samples or unlabelled 14-mer standards, there were three other types of tubes: 1). tracer tubes which had tracer added only and the radioactivity in the whole tracer was counted; 2). reference tubes in which the primary, the secondary antibodies and

tracer but neither samples nor unlabelled 14-mer standards were added and the tubes were centrifuged, drained before being counted; and 3). blank tubes in which the secondary antibody and tracer but neither primary antibody, samples nor standards were added and the tubes were centrifuged and drained before being counted. Thus the radioactive counts in the tracer tubes (1) represented that of the total tracer added, the counts in the reference tubes (2) reflected the maximal binding of the antibodies to the radiolabelled 14-mer, and the counts in the blank tubes (3) reflected the non-specific binding of the labelled 14-mer to the wall of the tubes or other materials in the tubes.

Total binding (the radioactive counts in the reference tube over that in the tracer tube) and non-specific binding (the radioactive count in the blank tube over that in the tracer tube) were calculated.

Routinely, assays were done in triplicate and the GR value for each sample represents the average of the triplicate determinations. For each type of tissue, all the samples under different glucocorticoid treatments were assayed at the same time (the same RIA run) to avoid inter-assay variations so that comparisons among different experimental conditions are more relevant.

M. GR RIA control experiments

The reliability of the RIA was monitored by using two inter-assay controls which were two liver cytosol samples from intact rats either uninjected (L1097) or injected with Dex for 4 days (L1099). Intra-assay control was conducted by assaying 7 aliquots of L1097 in one RIA run. In another experiment, various concentrations of the liver cytosol were tested to know how they would replace ^{125}I-14-mer bound to the antibody.

N. Detergent effects on GR RIA

Since detergent was added in the samples when they were homogenized, it was necessary to determine whether detergent could affect the GR measurement by RIA. Liver from an intact rat was homogenized by the method described in the section of

"preparation of RIA samples", except that no detergent was added when the liver was homogenized. The liver "cytosol" was adequately diluted, aliquoted, and determined for the GR levels by RIA. Before RIA was performed, Nonidet P-40 and deoxycholate were added in some of the aliquots. The GR levels were compared between cytosols with detergent and cytosols without detergent.

O. ANF RIA Procedure

To study the relationship between the GR levels and the magnitude of the responses of a target gene (ANF) to glucocorticoids, ANF concentrations were determined, by RIA, in the same heart cytosol samples as those assayed for GR levels. RIA buffer (pH 7.4) containing phosphate 0.1 M, NaCl 0.05 M, 0.1% BSA was used to dilute samples, antibodies and ^{125}I-ANF. RIA was performed in polystyrene tubes to which were added 100 μl of either known amount of ANF [synthetic ANF-(99-126) of the rat form, kindly provided by Dr. De Léan, Montreal] ranging from 2.0 to 1024 pg/tube or the unknown tissue sample preparations, 100 μl of RIA buffer containing 15,000 cpm of ^{125}I-ANF (Ong et al., 1987) and 100 μl of rabbit anti-rat ANF antibody diluted according to the instructions from Peninsula Labs. (Calif.). The purity of the tracer was well-described by Ong et al. (1987). After an one hour incubation at 22^{0}C, the precipitation reaction was carried out by the addition of 100 μl of ice-cold goat anti-rabbit antiserum at the final dilution of 1:200 (Biomega, Montreal). The mixture was incubated at 4^{0}C overnight (18-22 h). One ml of ice-cold RIA buffer was added before the tubes were centrifuged (5,000 g, 30 min.). The supernatant was discarded by draining. The pellets in the tubes were counted in an LKB-Wallac Compugamma counter (Sweden) and RIA data were calculated according to a software provided with the counter. Assays were done in triplicate and the ANF value for each sample represents the average of the triplicate determinations.

As in GR RIA experiments, tracer tubes, reference tubes and blank tubes were included in each ANF RIA run. Similar to GR RIA experiments, tracer tubes were

those tubes in which only ^{125}I-ANF was added and the radioactivity of the whole tracer was counted; reference tubes were those tubes in which antibodies and tracer but neither samples nor unlabelled ANF standards were added and the tubes were centrifuged and drained before being counted; and blank tubes were those tubes in which the secondary antibody and tracer but neither the primary antibody, samples nor standards were added, and the tubes were centrifuged and drained before being counted. Total binding (the radioactive count in the reference tube over that in the tracer tube) and non-specific binding (the radioactive count in the blank tube over that in the tracer tube) were calculated.

The cross-reactivity of the ANF antibody with other materials was less than 0.001% (Peninsula Laboratories, Calif.).

P. ANF RIA control experiments

The reliability of ANF RIA was monitored by using inter-assay controls, 63a and 64a (two different atrial cytosols), 63v and 64v (two different ventricular cytosols). 63a and 63v were from the heart of one rat, and 64a and 64v were from the heart of another rat. The specificity of ANF RIA was checked by assaying the liver cytosol (prepared in the same manner as for the cardiac tissues) as a negative control since liver is devoid of ANF peptide (Gardner et al., 1986a).

Q. Glucocorticoid Radioreceptor binding assay

Glucocorticoid receptor binding was determined using [^{3}H] dexamethasone. Briefly, the cytosol homogenates of liver, spleen and cardiac ventricle were incubated with $5x10^{-9}$ M [^{3}H] dexamethasone with and without a 100-fold molar excess of unlabelled dexamethasone at 4 ^{0}C overnight. Bound and unbound dexamethasone were separated by filtration through mini-Sephadex LH (Pharmacia, Uppsala, Sweden) columns (blue pipette tips filled with Sephadex). Specific binding was calculated by subtracting the counts obtained in the presence of unlabelled dexamethasone from those measured in its absence.

R. Statistical analysis

Unpaired Student t-test was used to compare means of two groups. When more than two means were concerned, analysis of variance and subsequent Duncan's test for multiple comparisons were applied. $P<0.05$ was considered to be significant.

S. Immunocytochemistry procedure

1. Preparation of tissue sections

After the rats were sacrificed, small pieces of the heart, testis and the submaxillary gland were immediately fixed in Bouin's solution at room temperature for 18 h. Then these tissues were routinely alcohol dehydrated and embedded in paraffin. The embedded tissues were sectioned at 4 μm, heat-mounted on previously gelatin-coated glass slides, and stored at room temperature until immunocytochemical processing.

2. GR antibodies used in immunocytochemistry

Two independent GR antibodies were used: the $14B_2$ which was an antiserum raised in rabbit to a synthetic 14 amino acid peptide corresponding to the N-terminal region of the human GR (Hollenberg et al., 1985), and BuGR1, a mouse monoclonal antibody to purified rat liver GR (Gametchu and Harrison, 1984).

3. Immunocytochemistry on heart tissues

The heart tissue sections were examined, by immunocytochemistry, for the cellular distribution of GR and for the co-existence of GR and ANF in the same cells. The tissue sections were first incubated with 10% normal goat serum for 20 min at 37^0C to block non-specific binding of the antibody to the tissue sections and then incubated with either $14B_2$ or BuGR1 (Gametchu and Harrison, 1984) overnight at 4^0C. Several antibody dilutions were tested, and the optimal antibody dilutions were found to be 1:400 for $14B_2$ and 1:20 for BuGR1. At these antibody dilutions, the immuno-staining signal was relatively high and the non-specific staining was relatively low. On the following day, the sections were washed with PBS before being incubated with biotinylated goat anti-rabbit IgG (Vector laboratory,

Burlingame, CA) for the sections previously incubated with $14B_2$, or the sections were incubated with biotinylated goat anti-mouse IgG (Vector laboratory, Burlingame, CA) for the sections previously incubated with BuGR1, for 1 hour at 22^0C. After the sections were washed thoroughly with PBS, they were incubated with a preformed complex (ABC) of avidin (having 4 biotin-binding sites) linked to 3 biotin molecules, each of which was conjugated to a peroxidase molecule. Thus the ABC complex was allowed to bind to the biotinylated goat anti-rabbit IgG or goat anti-mouse IgG, a reaction having an affinity constant ($10^{15}M^{-1}$) a million times higher than that for most antibody-antigen complexes. The peroxidase enzyme promoted the reduction of H_2O_2 and oxidized the chromogen 3,3'-diaminobenzidine (DAB; Sigma), resulting in an insoluble dark brown precipitate (Graham & Karnovsky, 1966).

In order to investigate whether GR and ANF were present in the same cardiac cells, a double labelling procedure was used. Subsequent to the immunoperoxidase reaction, the sections previously stained with BuGR1 were processed for immunofluorescent labelling for ANF. Briefly, after being extensively washed, the sections were incubated with a rabbit anti-rat antibody to ANF (Peninsula Labs, Calif.) overnight at 4^0C, washed with PBS, exposed to the fluorescein conjugated goat anti-rabbit IgG (Cappel Laboratories, Pa.) in the dark for 1 hour at 22^0C. The ANF antibody was diluted at 1:2,000. The sections were then washed in PBS overnight, mounted in a medium containing 0.1% p-phenylenediamine (Fisher Scientific Co.), 90% glycerol in PBS, PH 8.0, to preserve the fluorescence during microscopy (Johnson & de C. Nogueira Araujo, 1981), and examined by phase contrast microscopy. The heart tissues from 7 rats were used for each antibody labelling to repeat the experiment.

4. Immunocytochemistry on submaxillary glands

Submaxillary gland sections were examined for the cellular distribution of GR and the co-existence of EGF, α 2u globulin or ANF within the cells that contained GR. Two double labelling immunocytochemical procedures were used. The first

method consisted of staining serial or semiconsecutive histological sections with either one of the antibodies to GR, ANF, EGF, or α 2u globulin by an immunoperoxidase procedure. In the second method, the sections were processed for the immunoperoxidase localization of GR using BuGR1. Subsequently, they were re-incubated with rabbit antibody to either α 2u globulin, ANF or EGF after being thoroughly washed with PBS. The bound antibodies were revealed by a goat anti-rabbit antibody conjugated to fluorescein (Cappel Laboratories, Pa.). These rabbit antibodies were anti-ANF (Peninsula Laboratories, Calif.), anti-α 2u globulin (Antakly et al., 1982a), and anti-EGF (kindly supplied by Dr. P. Walker, Ottawa, Canada). The antibody dilutions were 1:600 for $14B_2$, 1:20 for BuGR1, 1:100 for the α 2u globulin antibody, 1:1000 for the ANF antibody, and 1:200 for the EGF antibody. To repeat the experiment, submaxillary glands from 6 rats were used for each antibody labelling.

5. Immunocytochemistry on testes

A double labelling procedure was applied to localize GR and ACTH in testicular tissues. Testis sections were first processed for GR localization using BuGR1 by the immunoperoxidase procedure. The BuGR1 dilution was 1:20. After subsequent washing, the same sections were re-incubated with a rabbit antibody to ACTH-(1-39) (Pelletier, 1977) and the bound antibody was revealed by fluorescein conjugated goat anti-rabbit antibody (Cappel Laboratories, Pa.). The dilution of the ACTH antibody was 1:1,000. Testes from six rats were used to repeat the experiment.

6. Immunocytochemistry control experiments

The specificity of the immuno-staining was routinely checked using the pre-immune or non-immune sera as controls and the dilutions of the above sera were the same as the corresponding antisera for the immunocytochemical labelling. In selected experiments, preadsorption controls were performed in which the GR antibody was preabsorbed at 4^0C for 24 hours with partially purified rat liver GR (Grandics et al.,

1984). Pituitary tissue sections prepared in the same manner were processed in parallel as internal controls for GR staining since the intermediate lobe, unlike the anterior one, is known to be devoid of GR (Antakly and Eisen, 1984).

CHAPTER III. RESULTS

Part I. GR levels quantified by RIA in rat tissues under various endocrinological conditions

A. Characterization of the $14B_2$ antibody

Using primary culture pituitary cells, a protein band of Mr of 94 KDa (Fig. 5A) was produced on SDS gel fluorogram by immunoprecipitation of [^{3}H] dexamethasone mesylate affinity-labelled GR with the $14B_2$ antibody. Western blot showed that, in HTC cells, the antibody reacted with a protein species having a Mr of 94 Kda (Fig. 5B).

B. Development of the GR RIA

1. ^{125}I-14-mer

The iodinated 14-mer was eluted as a single peak on reverse phase HPLC (Fig. 6). This iodinated 14-mer was stored at 4 ^{0}C and found to be stable for two months. Based on the suggestion by Parker (1976) that a minimum of 5,000 to 10,000 cpm of radioactive antigen per individual assay tube should be used in order to obtain adequate levels of bound radioactive antigen, the amount of iodinated 14-mer was set at about 20,000 cpm per assay tube in the RIA.

2. $14B_2$ antiserum

In order to know the optimal dilution of the $14B_2$ in the RIA, several antibody dilutions (1:10,000, 1:20,000, 1:40,000) were tested. When the normal rabbit and goat-anti-rabbit sera were set at the respective dilutions of 1:1,000 and 1:200, and the freshly prepared radioactive 14-mer was used at 20,000 cpm, a dilution of 1:20,000 for the $14B_2$ gave optimal results (Table 3). Under these conditions, 36.5% of the radioactive 14-mer was bound when no unlabelled 14-mer was added and this value was in the range (30%-50%) predicted for a highly sensitive RIA (Parker, 1976).

3. Standard curve, sensitivity of the RIA and affinity of $14B_2$ with the 14-mer

The standard curve is plotted as the ratio of bound to total radioactive 14-mer against the increasing concentrations of the unlabelled 14-mer on a semi-logarithmic

scale. A sigmoid curve was achieved; the linear part of the curve (bound/total of 0.07 to 0.31 in Fig. 7) was used to calibrate the GR concentrations in the unknown samples. The sensitivity of the RIA, defined as the lowest amount of the receptor which could be detected with 95% confidence (Rodbard, 1978), was 6 fmoles/tube (3.7×10^9 molecules/tube). The dissociation constant (Ka) of $14B_2$ with the 14-mer was calculated from the concentration of unlabelled 14-mer at half-saturation of the antibody and was 6×10^9 L/mole, suggesting a high affinity of $14B_2$ with the 14-mer.

Standard curves generated using sera from rabbit B (preimmune B) or following the first ($14B_1$) and second ($14B_2$) immunization booster injections and rabbit C after the first booster injection ($14C_1$) showed that $14B_2$ yielded the best binding (Fig. 8).

4. Affinity of $14B_2$ with liver cytosol GR

Various dilutions of liver cytosol that contained a high concentration of GR were used to compete for binding sites with the ^{125}I-14-mer. The competition curve was plotted as the bound/total radioactive 14-mer against increasing concentrations of liver cytosol (Fig. 9). As the concentration of liver cytosol increased, the binding of ^{125}I-14-mer to the antibody was inhibited. When the concentration of liver cytosol was 0.80 mg/ml at the logarithmic scale (equivalent to 6.30 mg/ml), 50% of the bound radioactivity was inhibited. The dissociation constant of the $14B_2$ with liver cytosol GR, calculated as the GR concentration in the liver "cytosol" (calibrated from the standard curve) which half-saturated the antibody, was 5×10^9 L/mole. This indicated a high affinity of the antibody with the receptor (Alberts et al., 1989).

A standard curve (Fig. 9) was produced in the same time as the liver cytosol competition curve and was used to calculate the GR levels in the liver cytosols. The GR level in the liver cytosol was 715 ± 65 fmoles/mg protein (mean $\pm$ SD, $n=5$ determinations). The average GR level detected in liver cytosol was similar to that reported by others (Ballard et al., 1974; Gregory et al., 1976; Turner, 1986).

5. Tissue cytosol dilution curves

Using increasing concentrations of liver, pituitary, and spleen cytosols, RIA displacement curves were generated and the slopes of these curves were similar to one another and to the standard curve (Fig. 10). This indicated that the specificity of the antibody to the synthetic peptide was the same as to the GR in these tissue cytosols tested.

6. Controls for RIA

The assay reproducibility was monitored by inter-assay controls. The inter-assay controls used were L1097 (a liver cytosol from a normal rat) and L1099 (a liver cytosol from a intact rat treated with Dex for 4 days) that were assayed in each RIA run to establish the assay reproducibility. The GR level in L1097 was 729.7 $\pm$ 83.6 fmoles/mg protein (mean $\pm$ standard deviation, n = 21 determinations); the GR level in L1099 was 463.9 $\pm$ 66.3 fmoles/mg protein (mean $\pm$ standard deviation, n = 21 determinations). The coefficients of variation in GR levels measured among 21 assays were 13.4% and 14.5% for L1097 and L1099, respectively. In addition, 7 aliquots of a liver cytosol (L1097) were assayed in a single RIA run as an intra-assay control and the coefficient of variation in GR levels was found to be 9.4%. These indicated that the assay results were reproducible. The difference between GR levels in L1097 and L1099 were significant ($p < 0.05$) by Student t-test and this indicated that GR levels were decreased after the rats were treated with Dex for 4 days.

7. GR levels measured by RIA and receptor binding assay

To confirm the reliability of the GR RIA, GR levels from the same tissue cytosols were measured by both RIA and receptor binding assay. As shown in Table 4, the GR levels in liver, spleen and cardiac ventricle cytosols measured by RIA and by receptor binding assay were similar. However, RIA is easier to perform and more efficient than the receptor binding assay in our hands.

C. GR extraction

1. Adding detergent to the sample in the homogenization process did not affect GR measurement

GR is known to exist in the cytoplasm and the nucleus of the target cells (Govindan et al., 1980; Fuxe et al., 1985; LaFond et al., 1988; Picard and Yamamoto, 1987; Antakly and Eisen, 1984). In order to extract GR from the rat tissues, the tissues were homogenized. To extract GR from the cytoplasm and also from the nucleus of the cell, detergent (Nonidet P-40 and deoxycholate) was added to the tissues in the homogenization process to rupture the cellular membranes. The detergent concentrations of 0.4% Nonidet P-40 and 0.06% deoxycholate were used to extract GR from the rat tissues (4 μl of pure NP40 and 0.6 μl of pure DOC were added to 1000 μl of homogenization buffer). More GR in the "cytosol" and less GR in the "particulate extract" was found when these detergent concentrations were used than when no detergent was used (these will be described in more detail in the following section).

The next experiment was designed to determine whether the detergent added to the RIA samples when homogenization was performed would affect the RIA result. The highest final concentration of the detergent in the RIA samples was 0.04% NP40 and 0.006% DOC when sample dilution factor was considered. For this reason, 0.04% NP40 and 0.006% DOC were selected for study of their influence on RIA results. A piece of liver was homogenized in the homogenization buffer that did not contain detergent. The homogenate was properly diluted and aliquoted. Detergent (0.04% NP40 and 0.006% DOC) was added to some of the aliquots when RIA was performed. As shown in Table 5, detergent at the concentrations used in this study did not affect the assay result.

2. "Cytosols" and "particulate extracts"

The extraction of cytosol GR from rat tissues was achieved by homogenization followed by two centrifugations: the first centrifugation yielded a pellet defined as the "particulate fraction" and a supernatant which was centrifuged with higher speed to yield a supernatant defined as "cytosol" and a pellet which was added to the "particulate fraction" pool. A high salt buffer was used to extract GR from the

"particulate fraction". After homogenization and subsequent centrifugation, a supernatant was yielded which was defined as the "particulate extract" (see Fig. 3).

To know if more GR could be extracted from rat tissue when detergent was added to the sample in the homogenization process, the following experiment was done. A piece of liver was cut into 14 parts. Seven parts (group A) were homogenized in the buffer containing 0.4% NP40 and 0.06% DOC and the other 7 parts (group B) were homogenized in the buffer which did not contain detergent. The GR in the "particulate fractions" was extracted by homogenization using a high salt buffer (0.01 M phosphate and 0.4 M NaCl). The "particulate fractions" having resulted from the two groups were handled differently. 0.4% NP40 and 0.06% DOC were added to the "particulate fractions" in group B but not to those in group A when they were homogenized. The reason for adding detergent to the "particulate fractions" in group B was that since no detergent had been used earlier in the homogenization of the liver tissue these "particulate fractions" might contain intact nuclei. Detergent would rupture the nuclear envelope and thus GR could be extracted from inside the nucleus. In the following text, however, the term "detergent was added" means that detergent was added to the tissue samples during the first homogenization process. Taking the above situation as an example, this would mean that detergent was added to the samples in group A but not to those in group B. The homogenization and subsequent centrifugation of the "particulate fractions" resulted in "particulate extracts". GR levels were measured, by RIA, in the "cytosols" and "particulate extracts". The total cellular GR level was calculated as the sum of the GR level in the "cytosol" and in the "particulate extract". The rationale of this experiment was that more GR would be extracted in the "cytosol" if the nuclear envelope was ruptured by the homogenization. As shown in Table 6, the total GR levels were about the same no matter if detergent was added to the tissue in the homogenization process. However, the proportion of the GR level in the "particulate extract" was decreased when detergent was added. When no detergent was added to

the tissue, the GR level in the "particulate extract" was 26.6% of the total GR level, but when detergent was added, the GR level in the "particulate extract" dropped to 6.0% of the total GR. The GR level (26.6% of the total cellular GR) in the "particulate extract" when no detergent was added in the homogenization seemed to represent the GR in the nucleus. This was consistent with the observation by Beato et al. (1974) that in the liver of normal rats, about 25% of the cellular GR sites was located in the cell nucleus. The GR extracted in the "cytosol" fraction when detergent was added in the homogenization process seemed to represent the GR in the cytoplasm and most of the GR in the nucleus. Because more GR was extracted in the "cytosol" fraction when detergent was added to the tissue sample in the homogenization process, detergent (0.4% NP40 and 0.06%) was used to extract GR from rat tissues in the subsequent experiments.

D. Dose response analysis of Dex on GR levels

In liver cytosols of intact rats previously treated with different doses of Dex ranging from 1 to 8 mg/kg for 4 days, the GR levels were decreased compared to the control rats with the maximal reduction of GR level occurring at 8 mg/kg (Fig. 11). The GR level was decreased by 53.2% at a Dex dose of 4 mg/kg which was chosen for further study.

E. Time course of Dex effects on GR levels

In liver cytosols of intact rats previously treated with Dex (4 mg/kg) for different time lengths ranging from 2 to 24 hours, significant reduction of GR levels occurred after 8 hours with the maximal reduction (36.7%) of the GR level at 24 hours of Dex treatment (Fig. 12). According to this result, Dex time course was set at 1, 4, and 8 days.

F. The action of peritoneal injection *per se* did not affect GR levels in rats

To determine the influence of glucocorticoids on GR levels in rat tissues, experiments were designed to compare GR levels between rats treated with Dex and control rats. The administration of Dex was through intraperitoneal injection. It is

known that stress affects glucocorticoid secretion and GR levels in rat tissues (Sapolsky et al., 1984). Since the injection itself could be a source of stress which could alter glucocorticoid secretion and thus might affect GR levels, it was necessary to establish whether GR levels could be affected by the action of injection.

Intact rats were either uninjected or injected with PBS daily for 1, 4 or 8 day(s). The method for PBS injection was exactly the same as that for Dex injection in the other experiments in which the effect of Dex on GR levels was studied. GR levels were measured by RIA in liver, spleen and the four compartments of the heart. Data were analyzed by analysis of variance and the probability of 0.05 was used as a significant level. The result showed no significant changes of GR levels in any of the tissues assayed between uninjected rats and injected rats (Fig. 13). Thus uninjected rats were used to compare with rats injected with Dex in all further experiments.

G. ADX and GR levels

In an initial experiment, the rats were either unoperated or adrenalectomized for 1, 2 and 3 week(s). GR levels were measured, by RIA, in the cytosols of liver, spleen, anterior pituitary and the four compartments of the heart. As shown in Fig. 14, the GR levels were increased after rats were adrenalectomized for 1 week and 2 weeks. In most cases, the maximal increase in the GR levels occurred after rats were adrenalectomized for 2 weeks. Whereas, at 3 weeks after adrenalectomy the GR levels were almost the same as those in normal rats for most tissues. For this reason, rats adrenalectomized for 2 weeks were used for further study.

A further experiment was carried out to assay the GR levels in more tissues than in the initial experiment. After rats were adrenalectomized for 2 weeks, GR levels were significantly increased in all the tissues assayed (Table 7). The increase of GR levels relative to normal GR levels was 100%, 89%, 53%, 99%, 65%, 60%, 65%, 29%, 21% and 40% in the left atrium, the right atrium, spleen, liver, the anterior pituitary, the left ventricle, the right ventricle, thymus, hippocampus and

hypothalamus, respectively. By Student t-test, the difference in GR levels between control rats and adrenalectomized rats was significant for every tissue examined ($p<0.05$). The result of this experiment confirmed that of the initial experiment shown in Fig. 14.

H. Dex treatment and GR levels

In some tissues, intact and adrenalectomized rats displayed a similar change in GR levels by Dex treatment. However, in other tissues, intact rats differed from adrenalectomized rats in the changes of GR levels after Dex treatment.

1. Tissues that displayed a decrease of GR levels after Dex treatment

In liver, the cardiac ventricles, the anterior pituitary, hypothalamus and hippocampus, GR levels were decreased by Dex treatment in both intact and ADX rats.

a). Liver

The GR level in the control rats was 703.6 ± 71.4 fmoles/mg protein (mean$\pm$SD, n=7 rats). After the rats were treated with Dex daily for 1, 4 and 8 day(s), the GR level was decreased by 53.6%, 52.3% and 55.4%, respectively (Fig. 15A). Compared to that in the control rats, the GR levels were found to be significantly decreased after the rats were treated with Dex for 1 day ($p<0.05$), 4 days ($P<0.05$) and 8 days ($P<0.05$).

Before Dex treatment, the GR level in the adrenalectomized rats was 1405.4 $\pm$ 294.9 fmoles/mg protein (mean$\pm$SD, n=7 rats). After the adrenalectomized rats were treated with Dex daily for 1, 4 and 8 day(s), the GR level was decreased by 41.2%, 45.9% and 46.8%, respectively (Fig. 15B). Compared to that in ADX rats before Dex treatment, the GR levels were significantly decreased after the rats were treated with Dex for 1 day ($p<0.05$), 4 days ($p<0.05$) and 8 days ($p<0.05$).

b). Cardiac ventricles:

The GR level in the left cardiac ventricles of the control rats was 163.1 ± 29.3 fmoles/mg protein (mean$\pm$SD, n=7 rats). It was decreased by 31.9%, 33.7% and

41.7% after the rats were treated with Dex daily for 1, 4 and 8 day(s), respectively (Fig. 16A). Compared to that in the control rats, the GR levels were significantly decreased after the rats were treated with Dex for 1 day ($P<0.05$), 4 days ($p<0.05$) and 8 days ($p<0.05$). The GR level in the right cardiac ventricles of the control rats was 164.7 ± 23.7 fmoles/mg protein (mean$\pm$SD, n= 7 rats). After the rats were treated with Dex daily for 1, 4 and 8 day(s), the GR levels were decreased by 26.2%, 38.2% and 42.1%, respectively (Fig. 16B). Compared to that in the control rats, the GR levels were found to be significantly decreased after the rats were treated with Dex for 1 day ($p<0.05$), 4 days ($p<0.05$) and 8 days ($p<0.05$).

Before the adrenalectomized rats were treated with Dex, the GR level in the left cardiac ventricle was 261.5 ± 25.9 fmoles/mg protein (mean$\pm$SD, n=7 rats). It was decreased by 32.6%, 38.3% and 44.4% after the rats were treated with Dex daily for 1, 4 and 8 day(s), respectively (Fig. 16C). Compared to that in the ADX rats before Dex treatment, the GR levels were significantly decreased after the ADX rats were treated with Dex for 1 day ($p<0.05$), 4 days ($p<0.05$) and 8 days ($p<0.05$). The GR level in the right cardiac ventricles of the adrenalectomized rats before Dex treatment was 270.3 ± 26.4 fmoles/mg protein (mean$\pm$SD, n=7 rats). After the rats were treated with Dex daily for 1, 4 and 8 day(s), the GR levels were decreased by 35.9%, 39.6% and 42.9%, respectively (Fig. 16D). Compared to that in the ADX rats before Dex treatment, the GR levels were significantly decreased after the ADX rats were treated with Dex for 1 day ($p<0.05$), 4 days ($p<0.05$) and 8 days ($p<0.05$).

c). Anterior pituitary

The GR level in the control rats was 182.2 ± 36.5 fmoles/mg protein (mean$\pm$SD, n=7 rats). After the rats were treated with Dex daily for 1, 4 and 8 day(s), the GR levels were decreased by 25.8%, 47.3% and 47.8%, respectively (Fig. 17A). Compared to that in the control rats, the GR levels were significantly decreased after the rats were treated with Dex for 1 day ($p<0.05$), 4 days ($p<0.05$)

and 8 days ($p<0.05$).

The GR level in the ADX rats before Dex treatment was 301.4 ± 21.7 fmoles/mg protein (mean$\pm$SD, n=7 rats). After the rats were treated with Dex daily for 1, 4 and 8 day(s), the GR levels were decreased by 43.3%, 42.3% and 49.7%, respectively (Fig. 17B). Compared to that in the ADX rats before Dex treatment, the GR levels were significantly decreased after the rats were treated with Dex for 1 day ($p<0.05$), 4 days ($p<0.05$) and 8 days ($p<0.05$).

d). Hypothalamus

The GR level in the control rats was 182.3 ± 32.2 fmoles/mg protein (mean$\pm$SD, n=7 rats). After the rats were treated with Dex for 1, 4 and 8 day(s), the GR levels were decreased by 30.2%, 36.3% and 31.9%, respectively (Fig. 18A). Compared to that in the control rats, the GR levels were significantly decreased after the rats were treated with Dex for 1 day ($p<0.05$), 4 days ($p<0.05$) and 8 days ($p<0.05$).

The GR level in the adrenalectomized rats before Dex treatment was 255.4 ± 33.2 fmoles/mg protein (mean$\pm$SD, n=7 rats). After the rats were treated with Dex daily for 1, 4 and 8 day(s), the GR levels were decreased by 29.4%, 34.1% and 45.1%, respectively (Fig. 18B). Compared to that in the ADX rats before Dex treatment, the GR levels were significantly decreased after the rats were treated with Dex for 1 day ($p<0.05$), 4 days ($p<0.05$) and 8 days ($p<0.05$).

e). Hippocampus

The GR level in the control rats was 193.6 ± 16.2 fmoles/mg protein (mean$\pm$SD, n=7 rats). After the rats were treated with Dex daily for 1, 4 and 8 day(s), the GR levels were decreased by 33.7%, 39.9% and 40.4%, respectively (Fig. 19A). Compared to that in the control rats, the GR levels were significantly decreased after the rats were treated with Dex for 1 day ($p<0.05$), 4 days ($p<0.05$) and 8 days ($p<0.05$).

The GR level in the adrenalectomized rats before Dex treatment was

234.8±18.6 fmoles/mg protein (mean±SD, n=7 rats). After the rats were treated with Dex daily for 1, 4 and 8 day(s), the GR levels were decreased by 32.9%, 30.3% and 43.6%, respectively (Fig. 19B). Compared to that in ADX rats before Dex treatment, the GR levels were significantly decreased after the rats were treated with Dex for 1 day ($p<0.05$), 4 days ($p<0.05$) and 8 days ($p<0.05$).

2. Dex treatment and GR level in spleen

The GR level in the control rats was 417.9±55.5 fmoles/mg protein (mean±SD, n=7 rats). After the rats were treated with Dex daily for 1, 4 and 8 day(s), the GR levels were increased by 57.8%, 68.8% and 75.8%, respectively (Fig. 20A). Compared to that in the control rats, the GR levels were significantly increased after the rats were treated with Dex for 1 day ($p<0.05$), 4 days ($p<0.05$) and 8 days ($p<0.05$).

The GR level in the adrenalectomized rats before Dex treatment was 638.6±34.6 fmoles/mg protein (mean±SD, n=7 rats). After the rats were treated with Dex daily for 1, 4 and 8 day(s), the GR levels were increased by 44.0%, 62.1% and 78.4%, respectively (Fig. 20B). Compared to that in the ADX rats before Dex treatment, the GR levels were significantly increased after the rats were treated with Dex for 1 day ($p<0.05$), 4 days ($p<0.05$) and 8 days ($p<0.05$).

3. Dex and GR levels in thymus

Dex treatment to either the intact or the ADX rats resulted in a biphasic effect on the GR levels in thymus with an initial increase of GR levels followed by a decrease.

The GR level in the control rats was 452.8±32.6 fmoles/mg protein (mean±SD, n=7 rats). The GR level was increased by 40.7% after the rats were treated with Dex for 1 day. However, after the rats were treated with Dex for 4 and 8 days, the GR levels were decreased by 36.7% and 54.0%, respectively (Fig. 21A). Compared to that in the control rats, the GR level was significantly increased after the rats were treated with Dex for 1 day ($p<0.05$), and the GR levels were

significantly decreased after rats were treated with Dex for 4 ($p<0.05$) and 8 days ($p<0.05$).

The GR level in the adrenalectomized rats before Dex treatment was 583.3 ± 37.3 fmoles/mg protein (mean$\pm$SD, n=7 rats). The GR level was increased by 52.8% after the rats were treated with Dex for 1 day. However, the GR level was decreased by 33.1% and 36.7% after the ADX rats were treated with Dex daily for 4 and 8 days, respectively (Fig. 21B). Compared to that in the ADX rats before Dex treatment, the GR level was significantly increased after the rats were treated with Dex for 1 day ($p<0.05$), and the GR levels were significantly decreased after the rats were treated with Dex for 4 ($p<0.05$) and 8 days ($p<0.05$).

Effect of glucocorticoid treatment on thymus weight: It was observed by the naked eye that the thymus size was reduced after the rats were treated with Dex. The thymus from each rat was weighed before it was processed for RIA sample preparation. The thymus weight was slightly decreased after the intact rats were treated with Dex for 1 day (Fig. 22A). However, it was decreased by 72.9% and 81.8% after the intact rats were treated with Dex daily for 4 and 8 days, respectively (Fig. 22A). Compared to that in the control rats, the thymus weight was significantly decreased after the rats were treated with Dex for 4 days ($p<0.05$) and 8 days ($p<0.05$). A similar phenomenon was observed in the adrenalectomized rats treated with Dex (Fig. 22B). After the ADX rats were treated with Dex daily for 1, 4 and 8 day(s), the thymus weight was decreased by 32.3%, 79.3% and 81.6%, respectively. Compared to that in the ADX rats before Dex treatment, the thymus weight was significantly decreased after the ADX rats were treated with Dex for 1 day ($p<0.05$), 4 days ($p<0.05$) and 8 days ($p<0.05$). In addition, the thymus weight was increased by 12.9% after the rats were adrenalectomized for 2 weeks. The finding that the thymus shrank after the rats were treated with glucocorticoid was in agreement with the observations by others (Sapolsky et al., 1984; Compton et al., 1987; Weissman, 1972; Rothenberg, 1980; Boersma et al., 1979; Ceredig and

Cummings, 1983; Alexandrová et al., 1989).

4. Dex and GR levels in the cardiac atria

In contrast to the cardiac ventricles in which the GR level was decreased by Dex treatment, the cardiac atria displayed an increase of GR level in the intact rats but a decrease of GR level in the ADX rats after Dex treatment.

The GR level in the left atrium of the control rats was 101.3±24.7 fmoles/mg protein (mean±SD, n=7 rats). After the rats were treated with Dex daily for 1, 4 and 8 day(s), the GR level was increased by 82.8%, 85.1% and 98.1%, respectively (Fig. 23A). The GR level in the right atrium of the control rats was 110.4±25.4 fmoles/mg protein (mean±SD, n=7 rats). After the rats were treated with Dex daily for 1, 4 and 8 day(s), the GR level was increased by 61.8%, 57.2% and 77.3%, respectively (Fig. 23B). In the left atrium, compared to that in the control rats, the GR levels were significantly increased after the rats were treated with Dex for 1 day ($p<0.05$), 4 ($p<0.05$) and 8 days ($p<0.05$). In the right atrium, compared to that in the control rats, the GR levels were significantly increased after the rats were treated with Dex for 1 day ($p<0.05$), 4 ($p<0.05$) and 8 days ($p<0.05$).

The GR level in the left atrium of the adrenalectomized rats before Dex treatment was 202.3±37.2 fmoles/mg protein (mean±SD, n=7 rats). After the rats were treated with Dex daily for 1, 4 and 8 day(s), the GR level was decreased by 32.2%, 35.1% and 44.1%, respectively (Fig. 23C). The GR level in the right atrium of the adrenalectomized rats before Dex treatment was 208.1±41.1 fmoles/mg protein (mean±SD, n=7 rats). After the adrenalectomized rats were treated with Dex daily for 1, 4 and 8 day(s), the GR level was decreased by 30.8%, 35.6% and 49.0%, respectively (Fig. 23D). In the left atrium, compared to that in the ADX rats before Dex treatment, the GR levels were significantly decreased after the adrenalectomized rats were treated with Dex for 1 day ($p<0.05$), 4 ($p<0.05$) and 8 days ($p<0.05$). In the right atrium, compared to that in ADX rats before Dex treatment, the GR levels were significantly decreased after the adrenalectomized rats

were treated with Dex for 1 day ($p<0.05$), 4 ($p<0.05$) and 8 days ($p<0.05$).

I. GR levels in the "particulate extracts" following adrenalectomy or Dex treatment

The total cellular GR level was composed of the GR levels in the "cytosol" and in the "particulate extract". Earlier, it was shown that the GR level in the "particulate extract" occupied only 6.0% of the total cellular GR level when detergent (0.4% NP40 and 0.06% DOC) was added to the liver tissue when it was homogenized. This result was reproduced by measuring the GR levels in the "cytosols" and the "particulate extracts" of liver and spleen tissues from some more rats. In the liver of normal rats, the GR level was 705.7 ± 74.0 fmoles/mg protein (mean$\pm$SD, n=7 rats) in the "cytosol" and was 58.6 ± 11.4 fmoles/mg protein (mean$\pm$SD, n=7 rats) in the "particulate extract". The total cellular GR level, calculated as the sum of the GR levels in the "cytosol" and the "particulate extract", was 764.3 fmoles/mg protein. Thus in the liver, the GR level in the "cytosol" made up 92.3% and the GR level in the "particulate extract" made up 7.7% of the total cellular GR level. In the spleen of the control rats, the GR level was 399.9 ± 71.6 fmoles/mg protein (mean$\pm$SD, n=7 rats) in the "cytosol" and 38.0 ± 11.4 fmoles/mg protein (mean$\pm$SD, n=7 rats) in the "particulate extract". The total cellular GR level, calculated as the sum of the GR levels in the "cytosol" and the "particulate extract", was 437.9 fmoles/mg protein. Therefore, in the spleen, the GR level in the "cytosol" made up 91.3% and the GR level in the "particulate extract" made up 8.7% of the total cellular GR.

The next question would be "would the amount of GR in the "particulate extracts" change after the rats were either treated with Dex or adrenalectomized and if so, would this change (probably due to the shift of GR from the "cytosol" pool to the "particulate fraction" pool or vice versa) significantly affect the GR level in the "cytosol" fraction? To answer these questions, the GR levels were measured, by RIA, in the "cytosols" and the "particulate extracts" of liver and spleen which were

from the rats under various glucocorticoid treatments. Earlier, it was shown that liver "cytosol" displayed a typical decrease in GR levels and spleen "cytosol" exhibited a typical increase in GR levels after the rats were treated with Dex. Thus these two tissues provided two examples in which to examine how GR levels in the "cytosols" and the "particulate extracts" would change after adrenalectomy or Dex treatment.

1. ADX and GR levels

As shown in Fig. 24, the GR level was increased by 67.0% in the liver "cytosol", by 63.8% in the "particulate extract" of liver, by 41.5% in the spleen "cytosol" and by 92.3% in the "particulate extract" of spleen after the rats were adrenalectomized. Similarly, McEwen et al. (1974) found that the hippocampal GR was increased in both the cytosol and cell nuclear fractions after the rats were adrenalectomized.

2. Dex treatment and GR levels

Liver: In intact rats, the "cytosol" GR level was decreased by 47.4%, 50.8%, and 55.6% after the rats were treated with Dex daily for 1, 4 and 8 day(s), respectively (Fig. 25A). The GR level of the "particulate extract" was decreased by 35.8%, 35.8%, and 49.1% after the intact rats were treated with Dex daily for 1, 4 and 8 day(s), respectively (Fig. 25C). The "cytosol" GR level in the ADX rats was decreased by 39.2%, 42.4%, and 44.2% after the rats were treated with Dex daily for 1, 4 and 8 day(s), respectively (Fig. 25B). The GR level in the "particulate extract" was decreased by 35.8%, 46.8%, and 49.1% after the ADX rats were treated with Dex daily for 1, 4 and 8 day(s), respectively (Fig. 25D). Thus the GR level in the "particulate extracts" were changed with the same tendency as that in the "cytosol". A similar phenomenon was previously observed by Berkovitz et al. (1988) that whole cell, cytosolic and nuclear GR declined in parallel after the human genital skin fibroblasts were incubated with Dex for 20 hours.

Spleen: In the intact rats, the "cytosol" GR level was increased by 52.3%,

60.9%, and 63.3% after the rats were treated with Dex daily for 1, 4 and 8 day(s), respectively (Fig. 26A). The GR level in the "particulate extract" was increased by 97.6%, 141.1%, and 163.3% after the intact rats were treated with Dex daily for 1, 4 and 8 day(s), respectively (Fig. 26C). In the ADX rats, the "cytosol" GR level was increased by 26.9%, 31.5%, and 54.2% after the rats were treated with Dex daily for 1, 4 and 8 day(s), respectively (Fig. 26B). The GR level in the "particulate extract" was increased by 44.6%, 51.8%, and 78.6% after the ADX rats were treated with Dex daily for 1, 4 and 8 day(s), respectively (Fig. 26D). Therefore, in the spleen, the change of GR levels in the "particulate extract" was in the same tendency as that in the "cytosol".

Since the change of GR levels in the "particulate extracts" was in the same direction as that in the corresponding "cytosols", the alteration of GR levels in the "particulate extracts" would not affect the conclusion on how the cellular GR level was changed based on the result regarding the changes of GR levels in the "cytosol". For this reason the data presented in this report related to the "cytosol" GR values.

Part II. GR levels and target gene responses

A. ANF RIA

1. Sensitivity of the assay

The synthetic ANF-(99-126) (the rat form) was labelled with ^{125}I (Ong et al., 1987). In the RIA, the ANF antibody (Peninsula Laboratories, Calif.) was incubated with ^{125}I-labelled ANF, and either various concentrations of the synthetic ANF or the cardiac samples containing ANF. This allowed a standard curve to be generated (Fig. 27). The standard curve was plotted as the percent bound/total radioactive ANF against the increasing concentrations of the unlabelled ANF on a logarithmic scale. A sigmoid curve was achieved; the linear part of the curve (bound/total of 0.08 to 0.29 in Fig. 27) was used to calibrate the ANF concentrations of the unknown samples. The sensitivity of the RIA, defined as the lowest amount of the ANF which could be detected with 95% confidence (Rodbard, 1978), was 16 pg/tube.

2. Control for RIA

The intra-assay variability of 8.9% was obtained. The inter-assay variabilities of 15.6% for 63a, 13.0% for 64a, 9.0% for 63v and 15.8% for 64v were observed (Table 8). This indicates that the results of the RIA experiments are reproducible (Thorell and Larson, 1978).

Liver "cytosols" from normal rats, prepared in the same manner as for the cardiac "cytosols", were assayed as a negative control since liver does not contain ANF (Gardner et al., 1986a). Only negligible level of ANF was detected in the liver cytosol [27.6±3.6 pg/mg protein, (mean±SD, n=7 rats)].

3. ANF levels in normal rats

In the present study, the average basal ANF levels (ANF levels in normal rats) were found to be 601.2 ng/mg protein for the left atrium, 807.4 ng/mg protein for the right atrium, 6.1 ng/mg protein for the left ventricle and 6.6 ng/mg protein for the right ventricle. Essentially, about 100-fold more concentrated ANF was observed in the atrial tissues than in the ventricular tissues. Similarly, Gardner et al. (1986a) observed that the concentration of ANF in atria was 100-fold higher than that in ventricles. The basal ANF levels in the ventricular and atrial tissues reported in the present study were similar to those of Gardner et al. (1986a). The technique that Gardner et al. applied was RIA using a 25 amino acid carboxyl-terminal fragment of rat ANF molecule [ANF-(102-126)].

B. ANF responses to Dex were related to the levels of GR

Intact rats were used. The same cardiac "cytosols" were assayed for both GR and ANF levels.

1. Left cardiac ventricle:

The GR level was decreased by 38.3%, 40.3% and 51.3% after the rats were treated with Dex daily for 1 day, 4 and 8 days, respectively (Fig. 28A). The absolute GR levels were 178.2±36.2 fmoles/mg protein (mean±SD, n=7 rats) for the control rats, and 122.5±31.5 fmoles/mg protein (mean±SD, n=7 rats), 113.4±34.8

fmoles/mg protein (mean±SD, n=7 rats), and 78.6±26.2 fmoles/mg protein (mean±SD, n=7 rats), for the rats treated with Dex for 1, 4 and 8 day(s), respectively. The ANF level in the control rats was 6.1±1.1 ng/mg protein (mean±SD, n=7 rats) (Fig. 28A). After the rats were treated with Dex, the ANF level was increased by 140.8%, 148.4% and 68.3% at 1, 4 and 8 day(s) after Dex injection, respectively.

When GR and ANF levels measured from the same cytosols over the length of Dex treatment were plotted, a linear correlation coefficient (r) of 0.919 (Fig. 28B) was obtained, indicating a correlation between the levels of GR and the response of ANF to Dex.

2. Right cardiac ventricle

The GR levels were decreased by 39.2%, 40.2%, and 50.8% after the rats were treated with Dex daily for 1, 4 and 8 day(s), respectively (Fig. 29A). The absolute GR levels were 172.6±38.9 fmoles/mg protein (mean±SD, n=7 rats) for the control rats, and 111.3±25.6 fmoles/mg protein (mean±SD, n=7 rats), 108.6±28.9 fmoles/mg protein (mean±SD, n-7 rats), and 78.4±24.1 fmoles/mg protein (mean±SD, n=7 rats) for the rats treated with Dex for 1, 4 and 8 day(s), respectively. The ANF level was 6.6±1.2 ng/mg protein (mean±SD, n=7 rats) in the control rats and it was increased by 122.2%, 89.2% and 50.0% after the rats were treated with Dex for 1, 4 and 8 day(s), respectively (Fig. 29A).

When GR and ANF levels measured from the same cytosols over the length of Dex treatment were plotted, a linear correlation coefficient (r) of 0.899 was obtained (Fig. 29B), indicating a correlation between GR levels and the ANF responses to Dex.

3. Left cardiac atrium:

An increase of GR level of 54.6%, 71.3% and 101.3% was observed after the rats were treated with Dex for 1, 4 and 8 day(s), respectively (Fig. 30A). The absolute GR levels were 110.2±38.2 fmoles/mg protein (mean±SD, n=7 rats) for

the control rats, and 168.9±30.6 fmoles/mg protein (mean±SD, n=7 rats), 180.4±26.1 fmoles/mg protein (mean±SD, n=7 rats), and 218.6±37.4 fmoles/mg protein (mean±SD, n=7 rats) for the rats treated with Dex for 1, 4 and 8 day(s), respectively. The ANF level in the control rats was 601.2±52.1 ng/mg protein (mean±SD, n=7 rats) and it was increased by 190.8%, 212.9% and 254.1% after the rats were treated with Dex for 1, 4 and 8 day(s), respectively (Fig. 30A).

When GR and ANF levels measured from the same cytosols over the length of Dex treatment were plotted, a linear correlation coefficient (r) of 0.939 was obtained (Fig. 30B), indicating a correlation between GR levels and the ANF responses to Dex.

4. Right cardiac atrium:

The GR level was increased by 62.3%, 81.9% and 114.3% after the rats were treated with Dex daily for 1, 4 and 8 day(s), respectively. The absolute GR levels were 106.8±27.2 fmoles/mg protein (mean±SD, n=7 rats) for the control rats, and 162.5±25.9 fmoles/mg protein (mean±SD, n=7 rats), 187.3±10.5 fmoles/mg protein (mean±SD, n=7 rats), and 224.9±39.4 fmoles/mg protein (mean±SD, n=7 rats) for the rats treated with Dex for 1, 4 and 8 day(s), respectively (Fig. 31A). The ANF level was 807.4±91.5 ng/mg protein (mean±SD, n=7 rats)` in the control rats and was increased by 133.3%, 151.1% and 202.3% after the rats were treated with Dex for 1, 4 and 8 day(s), respectively (Fig. 31A).

When GR and ANF levels measured from the same cytosols over the length of Dex treatment were plotted, a linear correlation coefficient (r) of 0.926 was obtained (Fig. 31B), indicating a correlation between the GR levels and the ANF response to Dex.

Part III. Immunocytochemical localization of GR in target tissues

A. Heart

The localization of GR was carried out by using two independent antibodies, the $14B_2$ and a well-established antibody, BuGR1 (Gametchu and Harrison, 1984).

The $14B_2$ antibody was raised in rabbit against a synthetic 14 amino acid peptide corresponding to the N-terminal region of the human GR (Hollenberg et al., 1985). BuGR1 was a monoclonal antibody raised in mouse against purified rat liver GR. The localization of ANF was carried out by using an antibody raised in rabbit against ANF (Peninsula Laboratories, Calif.).

Ventricular tissue and atrial tissue showed similar staining pattern. In both ventricle and atrium, the GR staining intensity was increased in ADX rats compared to intact rats (not shown). Fig. 32A shows a cardiac atrial section stained with BuGR1 using the immunoperoxidase procedure. This atrial section was from a rat adrenalectomized two weeks previously. Cardiac muscle cells contained immunocytochemical staining which was located predominantly in the cytoplasm. Endothelial and mesothelial cells were not stained. A similar phenomenon was described earlier by Antakly and Eisen (1984) in liver where endothelial cells were devoid of immunoreactive GR. Blood cells were also stained due to the massive presence of endogenous peroxidase in erythroid and myeloid cells. Fig. 32B is another atrial section stained with $14B_2$ by immunoperoxidase procedure. This section was also taken from a rat adrenalectomized for two weeks. Essentially a staining similar to that shown in Fig. 32A was observed in Fig. 32B.

In order to know whether GR and ANF were present in the same cardiac cells, an immunocytochemical double labelling procedure was used in which GR was detected by the immunoperoxidase method using BuGR1 (made in mouse) and the same section was subsequently stained for ANF by the immunofluorescence method using rabbit anti-rat ANF antibody (Peninsula Laboratories, Calif.). Fig. 32C is a micrograph showing ANF labelling by the immunofluorescence method on the same section which was previously stained with BuGR1 antibody for GR labelling (Fig. 32A). GR and ANF were found to be in the same cardiac cells. ANF was localized to the cytoplasm of the cardiac cells. Fig. 32D shows a control experiment in which the primary antibody was substituted with the non-immune rabbit serum and no

staining was observed, as expected.

B. Submaxillary gland

The distribution of GR in the submaxillary gland from normal rats was demonstrated by two independent antibodies, $14B_2$ and BuGR1, using immunoperoxidase method. GR was localized in the GCT cells, whereas the acinar cells were generally devoid of immunoreactive staining (Fig. 33A, 34A, 35A). A similar staining was observed using either BuGR1 (Fig. 34A, 35A) or $14B_2$ (Fig. 33A). GR was localized predominantly in the cytoplasm of the GCT cells, and to a lesser extent in the nucleus. By using a double-labelling immunocytochemical procedure, EGF (Fig. 33B), α 2u globulin (Fig. 34B) and ANF (Fig. 35B) were shown in the same GCT cells which contained GR. EGF, α 2u globulin, and ANF were localized to the cytoplasm of the GCT cells. When the submaxillary gland section was stained with non-immune rabbit serum or the $14B_2$ previously adsorbed with purified rat liver GR (Grandics et al., 1984), no staining was observed (Fig. 33C, 34C, 35C).

C. Testis

An immunocytochemical double-labelling procedure was performed to localize GR and ACTH (a POMC derived peptide) in the testis. The testicular tissue sections (from normal rats) were first processed for GR localization by an immunoperoxidase procedure using BuGR1 and the same sections were subsequently processed for ACTH staining by an immunofluorescein procedure. Leydig cells were strongly stained with BuGR1 whereas the seminiferous tubular cells were weakly stained (Fig. 36A). In Leydig cells, the GR was localized predominantly to the cytoplasm and to a lesser extent to the nucleus. The same Leydig cells which contained GR were shown to be ACTH-positive (Fig. 36B). ACTH was observed in the cytoplasm of the Leydig cells. In control experiments where the primary antibody was substituted with non-immune serum, no staining was observed (Fig. 36C).

D. Pituitary gland

Since the anterior lobe of the pituitary contains GR but the intermediate lobe of the pituitary is typically devoid of GR, pituitary tissue sections were used as controls for GR staining (Antakly and Eisen, 1984; Antakly et al., 1985; Antakly et al., 1987). In parallel with the immunocytochemical studies of other tissues (heart, testis, or the submaxillary gland), pituitary sections were processed for the immunocytochemical labelling of GR. Both BuGR1 and $14B_2$ were used. The two antibodies showed similar results, the anterior lobe of the pituitary being positively stained and the intermediate lobe of the pituitary being negatively stained (Fig 37A and B). In the anterior pituitary, about 70% cells were immunoreactive, with their cytoplasm, nucleus or both being stained. Fig. 37C is a pituitary section which was stained with non-immune rabbit serum. As expected, no staining was observed.

CHAPTER IV. DISCUSSION

Glucocorticoids have a major role in the regulation of numerous homeostatic and developmental processes. Since glucocorticoids have such wide-spread basic effects, a precise knowledge of their mechanism of action at the molecular level is of both biological and clinical significance. The actions of glucocorticoids are mediated by GR, whose structure and mechanism of action have been widely studied. Although GR is present almost in all mammalian tissues, it may not be expressed evenly in all the cells of glucocorticoid target tissues. The current study, by using immunocytochemical technique, demonstrated a cell-specific expression of GR in the testes, the submaxillary gland and the heart. In addition, the significance of this cell-specific expression of GR was investigated by colocalizing other substances in the same cells that contained GR. Given the nearly ubiquitous nature of GR expression and the increasingly large number of well documented glucocorticoid responses, regulation of GR expression itself, particularly by its cognate ligand, has become the subject of study. In regard to this issue, most studies resulted in a down-regulation of GR by its ligand although controversal results were also present. The current study, by examining systematically several rat tissues using RIA, demonstrated a tissue-specific regulation of GR by dexamethasone. The relevance of the Dex-caused GR regulation was investigated in the cardiac tissues and the result showed that the response of ANF to Dex was correlated to the GR levels.

A. Technique for studying GR regulation

Ligand-induced down-regulation of GR and its increase in response to the removal of glucocorticoid by surgical adrenalectomy have been widely studied by steroid binding assays (McEwen et al., 1974; Cidlowski and Cidlowski, 1981; Sapolsky et al., 1984; Sapolsky and McEwen, 1985; Reul et al., 1987). Although very useful, the receptor binding assay technique is tedious, usually necessitating large amounts of cells or tissues, and depends on the association of the receptor with its labile ligand. Other approaches to studying the regulation of steroid receptors

include examining the receptor mRNA levels and measuring the receptor protein levels using a receptor-specific antibody. However, receptor protein levels may reflect the activity of the receptor more closely than does the mRNA levels since the hormonal transduction is mediated by the receptor protein. In view of these possibilities, RIA was used to study the GR regulation in the current study.

During the course of the RIA development, the concentrations of the tracer and $14B_2$, the use of normal rabbit serum and goat anti-rabbit serum were established. Theoretically, the amount of the ^{125}I-labelled antigen used in the RIA should be large enough for the following two reasons. First, in the RIA (a competitive assay), the antibodies should already be close to saturation when only the ^{125}I-labelled antigen (no unlabelled antigen) is present. Otherwise, if unoccupied antibody binding sites remain, any unlabelled antigen added will bind to the empty sites without inhibiting the binding between ^{125}I-14-mer and the antibody. Second, the higher the radioactivity is in the sample , the more exact the measurement of the radioactivity is. Usually both the bound and free radioactivity will increase if a larger total radioactivity is added to the system. Thus the amount of the radioactivity should be as large as possible. However, there are certain limitations to this approach, mainly because of the nonspecific binding of the radioactivity to antibody precipitate or to the wall of the assay tubes. Such nonspecific binding is proportional to the total radioactivity of the sample. Thus it is necessary to choose the appropriate quantity of the radioactive 14-mer. Based on the suggestion by Parker (1976) that a minimum of 5,000 to 10,000 cpm of radioactive antigen per individual assay tube should be used in order to obtain adequate levels of bound-antigen radioactivity, the amount of iodinated 14-mer was set at about 20,000 cpm per assay tube in the RIA.

As mentioned above, optimal conditions for RIAs are found when the antibody sites are almost saturated by the radioactive antigens. This implies that the amount of the antibody should be as small as possible and that the antisera should be correspondingly diluted. Especially when antibody avidity is very high, antibodies

may be diluted, so that the radioactive antigen just saturates all binding sites, that is, almost all the antibody binding sites are bound when no unlabelled antigen is added. In most cases, however, the antibody avidity does not always permit the assay to work at perfect saturation, because the binding capacity cannot be saturated at the low concentrations of the radioactive antigen. Usually, the highest sensitivity of the assay occurs when there are binding sites available for 30% to 50% of the radioactive antigen, that is, when 30% to 50% of the added radioactivity is bound when no unlabelled antigen is added (Parker, 1976). In the present study, a dilution of the $14B_2$ to 1:20,000 was shown to yield 36.5% binding when only the radioactive 14-mer was added (Table 3) and this antibody dilution was used in the RIA.

In contrast to many other immunologic systems, no precipitates are formed during the primary reaction between the antigen and the primary antibody in RIAs. The reason for this is that the low concentrations of antibodies and antigens do not permit the lattice formation that is the structure of the immunologic precipitate. In order to circumvent this problem, a secondary antibody (goat anti-rabbit serum) at higher concentration was added in the reaction to precipitate the antibody and antigen complex. In this step the primary antibody ($14B_2$) acted as an antigen. The secondary antibody was made in goat as directed against the immunoglobulins of rabbit in which the $14B_2$ was produced. In addition, since the immunoglobulin making up the primary antibody of the RIA was diluted extensively, it is usually necessary to add more immunoglobulin from that same species to achieve optimal precipitation with the secondary antibody added. This is one of the reasons that normal rabbit serum was added in the assay buffer. Another reason for adding the normal rabbit serum in the assay buffer was to reduce nonspecific adsorption of the antibodies and antigens by the solid materials in the test tubes such as the inside wall of the test tube and the antibody-antigen precipitate. Such nonspecific adsorption would influence the assay result. For instance, the adsorption of radioactivity to the test tube wall or the reaction precipitate would increase the counting of the

radioactivity since the counting was conducted in the precipitate in the tube. In the present study, goat anti-rabbit serum and normal rabbit serum were used at the respective dilutions of 1:1,000 and 1:200 based on the observation by Antakly et al. (1990) that optimal binding result was achieved when these dilutions were utilized.

$14B_2$ was raised in rabbit against a synthetic 14-amino acid peptide (14-mer) derived from the N-terminal domain of the human GR (Hollenberg et al., 1985). The 14-mer corresponds to 171-184 amino acids of the human GR. The amino acid sequence of the 14-mer is conserved between rat and mouse GRs (Miesfield et al., 1986; Danielsen et al., 1986); in this region of the receptor, there are two amino acid differences between rat (Miesfield et al., 1986) and human GRs (Hollenberg et al., 1985). The antibody against the 14-mer reacted to rat tissue cytosol GR with the same specificity and a similar affinity as to the 14-mer (Fig. 7 and 9). Therefore, although raised against a 14 amino acid peptide of the human GR, this antibody was suitable for studying rat cytosol GR.

Steroid receptors contain three functional domains, the steroid-binding, the DNA-binding and the N-terminal domains. The N-terminal domain of the GR is the least conserved region among the various members of the steroid receptor family and shows less than 20% homology to other steroid receptors (Evans, 1988; Beato, 1989). A computer search of the gene bank showed no significant sequence homology between the 14-mer peptide and other proteins. Despite the structural and functional similarity between the GR and the mineralocorticoid receptor (Arriza et al., 1987), there is no homology between the 14-mer and the corresponding sequence in the mineralocorticoid receptor. Considerable heterogeneity of sequence for the N-terminal sequence exists also between GR, oestrogen receptor and progesterone receptor (Hollenberg et al., 1985; Miiesfeld et al., 1986; Green et al., 1986; Loosfelt et al., 1986). Thus, the antibody against the 14-mer is most likely specific to GR. Indeed, $14B_2$ did not show any cross-reactivity with other protein species. In the pituitary tissue extract or HTC cell extract, the antibody reacted with a single protein

species in immunoblots having an approximate molecular weight of 94 kDa and immunoprecipitation of [^{3}H]-dexamethasone mesylate affinity-labelled GR by $14B_2$ produced, on SDS gel fluorograms, a protein band with a molecular weight of 94 kDa (Fig. 5). The molecular weight of this protein species detected by $14B_2$ is in agreement with the GR described in rat liver (Wrange et al., 1984).

Antisera to synthetic peptides within the DNA-binding region of the progesterone and oestrogen receptors (Wilson et al. 1988) and GR (Urda et al. 1989) have all been described. In these cases, binding of the antibody to the steroid receptors inhibit interaction of the receptor with DNA. Despite their great utility in mapping the interaction of the receptor with DNA in vitro, these antibodies are not useful for quantitating receptor levels in tissues since these antibodies are not able to detect the DNA-binding form of the receptors. In addition, the DNA-binding region against which these antisera are generated is highly conserved among various steroid receptors (Evans, 1988; Beato, 1989) and the antisera would be expected to show high cross-reactivity with other steroid receptors. This is indeed the case with the antibodies prepared against the progesterone and oestrogen receptors which have been used to study the DNA-binding properties of the GR. The $14B_2$ antibody is against the least conserved region of the receptor and is specific to GR. From various observations, it is most likely that the $14B_2$ recognizes both steroid-bound and steroid-free receptors as well as the cytoplasmic and nuclear forms of the receptors (Antakly et al., unpublished observations). Thus the $14B_2$ is suitable for quantitating the cellular GR levels in tissue preparations.

The validity of the RIA method was also judged by other criteria, i.e., the purity of the tracer, the reproducibility of the results and the sensitivity of the RIA. The tracer (^{125}I labelled 14-mer) was demonstrated to be pure by HPLC elution of a single peak (Fig. 6). The reproducibility of the result was monitored by intra- and inter-assay controls. The observed interassay variance of 13.4%-14.5% and intra-assay variance of 9.4% were in the expected range for a reliable RIA experiment

(Thorell and Larson, 1978). The sensitivity of the RIA, defined as the smallest amount of the receptor which could be detected, was 6 fmoles/tube. The high sensitivity of the RIA makes it possible to measure small amount of GR in tissue preparations, especially in those tiny tissues such as the pituitary.

The reliability of the GR RIA was also examined by measuring GR levels, using both RIA and receptor binding assay (a conventional method to study steroid receptors), in tissue cytosols of liver, spleen and cardiac ventricles. The result showed that the GR levels measured by these two techniques were similar (Table 4), indicating that the RIA used in the present study for quantifying tissue GR levels was reliable. In normal rat tissues (Table 7), the highest GR level was found to be in the liver (about 700 fmoles/mg protein) with the relative abundance in the other tissues: thymus, 64.2%; spleen, 59.3%; hippocampus, 27.4%; the anterior lobe of the pituitary, 25.8%; hypothalamus, 25.8%; the right cardiac ventricle, 23.3%; the left cardiac ventricle, 23.1%; the right cardiac atrium, 15.6%; and the left cardiac atrium, 14.3%. This order is in concordance with that obtained by binding studies of Turner (1986). The basal GR levels quantitated by the RIA method in the present study were similar to those measured by binding assays of others (Olpe and McEwen, 1976; Ballard et al., 1974; Gregory et al., 1976; Boer and Oddos, 1979; Sapolsky et al., 1984; Turner, 1986; Alexandrová et al., 1989), further suggesting that the RIA method for measuring the tissue GR levels was to be trusted.

Although the advantage and the reliability of the GR RIA used in the present study are obvious, the RIA technique has certain limits. For example, information on gene regulation at the transcriptional level can not be obtained by the RIA technique. Thus the present study was not able to determine whether the regulation of GR expression occurred at the transcriptional level or posttranscriptional level.

B. GR regulation

The change in hormone receptor levels is an important aspect of physiologic control since the sensitivity of the target cells to a hormone signal is directly related

to the receptor concentration (Danielsen and Stallcup, 1984). In contrast to the largely accepted concept that GR is down-regulated by its ligand in the target tissues (for reviews, see Gustaffson et al., 1987; Svec, 1985), the present study showed a tissue-specific regulation of GR by its ligand. The GR levels could be increased or decreased by glucocorticoids depending upon the tissues examined and also upon the physiological conditions. The direction or the magnitude of GR regulation was not related to the basal GR level in the tissues. Similarly, studies by Turner (1986) and Kalinyak et al. (1987) failed to show a correlation between the initial GR concentration and the magnitude of the GR regulation.

The change in GR levels after glucocorticoid treatment cannot be attributed to a redistribution of the receptors between the "cytosolic fraction" and the "particulate fraction" (this fraction was presumed to contain GR associated with chromatin in the nucleus) as it was observed that the receptor levels were changed in parallel in these two fractions of liver and spleen (two examples) after the rats were treated with Dex. A similar phenomenon was previously described by Berkovitz et al. (1988) who showed that whole cell, cytosolic and nuclear GR declined in parallel after the human genital skin fibroblasts were incubated with Dex for 20 hours. Cidlowski and Cidlowski (1981) found that incubation of glucocorticoid-sensitive HeLa S_3 cells with glucocorticoids decreased GR levels both in the cytosol and nuclei. Also, the reduction in cytosolic receptors in the brain of the stressed rats (glucocorticoids were elevated in stressed rats) could not be explained by an equivalent increase in nuclear receptor number (Sapolsky et al., 1984). Thus there seemed to be a change of the total cellular receptor level rather than simply a redistribution of the receptor between the cytoplasm and the nucleus of the cell. After rats were adrenalectomized, an increase of GR levels was found both in the "cytosol" and the "particulate fraction" of liver and spleen. Similarly, McEwen et al. (1974) found that the hippocampal GR was increased in both the cytosol and cell nuclear fractions of the tissue after rats were adrenalectomized. This indicated that it was the entire population of GR which

increased and not a redistribution of the receptor between the cytoplasm and the nucleus after rats were adrenalectomized.

The present study showed that the GR levels were increased by adrenalectomy in all the rat tissues examined although the magnitude of the increase varied among different tissues (Table 7). The largest increase was shown by the left cardiac atrium (100%), followed by liver (99%), the right cardiac atrium (89%), the anterior pituitary (65%), the right cardiac ventricle (65%), the left cardiac ventricle (60%), spleen (53%), hypothalamus (40%), thymus (29%) and hippocampus (21%). The increase in the GR levels in the liver, cardiac ventricle, pituitary, hippocampus and hypothalamus was in agreement with the results obtained by others (Gregory et al., 1976; McEwen et al., 1974; Olpe and McEwen, 1976; Boer and Oddos, 1979). While the present study demonstrated a considerable increase of GR levels in the rat heart and pituitary, Turner's study (1986) showed only a slight increase of the GR level in the heart (left ventricle) and pituitary (whole pituitary) after the rats were adrenalectomized. This discrepancy may be due to the different length of ADX used in the two studies. Rats adrenalectomized for 2 weeks were examined in the present study whereas rats adrenalectomized for 4 days were used by Turner. Sheppard et al. (1990) showed that, after rats were adrenalectomized for 20 hours, the GR mRNA levels were increased in hippocampus but not in pituitary. The discrepancy between Sheppard's result and the result from the current study in pituitary GR regulation could be because of different time course of ADX or possible differential regulations of GR mRNA and protein (see below). Kalinyak et al. (1987) showed an increase of 40% and 80% in GR mRNA levels in brain and kidney respectively, but they failed to see any change in the receptor mRNA level in other tissues such as liver, heart, adrenal and testis after rats were adrenalectomized. However, the present study demonstrated a considerable increase in GR protein levels in the heart and the liver after rats were adrenalectomized for 2 weeks. It is possible that while the receptor mRNA level remained the same, the receptor protein level was increased probably

due to the increased translation efficiency of the receptor mRNA and/or the increased half-life of the receptor protein after rats were adrenalectomized. In this regard, GR protein has been shown to interact with regions in the GR cDNA which correspond to the 3' untranslated region (Okret et al., 1986). This 3' untranslated region may be involved in posttranscriptional regulation, determining mRNA stability (Sham and Kamen, 1986; Rahmsdorf et al., 1987) or translational efficiency (Liebhaber and Kan, 1982; Miller et al., 1984). The half-life of the GR protein has been shown, by a number of investigators, to be decreased after cultured cells are treated with glucocorticoids (Svec and Rudis, 1981; Raaka and Samuels, 1983; McIntyre and Samuels, 1985; Dong et al., 1988; Hoeck et al., 1989). Conversely, the half-life of the GR protein might be increased by removal of the hormones (adrenalectomy).

The data showing that GR levels are back to baseline after the rats were adrenalectomized for 3 weeks is interesting. After prolonged adrenalectomy, other steroid receptors might also have been regulated as estrogen receptor (Freyschuss et al., 1991) and androgen receptor (Smith et al., 1984) have been shown to be regulated by glucocorticoids. The regulated levels of these other steroid receptors may affect GR expression and normalize the GR levels after 3 weeks of adrenalectomy. It was also possible that alternative sites for corticosterone synthesis might have appeared after prolonged adrenalectomy and thus, the GR levels were normalized to baseline. However, these hypotheses need to be verified by further experiments.

The loss of epinephrine in the rats after ADX might not have contributed to the regulation of GR levels as epinephrine has been shown not to affect GR number in liver cytosols (Mi et al., 1992). Mineralocorticoids decrease GR level through mineralocorticoid receptors (O'Donnell and Meaney, 1994). Conversely, removal of mineralocorticoids may result in an increase of GR level. However, ADX-induced GR expression can be reversed by glucocorticoid but not by aldosterone (Holmes et al., 1995), suggesting that ADX-induced GR expression is caused by glucocorticoids

but not by mineralocorticoids. Thus it is not clear whether mineralocorticoids have contributed to the ADX-induced GR expression. With regard to GR regulation by oestrogen, controversal data have been found in the literature (Camacho-Arroyo et al., 1994; Peiffer et al., 1994). In addition, the minimal amount of sex hormone that is present in the adrenal glands might not account for the GR regulation by ADX. Since the increased GR mRNA and protein expression after ADX can be reversed by the addition of glucocorticoids (Kalinyak et al., 1987; Reul et al., 1989; Ahima and Harlan, 1991; Holmes et al., 1995), the increased GR expression after ADX is at least partially controlled by the concentration of glucocorticoids.

After rats were treated with Dex, the GR levels were decreased in liver, anterior pituitary, hypothalamus, hippocampus, and the cardiac ventricles. The time response curve (Fig. 12) showed that down-regulation was relatively slow, requiring 24 hours of exposure to glucocorticoids to reach 36.7% of GR reduction. The finding of relatively slow down-regulation of GR levels has been supported by other investigators (Svec and Rudis, 1981; Cidlowski and Cidlowski, 1981; Danielsen and Stallcup, 1984). The data in the present study support the observation by Okret et al. (1986) that dexamethasone mediated down-regulation of GR levels in rat liver was not accompanied by an initial induction of GR although continuous treatment of rat hepatoma cells with dexamethasone resulted in an initial increase of both GR mRNA and protein levels followed by a decrease of GR mRNA and protein levels (Okret et al., 1986). The discrepant findings between in vivo and in vitro studies may reflect independent or combined cell-specific differences in gene regulation, receptor half-life and/or the absence of other important in vivo regulatory hormones. The decrease in hippocampal GR level ranging from 30-40% is similar to the results obtained by Tornello et al. (1982) and Sapolsky et al. (1984). The present study showed that the GR protein level in the liver was decreased by 41-55% after rats were treated with Dex. This number was similar to that observed for the reduction of the hepatic GR mRNA level after rats were treated with Dex (Kalinyak et al., 1987).

The down-regulation of GR level by glucocorticoids is important for the negative modulation of the hormone action. For instance, the glucocorticoid-induced decrease in GR levels was associated with a reduced response of a target gene tyrosine aminotransferase (TAT), a key enzyme in glyconeogenesis (Shirwany et al., 1986). Down-regulation of GR in hippocampus may be expressed as more excitability as hippocampal mineralocorticoid receptor activation maintains excitability, while GR occupancy suppresses excitability (De Kloet et al., 1993). Thus, by means of reducing GR levels, the magnitude of the hormone action is controlled.

In the spleen, an increase of the GR level by Dex was found in both intact and ADX rats. This was in sharp contrast to the result obtained by Kalinyak et al. (1987) who showed that the splenic GR mRNA level was decreased after intact rats were treated with Dex. In Kalinyak's study, the GR mRNA levels were measured after rats were treated with Dex for 6 hours. In the present study, the GR protein levels were quantitated after rats were treated with Dex for 1 day (24 hours), 4 and 8 days. It is possible that Dex treatment might have resulted in an initial decrease of the GR level (for example, 6 hours after Dex treatment) and a later increase of the GR level (after 24 hours). The complicated autoregulatory pattern of the GR expression has been shown in the rat HTC cell line (Okret et al., 1991). In this case, continuous presence of dexamethasone in the medium led to a transient 2-fold increase in GR mRNA levels (peak 6 hours after addition of hormone) followed by about 80% down-regulation of the GR mRNA 18-42 hours later. Such a biphasic pattern of glucocorticoid receptor mRNA regulation on Chinese hamster ovary-derived cell line was not reflected at the level of receptor protein, suggesting that both transcriptional and translational control mechanisms may be involved in ligand-dependent receptor regulation (Bellingham et al., 1992). Thus the discrepancy in GR regulation in the spleen between Kalinyak's study and the current study may reflect not only a difference in time course of Dex treatment but also a difference in the level of

receptor expression studied. In addition, it is also possible that the differential regulation of GR expression between the present study and Kalinyak's study is due to different dexamethasone dose used since a comprehensive dose-responsive profile has not been available. The dexamethasone dose was 4 mg/kg body weight in the present study and 7 mg/kg body weight in Kalinyak's study. Although the present study showed, in the liver, the GR level was decreased by Dex treatment at doses ranging from 1 to 8 mg/kg (Fig. 11), a dose response curve was not performed in the spleen cytosol. GR expression may respond to different doses of glucocorticoids with a complicated pattern, as a biphasic dose response has been observed for oestrogen receptor regulation by oestrogen in MCF-7 cells (Ree et al., 1989). An increase in oestrogen receptor mRNA was seen with low concentration of oestrogen treatment while a decrease in oestrogen receptor mRNA level was obtained with higher concentration of the hormone. The data showing that GR level was increased not only by Dex but also by ADX in the spleen might be explained by the presence of other important adrenal regulatory hormones. This possibility is supported by the studies of O'Donnell and Meaney (1994) showing that mineralocorticoids regulate GR level. The present finding is the first to show an increase of GR levels by glucocorticoids in any normal tissue. In the progress of this work, several groups showed an increase in the GR levels by glucocorticoids in a human leukemic cell line (Eisen et al., 1988; Antakly et al., 1989; Denton et al., 1993). Ligand induction of receptor levels may indicate a novel concept in the regulation of cell function in which cells reach the maximal response to hormone by increasing the concentration of the hormone receptors. Glucocorticoids have been well known to have inhibitory effects on lymphoid tissues (Munck and Crabtree, 1981; Compton and Cidlowski, 1987). The induction of splenic GR by glucocorticoids might accelerate this inhibitory effect since the GR levels seem to determine the cellular response to the hormone (reviewed by Gustafsson et al., 1987).

In the thymus, the GR level was shown to be increased after rats were treated

with Dex for 1 day, but decreased after rats were treated with Dex for 4 and 8 days. In addition, the thymus weight was slightly decreased (13%) after rats were treated with Dex for 1 day and significantly decreased (72-82%) after rats were injected with Dex for 4 and 8 days. The extent of the decrease in thymus weight after rats were treated with glucocorticoids for prolonged lengths of time (4 and 8 days) was in good agreement with the observation by others (Vollmar and Schulz, 1991; Alexandrova et al., 1989). The decrease in thymus weight might be due to a glucocorticoid induced lysis of thymocytes (Compton et al., 1987; Munck and Crabtree, 1981). It is known that thymocyte subpopulations respond to Dex with different sensitivities (Boersma et al., 1979). Boersma et al. (1979) characterized three mouse thymocyte subpopulations by size and density. They found that the small cells of intermediate density were the most sensitive to Dex, the medium-sized low density cells were relatively resistant to Dex, and the sensitivity of the small cells of high density to Dex was between the above two cell types. They also found that after Dex treatment the proportion of medium-sized low density cells (the Dex-resistant thymocytes) was increased. The Dex caused involution of thymus gland and changes in thymocyte subpopulations may at least partially be responsible for the Dex induced changes in GR levels observed in the present study. It is most likely that the small cells of intermediate and high density contain higher GR levels than the medium-sized low density cells do, since, in most cases, the cellular sensitivity to glucocorticoids is proportional to the GR concentration in the cells (Reviewed by Gustafsson et al., 1987). If this is true, the overall GR level in the thymus would be decreased simply because of the decreased proportion of the small cells of intermediate and high density, and the accordingly increased proportion of the medium-sized low density cells after rats were treated with Dex for 4 and 8 days. The initial increase in the GR levels (after rats were treated with Dex for 1 day) may be due to an increased GR concentration in the Dex-sensitive thymocytes. This increased GR levels may be necessary for the maximal response of the Dex-sensitive thymocytes to further Dex

treatment.

In the cardiac atrium, the GR level was increased by Dex in intact rats and decreased by Dex in ADX rats. The differential regulations of GR levels between intact and ADX rats might be caused by some secretory products from the adrenal glands. In addition to glucocorticoids, adrenal glands also secrete mineralocorticoids, catecholamines, enkephalins and a minimal amount of sex hormones. Although glucocorticoids and mineralocorticoids are secreted mainly from the adrenal glands, other secretory products are also produced by organs besides the adrenal glands. For example, sex hormones are secreted by sex organs, enkephalins by central nervous tissue, and catecholamines by central nervous tissue and postganglionic sympathetic nerves. As a result, sex hormones, enkephalins and catecholamines are still present in the adrenalectomized rats and these substances may not be responsible for the differential GR regulations between the intact and the ADX rats observed in the present study. The cardiac heart contains mineralocorticoid receptors (Arriza et al., 1987; Myles and Funder, 1994). In the cardiac atria, there may be a tissue-specific factor that reverses the glucocorticoid induced GR regulation under the influence of mineralocorticoids or other unknown adrenal secretory products. However, this remains to be verified. For the first time, the present study has demonstrated an increase of the atrial GR level by Dex in intact rats. The present study is also the first to examine GR regulation in atrial versus ventricular tissues. The distinct regulation of GR levels in the ventricles (the GR levels were decreased by glucocorticoids) and atria (the GR levels were increased by glucocorticoids in the intact rats) indicates that ventricular and atrial cells represent distinct cell types although they share a common embryological origin. The distinct cell types between atria and ventricles can be further evidenced by the observation by Kennedy and Ziegler (1991) that the synthesis of epinephrine is increased in atria but not in ventricles by dexamethasone treatment.

The glucocorticoid induced down-regulation of GR is probably a primary

response mediated by GR, since it also occurs in the presence of a protein synthesis inhibitor, cycloheximide (Svec and Rudis, 1981; McIntyre and Samuels, 1985; Okret et al., 1986; Burnstein et al., 1994). Regulation of GR protein in the cell may occur at various levels- transcriptional or posttranscriptional, the latter involving nucleo-cytoplasmic transport, changes in the GR mRNA stability, translation efficiency and protein turnover. Govindan et al. (1991) reported negative regulation of CAT activity by glucocorticoids after transfection of Hela cells with plasmids containing the human GR promoter fused to the CAT structural gene, indicating that the GR down-regulation by glucocorticoids was attributed to a decreased rate of transcription of the GR gene. Dong et al. (1988) found that the half-life of the GR mRNA in HTC cells was unaffected by Dex treatment. These authors concluded that the decrease in the level of the GR mRNA was due to a reduced transcription rate of the GR gene as assessed by nuclear run-on transcription experiments using a cDNA probe corresponding to the 3'-untranslated end of the rat GR mRNA. Similarly, Rosewicz et al. (1988) showed that, in human IM-9 and rat pancreatic acinar AR42J cells, the half-life of the GR mRNA was not influenced by glucocorticoid whereas the rate of the GR transcription was decreased by nuclear run-on assay using a GR cDNA probe. A primarily transcriptional response in AtT-20 cells treated with hormone has also been demonstrated (Vig et al., 1994).

In the case of heterologous genes (i.e., MMTV) positively regulated by glucocorticoids, the GR has been shown to recognize specific DNA contact regions in vitro that represent positive glucocorticoid response elements in vivo (Yamamoto, 1985). Studies toward the identification of such GR binding sites on the GR gene would be necessary in order to understand the mechanism of GR regulation by glucocortiocoids.

GR-specific binding sequences within the 3-nontranslated region of rat GR cDNA were reported by Okret et al. (1986). This particular fragment contains three copies of the degenerate consensus sequence, 5' AGAACA(G)A 3', previously

emphasized for specific GR-DNA interaction in, for instance, MMTV (Payvar et al., 1983; Scheidereit et al., 1983). Thus, it has been suggested that the down-regulation of GR by glucocorticoid hormones is caused by the interaction of the GR protein with its own gene in analogy to the postulated model for other gene regulation by glucocorticoids. It is noteworthy to mention that GR-specific contact regions have been described not only upstream of the MMTV promoter in the 5' long terminal repeat but also within discrete regions far from the promoter and within transcribed and translated portions of the MMTV element (Payvar et al., 1983). Similarly, GR-specific binding regions identified in the transcribed part of the GR gene (Okret et al., 1986) might be responsible for GR regulation. Although the GR binding 3'-untranslated region found on the GR cDNA is located tens of kb away from the GR gene promoter, chromosomal arrangement might place it in close proximity to other important gene regulatory regions (Ptashne, 1986). It is also possible that the binding of GR to this region may affect the GR mRNA stability (Shaw and Kamen, 1986; Rahmsdorf et al., 1987) or the translation efficiency of the GR mRNA (Liebhaber and Kan, 1982; Miller et al., 1984). However, the role of these GR-binding sequences in GR regulation needs to be tested by mutational experiments. Not only were GR-binding regions found on rat GR cDNA (Okret et al., 1986), human GR cDNA was also shown to contain a fragment, from base pair +527 to +1526, which was responsible for the glucocorticoid reduction of GR levels (Burnstein et al., 1990; 1991). This fragment contained some sequences with homology to a potential negative GRE in POMC promoter region (Drouin et al., 1989).

Since the human GR gene was cloned (Encío and Detera-Wadleigh, 1991; Leclerc et al., 1991), it has been possible to examine whether the upstream region of the GR gene contained GR-specific binding sequences. Leclerc et al. (1991) examined the 5' flanking region of the human GR gene and identified regulatory sequences responsible for glucocorticoid reduction of GR. These sequences are rich in G+C content which are known to be involved in regulation of many housekeeping

genes such as GR, as well as a number of cellular oncogenes, but they do not contain any typical steroid response element. This indicates that the typical steroid response element may not be necessary for the regulation of GR in response to glucocorticoid hormones. A protein factor was found to interact with the human GR gene fragment implicated in homologous down-regulation (Leclerc et al., 1991), suggesting that GR down-regulation is a complex process.

It is not clear how, if tissue-specific regulation is a primary transcriptional response, such regulation is accomplished. One possible explanation is suggested by the study of Strahle et al. (1992), who found three different promoters for the mouse GR gene, one of which appeared to be used only in lymphoid cells in which up-regulation of GR was observed. Up- or down-regulation may be the result of regulated expression from unidentified promoter regions. Alternatively, the tissue-specific regulation may result from complex interactions between the GR and other transcription factors acting through protein-protein interactions (Yang-Yen et al., 1990; Schule et al., 1990; Konig et al., 1992), overlapping cis-acting elements (Imai et al., 1990; Akerblom et al., 1988; Stromstedt et al., 1991; Zhang et al., 1991) or a combination of both (Strahle et al., 1988; Diamond et al., 1990). For instance, the expression of the glycoprotein hormone alpha gene is regulated divergently by glucocorticoids in different cell types. Coexpression of the GR with an alpha-CAT reporter gene caused activation of alpha promoter activity in fibroblasts, but repression in JEG-3 choriocarcinoma cell, indicating that cell-specific factors dictate positive versus negative regulation of this promoter by GR. This supports a mechanism in which GR-mediated repression in JEG-3 cells occurs by receptor interference with transactivating potential of enhancer-binding protein or associated transcription factors (Chatterjee et al.,1991).

Posttranscriptional mechanisms of control in GR homologous regulation may be also present. In COS-1 monkey kidney cells transiently transfected with a GR expression vector, in which GR transcription is under the control of Rous sarcoma

virus promoter (Burnstein et al., 1990), and in stably transfected Chinese hamster ovary cells expressing the GR under the control of either the Rous sarcoma virus (Burnstein et al., 1991b) or human metallothionein-IIa (Alksnis et al., 1991) promoters, there was a decrease in GR mRNA after steroid treatment, suggesting posttranscriptional regulation of GR expression. Studies using dense amino acid labelling and immunochemical detection suggest that the half-life of the GR protein is decreased after steroid treatment (Dong et al., 1988; McIntyre and Samuels, 1985). The half-life of GR protein was shown to be decreased by glucocorticoid treatment in AtT-20 cells (Svec and Rudis, 1981), in GH_1 pituitary tumour cells (Raaka and Samuels, 1983; McIntyre and Samuels, 1985), and in rat hepatoma culture cells (Dong et al., 1988; Hoeck et al., 1989). In contrast, Distelhorst and Howard (1989) showed that GR half-life in S49 mouse lymphoma cells was not affected by glucocorticoids. The discrepancy between the results from different laboratories may represent a difference in receptor regulation in different types of cells.

Thus GR regulation by glucocorticoid hormones may be a complicated process which can occur at the transcriptional level (GR gene contains GREs), and the posttranscriptional level (stability of mRNA, translation efficiency of mRNA and/or degradation of GR protein) in some cells. The GR regulation observed in the present study may also reflect these mechanisms. The tissue-specific regulation of GR might be due to a direct or indirect interaction of the GR with tissue-specific factors. Alternatively, tissue-specific regulation may be the result of regulated expression from unidentified promoter regions as suggested by studies of Strahle et al., (1992). Studies toward the identification of tissue-specific factors will be necessary to understand the mechanisms of the differential GR regulation.

In summary, the present study demonstrated a tissue-specific regulation of GR levels by glucocorticoids. The receptor can be up- or down-regulated depending on the tissues examined. While most hormone receptors are decreased by their own ligands, there are other instances in which receptors are increased by the homologous

ligands. Estrogens (Reigel et al., 1987), androgens (Kaufman et al., 1981; Syms et al., 1985), and vitamin D_3 (Costa et al., 1985; McDonnell et al., 1987; Mangelsdorf et al., 1987) have all been described to induce their respective receptors. The receptors for prolactin (Posner et al., 1975), epidermal growth factor (Hudson et al., 1989), and interleukin 2 (Depper et al., 1985) are also known to be increased by their respective ligands. In addition, oestrogen increases oestrogen receptor mRNA levels in rat liver and pituitary but decreases oestrogen receptor levels in uterus (another example of tissue-specific regulation of the receptor) (Shupnik et al., 1989). Dex treatment was reported to increase GR mRNA in cerebral cortex cells but decrease GR mRNA in hypothalamus cell (Pepin et al., 1990). Administration of Dex to rats significantly reduced GR mRNA levels in hippocampal subfields CA1-2 and CA-3, but had no effect on GR mRNA levels in the dentate gyrus (Herman et al., 1989). Denton et al. (1993) observed a cell-specific GR regulation in that the GR mRNA and protein levels were decreased in a human leukemic B-cell line but were increased in a human leukemic T-cell line by dexamethasone treatment. That the GR regulation by glucocorticoids is complex is also supported by the biphasic variation in GR expression (Okret et al., 1991; Bellingham et al., 1992; Vig et al., 1994). A positive correlation has been established between the concentration of receptor and the magnitude of the biological response (Bourgeois and Newby, 1977; Gehring et al., 1984; Vanderbilt et al., 1987; Dong et al., 1990). Regulation of receptors may be a physiological means of modulating cellular responses to the hormone. In systems in which the GR is down-autoregulated, such regulation provides a convenient means for attenuating a steroid-induced response and thus, contributes to the overall mechanism of negative feedback control. In contrast, up-regulation would tend to amplify the response. In cells in which the role of the steroid is to promote differentiation or initiate programmed cell death, such an amplification would contribute to and perhaps even be required for a full response, as might be the case for the GR regulation in the spleen and the thymus. Indeed, of two human myeloma

cell lines established from the same patient, the one that showed no up-regulation was glucocorticoid resistant while the one whose growth was inhibited by steroid treatment showed significant up-regulation (Gomi et al., 1990). Also, Gomi et al. (1990) found that glucocorticoid induced GR mRNA in glucocorticoid-sensitive myeloma cells and correlated this induction with glucocorticoid-evolved cell lysis. However, the fact that up-regulation was observed in CEM-C1 cells, whose growth is unaffected by the presence of Dex, but which contain functional receptors (Zawydiwski et al., 1983), clearly demonstrates that such regulation is not by itself sufficient to induce growth arrest and cell death. In addition, up-regulation of GR expression was observed, in the present study, in the cardiac atrium, which presumably are already terminally differentiated and for which there is no evidence for glucocorticoid-induced apoptosis.

GR regulation may be important in the pathophysiology of Cushing's Syndrome and Addison's Disease. Cushing's Syndrome is a condition in which an inappropriate secretion of glucocorticoids is present. If the GR levels are down-regulated in most tissues by the high levels of glucocorticoids in Cushing's Syndrome, such a regulation may make the cells resistent to the high levels of glucocorticoids and thus may dimish the manifestations of Cushing's Syndrome. However, binding studies showed that both the GR binding sites and the binding affinity were the same in patients with Cushing's Syndrome as in the normal subjects (Brentani et al., 1986). Addison's Disease is a condition in which the adrenal secretion of glucocorticoids is insufficient. If the GR levels are up-regulated by the decreased glucocorticoid levels in Addison's Disease, such a regulation may make the cells more sensitive to glucocorticoid treatment. However, the mononuclear leukocytes from Addison's Disease showed lower GR binding sites than those from the normal subjects (Brentani et al., 1986). Thus the cellular sensitivity to glucocorticoids, in at least the mononuclear monocytes, may not be higher in Addison's Disease than in the normal subjects. Athough in most systems the GR

levels are regulated by glucocorticoids and such a regulation is correlated with the cellular response to the steroid hormone, the GR regulation in Cushing's Syndrome and Addison's Disease may be complicated by the presence of other factors in these disease conditions.

C. GR levels and ANF responses to glucocorticoids

The relevance of glucocorticoid regulation of GR levels for ANF expression was investigated in the cardiac tissues. Glucocorticoids stimulate cardiac ANF biosynthesis and secretion in vivo and in vitro (Matsubara et al., 1987a; 1987b; Shields and Glembotski, 1988; Shields et al., 1988; Weidmann et al., 1988; Nemer et al., 1988). The glucocorticoid regulatory elements within the ANF gene has been identified (Seidman et al., 1984b; Greenberg et al., 1984). Indeed, distal cis-acting promoter sequences have been shown to mediate glucocorticoid stimulation of cardiac ANF gene transcription (Argentin et al., 1991). The cardiac tissues contain GR and synthesize ANF, thus providing a model system to study the relationship between the GR levels and the ANF responses to glucocorticoids. After Dex treatment, the ANF levels were increased to 2-3 fold in both ventricular and atrial tissue of the intact rats. The 2 to 3-fold increase of the ANF levels in response to glucocorticoid treatment was not unexpected since, unlike the widely studied MMTV promoter which is dramatically (30-50-fold) induced by glucocorticoids, most cellular genes that are glucocorticoid sensitive, such as the phosphoenolpyruvate carboxykinase and the ANF genes, are usually induced to lesser extent (about 3-fold) (Argentin et al., 1991). The magnitude of ANF responses to Dex was correlated to the GR levels. This result suggests that the stimulatory effect of Dex on ANF production is mediated by the cardiac GR. This possibility is supported by the double labelling immunocytochemical experiment demonstrating that GR and ANF are present in the same cardiac cells (Fig. 32). It is also supported by the observation that a specific GR agonist, RU28362, mimics the actions of Dex on atrial cells (Philiber and Moguilewsky, 1983). The inhibition of both Dex-stimulated and RU28362-stimulated

ANF biosynthesis by the specific glucocorticoid antagonist RU38486 (Gagne et al., 1985) supports this possibility. The present study is the first to show a correlation between the GR concentration and the extent of the ANF response to glucocorticoids. Taken together, these results suggest, most likely, a direct action of Dex on ANF regulation at the level of the heart. In this regard, it is interesting to note that the glucocorticoid induced rise in plasma ANF in normal men was not secondary to sodium and volume retention (Weidmann et al., 1988). Also, Dex seemed to stimulate ANF synthesis in the cardiac cells that contained ANF, as initially suggested by in situ hybridization study of Gardner et al. (1988a) which showed that Dex did not recruit additional cells into the ANF expressing pool but did increase the level of ANF expression within individual cells. The same authors also demonstrated that the effect of glucocorticoids on ANF mRNA levels resulted from both the increased half-life of ANF mRNA and enhanced ANF gene transcription. It is interesting to note that the glucocorticoid stimulation of ANF promoter activity is restricted to cardiac cells, suggesting that glucocorticoid responsiveness of the ANF promoter requires the activity of cardiac-specific regulatory elements within the promoter (Argentin et al., 1991). Factor(s) that bind these elements may interact cooperatively with the glucocorticoid receptor to confer hormone responsiveness.

It has been recognized that a prerequisite for glucocorticoid response is the presence of the intracellular GR, since the hormonal response is mediated by the GR protein (Grove et al., 1980). However, whether there is a relationship between the cellular sensitivity to glucocorticoid hormones and the concentration of GR has been a heated subject. The finding that ANF responses to Dex are correlated to the cellular GR levels reinforces the notion that GR concentration determines the magnitude of the biological responses of cells to glucocorticoids (Gustafsson et al., 1987; Bellingham et al., 1992; Fukawa et al., 1994; Bourgeois and Newby, 1979; Vanderbilt et al., 1987), suggesting that GR is a limiting factor in controlling the responses of a target gene to the hormone. Thus, by regulating the receptor level,

the response of a target gene to the hormone can be regulated. This was true for the relationship between the glucocorticoid reduced GR level and the smaller magnitude of TAT response to glucocorticoid hormones in rat liver (Shirwany et al., 1986). The TAT gene contains GRE and is subject to glucocorticoid induction (Evans, 1988). Shirwany et al. (1986) observed that Dex administration to rats led to a rapid increase in hepatic TAT; at 24 h after Dex injection, GR levels were 50% of baseline, and readministration of Dex at 24 h after the initial injection produced a peak level of TAT that was only 22% of that initially obtained.

Glucocorticoids increase the levels of prorenin (Krakoff and Elijovich, 1981) and renin substrate (Saruta et al., 1986), thus causing sodium and water retention, and hypertention. The induction of ANF by glucocorticoids is of physiological importance since ANF exhibits natriuretic, diuretic and vasorelaxant functions which can reverse the glucocorticoid induced water retention and hypertension.

D. Localization of GR in the target tissues

The specificity of the GR immunocytochemistry was tested by several methods. First, the specificity of the immunocytochemical staining by $14B_2$ was confirmed by a well-established monoclonal antibody (BuGR1, Gamecthu and Harrison, 1984) since the two antibodies produced similar immunocytochemical staining on several rat tissues. Second, the specificity of the immunocytochemical staining was monitored by several controls as will be described below. The presence of possible contaminating antibodies was excluded by an antibody adsorption experiment which showed that adsorption against partially purified rat liver GR removed the staining. In other cases, the substitution of the primary antibody with the non-immune or pre-immune sera produced negative staining, and this further confirmed the specificity of the antibody and also the specificity of the immunocytochemical staining. The $14B_2$ specificity was also tested on pituitary tissue sections which were used as internal controls (Fig. 37). The result showed that the intermediate lobe of the pituitary, unlike the anterior one, was devoid of

immunocytochemical staining for GR, suggesting that the antibody was specific since it is known that the anterior lobe of the pituitary contains GR but the intermediate lobe is typically devoid of GR (Antakly et al., 1985). It is noteworthy to mention that the intermediate pituitary lobe contains otherwise detectable levels of oestrogen receptor (Pelletier et al., 1988). Thus the antibody did not cross-react with an other steroid receptor such as the receptors for oestrogen. In the anterior pituitary, about 70% cells were immunoreactive, with their cytoplasm, nucleus or both being stained. Their number exceeded that of corticotrophs which was previously shown to represent only 5-10% of the cell population of the anterior lobe (Gee and Roberts, 1983; Gee et al., 1983). This is not surprising since glucocorticoids also inhibit prolactin secreting cells (Sakai et al., 1988) and stimulate growth hormone secreting cells (Kohler et al., 1969; Kohler et al., 1968). The latter two types of cells acount for approximately 60% of the anterior pituitary cells (Moriarty, 1973). Other antibodies used in the immunocytochemistry were specific to the respective substances studied. BuGR1 was a monoclonal antibody against purified rat liver GR and has been well-characterized to be specific to GR (Gametchu and Harrison, 1884). The ANF antibody was commercially obtained from Peninsula Laboratories and the cross-reactivity of this antibody with other materials was less than 0.001% (Peninsula Laboratories, Calif.). The anti-α 2u globulin antibody was monospecific to α 2u globulin (Antakly et al., 1982b). The EGF antibody is specific to EGF as this antibody immunoreacted with a single protein species, eluted by Sephadex G-200, with a molecular weight of 6,400 dalton which was in agreement with the known molecular weight of the biological active EGF (Walker et al., 1981). The ACTH antibody is specific to ACTH and did not cross-react with other proteins as checked by radioimmunoassay (Pelletier et al., 1977).

The general presence of GR in mammalian tissues has been well-described (Ballard et al., 1974). However, whether GR is expressed homogenously in all the cells within target tissues remains to be an open question. The present study

demonstrated a cell-specific expression of GR in glucocorticoid target tissues.

The immunoreactive cells in the submaxillary gland consisted exclusively of GCT cells that secrete many physiologically important polypeptides, including epidermal and nerve growth factors (Barka, 1980), and ANF (Gutkowska and Nemer, 1989). α 2u globulin, a peptide with unknown function originally thought to be exclusively produced in the liver, was also found to be synthesized in the GCT cells of the submaxillary gland (Antakly et al., 1982b). Glucocorticoids influence the growth, differentiation, and secretory activity of the GCT cells (Walker, 1982; Walker et al., 1981; Gresik et al., 1981). However, the effects of glucocorticoids on the GCT cells were thought to be mediated through androgen receptors since androgen receptor had been well documented in these cells but little information had been available for the presence of GR therein (Verhoeven, 1979; Nemoto et al., 1985; Ohara-Nemoto et al., 1988; Nemoto et al., 1986; Minetti et al., 1986; Kyakumoto et al., 1986 and 1987; Sato et al., 1986). The localization of GR in the GCT cells suggests that the actions of glucocorticoids in the GCT cells are at least partially mediated via GR. This possibility is supported by studies of Gerald et al. (1986) that adrenalectomy and glucocorticoid administration affect specific gene products in the GCT cells in a different manner than androgens. In double-labelling experiments, GR was co-localized in the same GCT cells which contained EGF, α 2u globulin and ANF. In the heart, ANF is stimulated by glucocorticoids (Shields et al., 1988; Nemer et al., 1988; Weidmann et al., 1988), and its colocalization with GR in the same cells of the submaxillary gland suggests that glucocorticoids may affect ANF gene expression in this tissue. However, this hypothesis remains to be verified. Glucocorticoids have been known to reduce EGF activity in mouse submaxillary gland (Walker et al., 1981) and to inhibit EGF secretion from a human submandibular gland cell line (Kurokawa et al., 1988). The colocalization of EGF with GR in the GCT cells suggests that glucocorticoids may affect GCT cell growth through a direct effect on EGF expression as EGF receptor is present in these cells

(Kurokawaet al., 1987). Indeed, glucocorticoid treatment results in reduction of EGF secretion and suppression of DNA synthesis in these cells to a similar extent (Kurokawa et al., 1988). The finding that GR is contained in the same cells as α 2u globulin is not surprising as hydrocortisone has been shown to superinduce α 2u globulin mRNA levels in the male rat submaxillary gland (Gubits et al., 1984).

Collectively, the presence of GR in the GCT cells suggests that the effects of glucocorticoids on the GCT cells are mediated, at least partly, by GR. Finally, the absence of GR in the acinar cells could explain the known lack of glucocorticoid effects on the acinar portion of the rat submaxillary gland (Bixler et al., 1957). A similar phenomenon was observed in the intermediate pituitary gland, where lack of response to glucocorticoids is associated with the absence of GR (Antakly et al., 1985).

GR was localized in the myocytes of ventricles and atria. ANF was found in the same cardiac myocytes which contained GR. Glucocorticoid induction of cardiac ANF synthesis has been well described (Nemer et al., 1988; Sheilds et al., 1988; Weidmann et al., 1988). The presence of a putative glucocorticoid regulatory sequence on the ANF gene indicates a direct action of the steroid on the ANF gene expression (Argentin et al., 1985; Nemer et al., 1984; Argentin et al., 1991). In the present study, it is shown that GR concentration correlates with the magnitude of ANF responses to glucocorticoids in the heart tissues. Together with these data, the colocalization of GR and ANF in the same cardiac myocytes suggests that the stimulatory effect of glucocorticoids on the cardiac ANF synthesis is mediated by the cardiac GR.

The localization of GR largely to the Leydig cells is consistent with the known inhibitory effect of glucocorticoids on testosterone synthesis in the Leydig cells (Saez, 1977; Bambino and Hsueh, 1981; Monder et al., 1994). The presence of a small amount of GR in the seminiferous tubules may explain the suppressive action of glucocorticoids on plasminogen activation in the Sertoli cells (Jenkins and Elison,

1986). In a previous study, we showed that POMC mRNA was synthesized at a low level in testicular tissue (Tremblay et al., 1988). It was interesting to note that the POMC transcription was repressed by glucocorticoids in the anterior lobe of the pituitary, but was not regulated by the same hormone in the testis and the intermediate lobe of the pituitary (Tremblay et al., 1988). Since the lack of glucocorticoid regulation of POMC gene in the intermediate lobe of the pituitary was associated with the absence of the GR in that tissue (Antakly and Eisen, 1984; Antakly et al., 1985), it was tempting to think that the non-responsiveness of testicular POMC to glucocorticoids might be also due to the lack of GR in the same testicular cells which contained POMC. Thus it was necessary to localize GR and POMC in the testicular tissue. By using an immunocytochemical double-labelling procedure, GR and ACTH (a POMC derived peptide) was co-localized in the same Leydig cells of the testis. Thus, although GR and POMC are present in the same Leydig cells of the testis, glucocorticoids do not affect POMC expression. This suggests that the presence of GR does not always confer the hormone effect on POMC gene expression. Glucocorticoid regulatory sequences were identified on the promoter region of the POMC gene (Drouin et al., 1989). Since POMC appears to be encoded by a single copy gene (Eberwine and Roberts, 1983; Drouin et al., 1985), an explanation for the differential regulation of POMC gene in different tissues becomes complicated. There are at least two possibilities. First, an alternative mode of transcriptional initiation of the POMC gene may determine the non-responsiveness of the POMC gene to glucocorticoids in the testicular tissue. Unlike in the pituitary tissues, the POMC transcripts in the testis do not contain sequences transcribed from exon 1 and exon 2, and appear to initiate within exon 3 of the POMC gene (Jeannotte et al., 1987; Lacaze-Masmonteil et al., 1987). Second, tissue-specific factors may determine the tissue-specific regulation of POMC by glucocorticoids.

GR is largely cytoplasmic in the absence of glucocorticoids and translocates into the nucleus following steroid binding (Govindan et al, 1980; Antakly and Eisen,

1984; Fuxe et al., 1985; LaFond et al., 1988; Wikstrom et al., 1987; Picard and Yomamoto, 1987). This notion was supported by the result of the present study by showing that GR is present mainly in the cytoplasm of target cells in intact rats. In the present study, rats were sacrificed in the morning. Glucocorticoid levels in these rats were probably very low at the time when the rats were sacrificed due to the diurnal rhythm of corticosterone secretion as in rats; corticosterone secretion is increased in the evening and decreased in the morning (Marotta et al., 1975). The primarily cytoplasmic localization of GR was evidenced by a recent study using chemical crosslinking of intact HTC cells, showing that GR was distributed between cytosol and nuclei in a ratio which is about 2:1 (Rossini and Malaguti, 1994). However, controversal data also exist regarding the subcellular localization of GR. A study by Yokote et al. (1991) showed, by immunohistochemistry, that GR in the pituitary was confined to the nucleus in intact rats and GR immunoactivity in the pituitary cells was lost in ADX rats. Since ADX induces GR level in pituitary (Turner, 1986; result from the present study), the loss of the GR immunoactivity after ADX observed by Yokote et al. was unlikely due to a loss of GR protein in the cells, but most probably due to the possibility that the antibody used by these authors was unable to react with cytoplasmic GR but with nuclear GR only as GR is cytoplasmic in the absence of steroid hormone (Fuxe et al., 1985; LaFond et al., 1988). By immunohistochemical technique using a monoclonal antibody against the rat liver GR, Van Eekelen et al. (1987) localized GR in the cell nucleus in the brain of intact rats. The discrepancy between the result of this study and the current study in receptor localization may be explained by tissue differences and/or differences in epitope recognition by different antibodies or by the fact that endogenous glucocorticoid levels were high in these other studies.

While GR has been detected in most tissue extracts studied to date, at both the protein (Ballard et al., 1974) and the mRNA levels (Kalinyak et al., 1987), it is becoming increasingly evident that a cellular heterogeneity exists relative to the

expression of the receptor. In earlier studies, Antakly and Eisen (1984) showed that GR staining is specific to hepatic parenchymal, but not Kupffer, cells of the liver. In the pancreas, Fischer et al. (1990) have reported that GR is localized exclusively in the β-cells of the islets, but not in the exocrine acinar cells. Thus, the receptor is not ubiquitously expressed as previously believed. The data in the present study demonstrated a cell-specific localization of GR in the rat heart, testis and submaxillary gland, and provided another example for the cell-specific expression of the GR.

E. Future studies

The present study showed that while in most tissues GR protein levels were decreased by its own ligand, in some other tissues GR protein levels were increased, suggesting a tissue-specific regulation of GR. The glucocorticoid regulation of GR is probably a primary response mediated by GR, since it also occurs in the presence of a protein synthesis inhibitor, cycloheximide (Svec and Rudis, 1981; McIntyre and Samuels, 1985; Okret et al., 1986; Burnstein et al., 1994). Since only the GR protein levels were examined, whether the GR regulation observed in the present study occurred at the transcriptional level and/or the posttranscriptional level can not be determined. In order to understand whether the GR regulation observed in the present study occurred at the transcriptional level, the GR mRNA levels will need to be measured under various endocrinologic conditions. From the literature, it is known that glucocorticoids regulate GR expression at both the transcriptional and the posttranslational level (Govindan et al., 1991; Dong et al., 1988; Rosewicz et al., 1988; McIntyre and Samuel, 1985). Thus the GR regulation observed in the current study may also reflect these two mechanisms. Examination of the 5' flanking region of the human GR gene has revealed regulatory sequences responsible for glucocorticoid regulation of GR (Leclerc et al., 1991). At the transcriptional level, the tissue-specific regulation of GR by glucocorticoids requires the activity of tissue-specific regulatory factors interacting with regulatory elements within the GR gene.

Such tissue-specific hormonal regulation has been observed for some hepatic and pituitary genes such as tyrosine amino transferase (Strahle et al., 1988) and prolactin (Simmons et al., 1990). In the case of prolactin, it has been shown that the oestrogen receptor binds to a distal promoter element and interacts cooperatively with the pituitary-specific transcription factor, Pit-1, to induce prolactin expression. In order to understand the mechanism of the tissue-specific regulation of GR, it will be necessary to identify cis-elements within the receptor gene that are crucial for the regulation in GR mRNA synthesis and to identify tissue-specific factors that interact with the GR gene for GR regulation.

F. Conclusion and summary

By using radioimmunoassay, GR concentration has been measured in several rat tissues. In contrast to the largely accepted notion that glucocorticoids decrease GR levels in the target tissues, the present study demonstrated that regulation of GR by glucocorticoids varied with tissue type: GR could be increased or decreased by glucocorticoids depending on the tissues examined. GR levels were decreased by glucocorticoid treatment in liver, cardiac ventricles, pituitary, hypothalamus, hypothalamus and the cardiac atria of the adrenalectomized rats, but were increased in the cardiac atria of the intact rats, thymus (after the first day of glucocorticoid treatment) and spleen. The GR levels determined the response of a target gene, ANF, to glucocorticoids in cardiac tissues. Thus by regulating the receptor level, the magnitude of the target gene response to the hormone can be modulated. By immunocytochemical study, GR was localized to the cardiac myocytes, to the Leydig cells of the testis, and to the granular convoluted tubular cells of the submaxillary gland. Thus GR expression in target tissues is cell-specific. The cardiac cells containing GR colocalize with ANF, suggesting that glucocorticoid regulation of ANF occurs, at least partly, at the level of the heart. Together with the result that ANF responses correlated with the GR levels, the colocalization of GR and ANF in the same cardiac cells suggests that glucocorticoid stimulatory effect on ANF

expression is mediated by the cardiac GR. The Leydig cells of the testis containing GR colocalize with ACTH, suggesting that the presence of GR in the same cells does not always confer the regulation of POMC gene by glucocorticoids as POMC gene is inhibited by glucocorticoids in the anterior lobe of the pituitary but not regulated in the testis (Tremblay et al., 1988). GR was colocalized with EGF, ANF and α 2u globulin in the GCT cells of the submaxillary gland, suggesting roles of glucocorticoids in regulating these substances therein.

G. Original contributions

There are three original findings in the present study. First, the present study provides the first evidence for an increase of the GR level by glucocorticoids in normal rat tissues (spleen, thymus and cardiac atria) and demonstrates a tissue-specific regulation of the GR. Thus the receptor level is not only decreased in some tissues in response to its ligand by a mechanism of negative feed-back but is also increased in other tissues in which the enhanced receptor expression may be required for the tissues to obtain maximal response to the hormone. The tissue-specific regulation of GR implies that there are tissue-specific factors which may interact with the regulatory sequences on the GR gene and cooperate with or antagonize the GR protein to control GR expression. Second, the present study is the first to show a correlation between the GR levels and the ANF responses to glucocorticoids in cardiac tissue. This shows that the GR concentration determines the magnitude of a target gene response to glucocorticoids and confirms that the stimulatory effect of glucocorticoids on ANF synthesis is mediated by GR. Thus by changes in the GR level, the response of a target gene can be modulated. Third, the immunocytochemical study has demonstrated, for the first time, that GR is co-localized with ANF in the cardiac myocytes, with ACTH in the Leydig cells of the testis, and with EGF, ANF and α 2u globulin in the GCT cells of the submaxillary gland. The colocalization of GR with ANF in the cardiac cells, together with the finding that GR levels determine the ANF response to glucocorticoids (the present

study) and the identification of glucocorticoid regulation sequences on the ANF gene (Seidmen et al., 1984b; Greenberg et al., 1984; Argentin et al., 1991), suggests that glucocorticoid regulation of ANF synthesis is mediated by GR. The colocalization of GR and ACTH in the Leydig cells of the testis, together with the result that POMC (the precursor of ACTH) is inhibited by glucocorticoids in the anterior lobe of the pituitary but is not regulated in the testis (Tremblay et al., 1988), suggests that the presence of the receptor in the POMC containing cells does not always guarantee a response of the POMC gene to glucocorticoids and other factors may influence the responsiveness of the POMC gene to the steroid hormone. The colocalization of GR with ANF, EGF and α 2u globulin in the GCT cells of the submaxillary gland suggests that glucocorticoids may have regulatory effects on these molecules in this tissue. The demonstration of GR in the GCT cells of the submaxillary gland also suggests that glucocorticoid effects on the submaxillary gland are, at least partly, mediated by GR, not as previously thought that the glucocorticoid effects on the GCT cells were mediated by the androgen receptor (Sato et al., 1981; Maruyama and Sato, 1986).

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Table 1. Literature review on the GR regulation by ADX

Animal species	Tissue (Cell)	Time Post-ADX	GR levels	Assay method	Reference
Male rat	Heart	12-48 h.	↑ 40%	Binding assay	Gregory et al., 1976
	Liver		↑ 20%	Binding assay	
Male rat	Hippocampus	3 d., 7 d.	↑ 50%	Binding assay	McEwen et al., 1974
Male rat	Hippocampus	3 d.	↑ 75%	Binding assay	Olpe and McEwen, 1976
	Pituitary		↑ 22%	Binding assay	
	Hypothalamus		↑ 45%	Binding assay	
	Septum		↑ 80%	Binding assay	
	Amygdala		↑ 56%	Binding assay	
	Cerebral cortex		↑ 65%	Binding assay	
Male rat	Skeletal muscle	2 w.	↑ 50%	Binding assay	Mayer and Rosen, 1979
Male rat	Cardiac ventricle	6 d.	↑ 550%	Binding assay	Boer and Oddos, 1979
	Liver	6 d.	↑ 180%	Binding assay	
Male rat	Liver	4 d.	↑ 85%	Binding assay	Turner, 1986
	L. cardiac ventricle		↑ 18%	Binding assay	
	Kidney		↑ 529%	Binding assay	
	Hippocampus		↑ 46%	Binding assay	

Table 1. (cont'd)

Animal species	Tissue (Cell)	Time post-ADX	GR levels	Assay method	Reference
Male rat	Cerebral cortex	4 d.	↑ 96%	Binding assay	Turner, 1986
	Amygdala-entorhinal cortex		↑ 106%	Binding assay	
	Hypothalamus		↑ 27%	Binding assay	
	Pituitary		↑ 24%	Binding assay	
Male rat	Liver	2 w.	mRNA -	RNA blot hybrid	Kalinyak et al., 1987
	Lung		mRNA -	RNA blot hybrid	
	Brain		mRNA ↑ 40%	RNA blot hybrid	
	Kidney		mRNA ↑ 80%	RNA blot hybrid	
Female rat	Hypothalamus	14 d.	mRNA -	RNA blot hybrid	Peiffer et al., 1991
	Hippocampus		mRNA ↑196%	RNA blot hybrid	
	Amygdala		mRNA ↑140%	RNA blot hybrid	

↑% means that the GR levels were increased by % after the rats were adrenalectomized. mRNA - means that the GR levels were unchanged after the rats were adrenalectomized. mRNA ↑% means that the GR mRNA levels were increased by % after the rats were adrenalectomized. h., hour(s); d., day(s); w., week(s); hybrid., hybridization; ADX, adrenalectomy.

Table 2. Literature review on the GR regulation by glucocorticoids

Animal species	Tissue (Cell)	Glucocorticoid treatment	GR levels	Assay method	Reference
Human	CEM-7 cells	Dex 1 μM, 18 h.	mRNA ↑ Protein ↑	In situ hybrid Immunocytochem	Antakly et al., 1989
Human	Skin Fibroblast	Dex 25 nM, 24 h.	↓ 50%	Binding assay	Berkovitz et al., 1988
Human	Fibroblast	Dex 1μM, 12 h.	mRNA ↓ 60%	RNA blot hybrid	Bronnegard et al., 1991
Human	HeLa S_3 cells Cos 1 cells (transfected with a human GR vector)	Dex 1 μM, 24 h. Dex 1 μM, 24 h.	mRNA ↓ mRNA ↓	RNA blot hybrid RNA blot hybrid	Burnstein et al., 1991
Human	HeLa S_3 cells	Dex nM, 24 h.	↓ 40%	Binding assay	Cidlowski and Cidlowski, 1981
Rat Mouse	HTC cells W7 lymphoma cells	Dex 1 μM, 36 h. Dex 1 μM, 15 h	↓ 64% ↓ 33%	Binding assay Binding assay	Danielsen and Stallcup, 1984
Human	Leukemic B cells Leukemic T cells	Dex 1 μM, 16-18 h. Dex 1 μM, 16-18 h.	↓ 65-80% ↓ 800%	RNA blot hybrid	Denton et al., 1993
Male rat	Liver HTC cells	Dex 4 mg/kg, 18 h. Dex 4 mg/kg, 24 h. Dex 0.5 μM, 48 h Dex 0.5 μM, 48 h	mRNA ↓ 90% Protein ↓ 80% mRNA ↓ 80% Protein ↓ 60%	RNA blot hybrid Immunoblotting RNA blot hybrid Immunoblotting	Dong et al., 1988

Table 2. (Cont'd)

Animal species	Tissue (cell)	Glucocorticoid treatment	GR levels	Assay method	Reference
Human	CEM-7 cells	Dex 1 μM, 18 h	mRNA ↑ Protein ↑	RNA blot hybrid Immunoblotting	Eisen et al., 1988
Mouse	NIH 3T3 cells	Dex 1 μM, 3 h Dex 1 μM, 20 h	mRNA ↓ 75% Protein ↓ 80%	RNA blot hybrid Immunoblotting	Hoeck et al., 1989
Male rat	Liver Lung Kidney Brain Adrenal gland Spleen	Dex 7 mg/kg, 6 h.	mRNA ↓ 40% mRNA ↓ 50% mRNA ↓ 60% mRNA ↓ 60% mRNA ↓ 60% mRNA ↓ 40%	RNA blot hybrid RNA blot hybrid RNA blot hybrid RNA blot hybrid RNA blot hybrid RNA blot hybrid	Kalinyak et al., 1987
Human	T cell	Cortisol 10 μM, 24 h	↓ 50%	Binding assay	Lacroix et al., 1984
Male rat (2 weeks post-ADX)	Skeletal muscle	Hydrocortisone,10 mg/kg body weight daily for 5 d	↓ 90%	Binding assay	Mayer and Rosen, 1978
Human	Skin fibroblasts	Dex 10 μM, 9 d.	↓ 87%	Binding assay	Oikarinen et al., 1987
Rat	HTC cells	Dex 0.5 μM, 24 h.	mRNA ↓ 50%	RNA blot hybrid	Okret et al., 1986
Female rat	Hypothalamus Hippocampus Amygdala	Dex 1 mg/kg, 14 d.	mRNA - mRNA ↓ 30% mRNA ↓ 55%	RNA blot hybrid RNA blot hybrid RNA blot hybrid	Peiffer et al., 1991

(To be continued)

Table 2. (Cont'd)

Animal species	Tissue (Cell)	Glucocorticoid treatment	GR levels	Assay method	Reference
Rat	Hypothalamus Cerebral cortex	Dex 1 mg/kg, 72 h.	↓ 30-40% ↑ 1.4 folds	RNA blot hybrid	Pepin et al., 1990
Human Rat	IM9 cells AR 42J cells	Dex 10 nM, 24 h. Dex 10 nM, 24 h.	mRNA ↓ 50% mRNA ↓ 50%	RNA blot hybrid RNA blot hybrid	Rosewicz et al., 1988
Male rat	Amygdala Hypothalamus Pituitary Hippocampus	Stress, 3 w.	↓ 39% ↓ 17% ↓ 10% ↓ 26%	Binding assay Binding assay Binding assay Binding assay	Sapolsky et al., 1984
Human	Lymphocytes	Dex 1 mg daily for 1 w.	↓ 30%	Binding assay	Schlechte et al., 1982
Human	HeLa S_3 cells	Dex 1μM, 24-48 h.	Protein ↓ Protein ↓ mRNA ↓	Affinity labelling Western blotting Northern blotting	Silva et al., 1994
Mouse	AtT-20 pituitary tumor cells	Dex 10 μM, 96 h.	↓ 75%	Binding assay	Svec and Rudis, 1981
		TA, 1 μM, 0-72 h.	mRNA ↓ Protein ↓	Northern blotting Northern blotting	Vig et al., 1994
Male rat	Hippocampus	Corticosterone, 80-100 μg/kg daily for 3 w.	↓ 50%	Binding assay	Tornello et al., 1982
Sheep fetus	Hypothalamus Pituitary	ACTH, 100 h.	↓ 60% ↓ 40%	Binding assay Binding assay	Yang et al., 1990

↓% means that the GR level was decreased by % after the animals or the cells were treated with glucocorticoids. mRNA ↓% means that the GR mRNA level was decreased by % after the animals or the cells were treated with glucocorticoids. Protein ↓% means that the GR protein level was decreased by % after the animals or the cells were treated with glucocorticoids. h., hour(s); w., week(s); d., day(s); hybrid., hybridization; immunocytochem., immunocytochemistry.

Table 3. Test of the $14B_2$ dilution in the RIA

Dilution of $14B_2$	Total binding (%)	Non-specific binding (%)
1:10,000	69.2	2.1
1:20,000	36.5	1.8
1:40,000	13.9	1.4

Total binding was calculated as the ratio of the radioactive count of the reference tubes* over that of the tracer tubes** and non-specific binding as the ratio of the radioactive count of the blank tubes*** over that of the tracer tubes. Values shown are the average of three determinations.

*The reference tubes were those to which the tracer, the primary and the secondary antibodies, but no unlabelled antigen was added. **The tracer tubes were those which were counted for the total tracer added. ***The blank tubes were those to which the tracer, the secondary antibody, but neither the primary antibody nor unlabelled antigen was added.

Table 4. Comparison between RIA and receptor binding assay

Tissue cytosols	n	GR (fmoles/mg protein) Mean ± SD		P
		RIA	Receptor binding	
Liver	8	729.3 ± 93.1	713.4 ± 84.2	NS
Spleen	8	438.6 ± 54.2	449.5 ± 51.3	NS
Cardiac ventricle	8	109.5 ± 14.8	102.7 ± 16.9	NS

NS, not significant by Student's t-test.

Table 5. Test of detergent effect on the GR RIA

Samples	n	Detergent concentration (%)		GR (fmoles/mg protein)
		NP40	DOC	Mean ± SD
L1114	9	0	0	716.2 ± 89.3
L1114	9	0.04	0.006	708.6 ± 82.5

No difference was found in GR levels between detergent treated and detergent free samples (by Student's t-test).

Table 6. Effect of detergent on GR extraction

Samples	Detergent	GR (fmoles/mg protein) Mean ± SD			n
		Cytosol	Particulate extract	Total	
L1115	-	465.0 ± 59.6	168.5 ± 19.2	633 ± 72.6	7
L1115	+	604.3 ± 67.2	39.2 ± 5.1	643 ± 71.5	7

The GR level was higher when detergent was used than when no detergent was used in the cytosol and the GR level was lower when detergent was used in the particulate extract. The total GR level (the sum of the GR levels in the cytosol and the particulate extract) was not different whether detergent was used or not. Student's t-test was used.

Table 7. Effect of adrenalectomy on GR levels

Tissue	GR levels (fmoles/mg protein (mean ±SD)		Increment in GR levels (%)
	Control	ADX	
Left atrium	101±25	202±37*	100
Right atrium	110±25	208±41*	89
Spleen	417±55	638±34*	53
Liver	703±71	405±29*	99
AL pituitary	182±37	300±40*	65
Left ventricle	163±29	261±26*	60
Right ventricle	164±24	270±26*	65
Thymus	452±33	583±37*	29
Hippocampus	193±16	234±19*	21
Hypothalamus	182±32	255±33*	40

RIA was used to measure GR levels.

* indicates that GR levels between the control and the ADX rats were significantly different by Student's t test.

n=7.

Table 8. Interassay controls of ANF RIA

Sample	No. of determinations	ANF (ng/mg protein) (mean ± SD)	Coefficient of variation (%)
63a	7	599.5 ± 93.5	15.6
64a	7	612.3 ± 79.3	13.0
63v	7	6.7 ± 0.6	9.0
64v	7	6.3 ± 1.0	15.8

63a and 64a are two different atrial cytosols, and 63v and 64v are two different ventricular cytosols.

Figure 1. Glucocorticoid action within responsive cells. Steroid (S) enters the target cell by simple diffusion. In the cytoplasm of the cell, steroid binds to its receptor (R). This binding triggers a conformational change in the receptor which enhances the affinity of the receptor for chromatin. This phenomenon is referred to as activation of the receptor. The activated receptor-steroid complex then translocates into the nucleus to influence the specific gene transcription. It can stimulate (+) or inhibit (-) gene transcription depending on the particular genes involved. The transcribed mRNA codes for protein which is in turn responsible for the hormonal response.

GLUCOCORTICOID - RESPONSIVE CELL

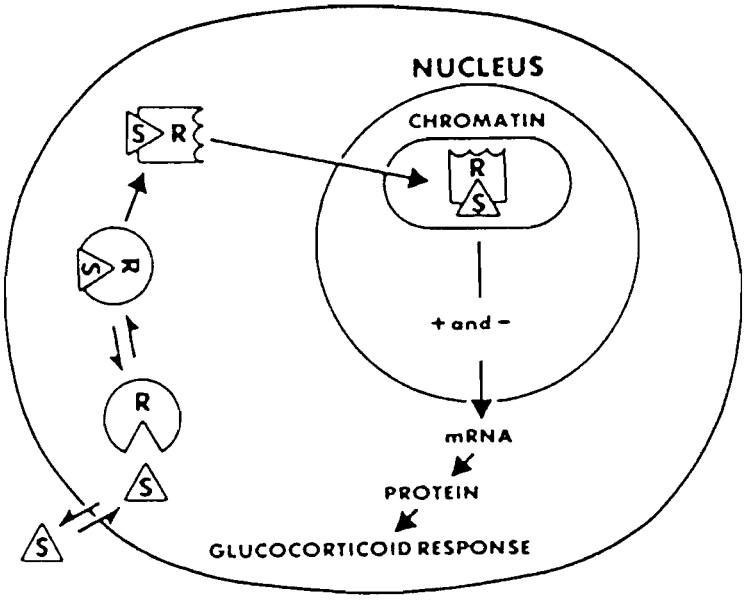


Figure 2. Structural properties of the GR. GR contains three functional domains: a C-terminal steroid-binding domain (steroid), a central DNA-binding domain (DNA), and a N-terminal domain. The N-terminal domain is rich in acidic amino acids and this region of the receptor may have modulatory effect on the transactivation, DNA binding, or both. The DNA-binding domain is rich in Cys, Lys and Arg residues. Shown here is the structure of the human GR. The numbers indicate amino acid position. NH_2- indicates the N-terminus and -COOH indicates the C-terminus of the receptor protein.

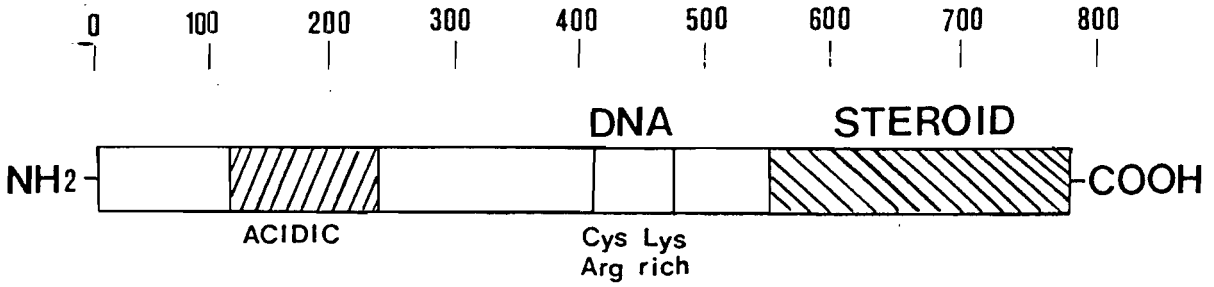

0
100
200
300
400
500
600
700
800
DNA
STEROID
NH2
COOH
ACIDIC
Cys Lys
Arg rich

Figure 3. Procedure for preparation of RIA samples. To extract GR from the rat tissue, the tissue was homogenized in an isotonic buffer containing Tris HCI (pH 7.2) 25 mM, NaCl 10 mM, EDTA 1 mM, $MgCl_2$ 2 mM, dithiothreitol 1 mM, glycerol 5% and leupeptin 10 nM (0.4% NP40 and 0.06% DOC were added to the tissue when homogenization was performed). The homogenate was centrifuged at 12,000xg for 5 min. This centrifugation resulted in a pellet that was referred to as the "particulate fraction" and a supernatant that was centrifuged at 128,000xg for 10 min. The 128,000xg supernatant was referred to as "cytosol" and the pellet was added to the "particulate fraction". To extract GR from the "particulate fraction", the "particulate fraction" was homogenized in a high salt buffer containing 0.01 M phosphate (pH 7.4) and 0.4 M NaCl. The homogenate was centrifuged at 12,000xg for 5 min and the resulting supernatant was centrifuged at 128,000xg for 10 min. The 128,000xg supernatant was referred to as the "particulate extract".

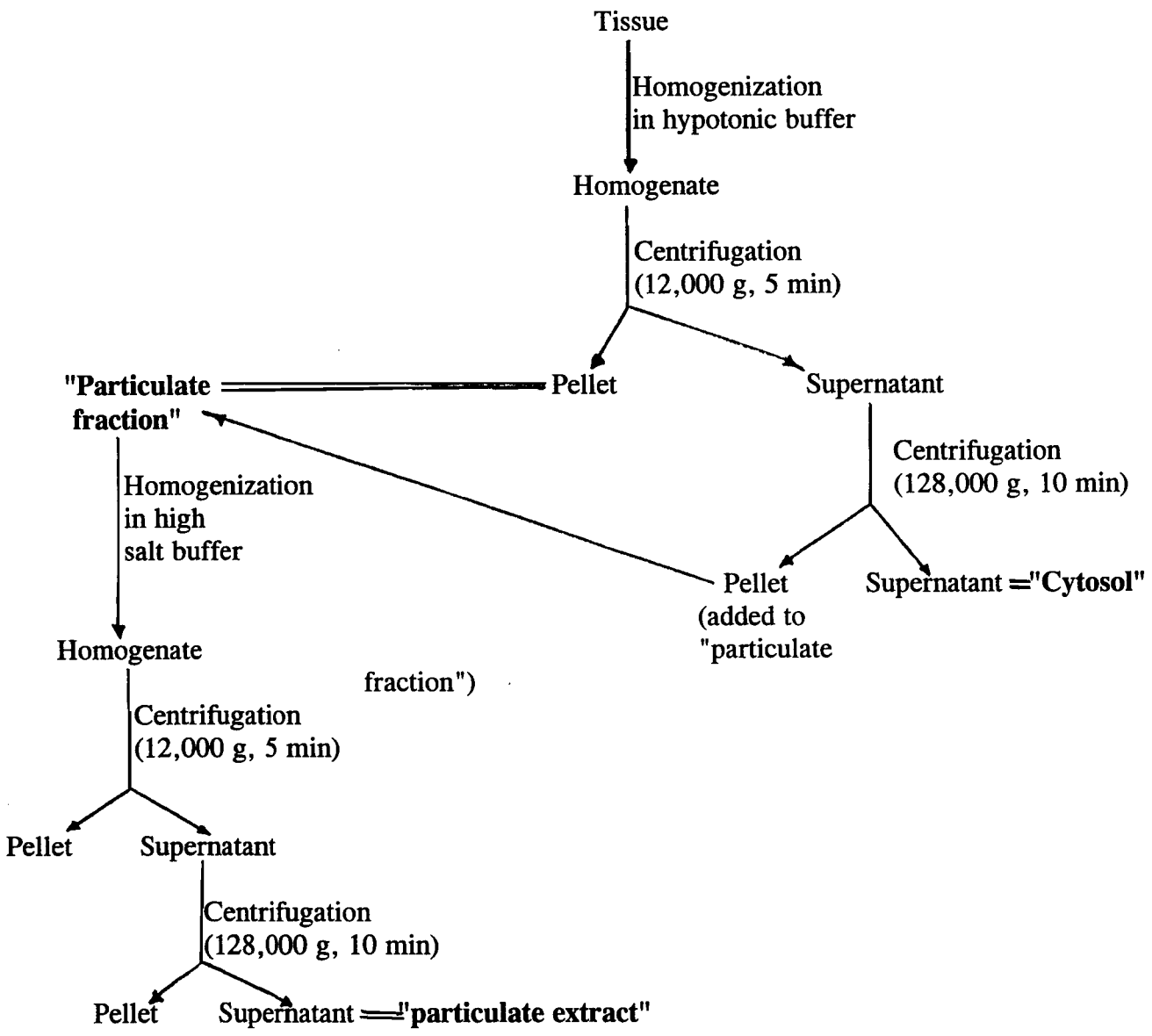

Tissue
Homogenization
in hypotonic buffer
Homogenate
Centrifugation
(12,000 g, 5 min)
"Particulate
fraction"
Pellet
Supernatant
Centrifugation
(128,000 g, 10 min)
Homogenization
in high
salt buffer
Pellet
(added to
"particulate
fraction")
Supernatant ="Cytosol"
Homogenate
Centrifugation
(12,000 g, 5 min)
Pellet
Supernatant
Centrifugation
(128,000 g, 10 min)
Pellet
Supernatant ="particulate extract"

Figure. 4. Principle of radioimmunoassay. In the RIA, the interaction between the antigen and the antibody results in a antigen-antibody complex. In order to precipitate the antigen-antibody complex, a secondary antibody is added (the secondary antibody is anti-immunoglobulin of the species in which the primary antibody was produced and therefore from a different species). The amounts of the antibody and the radioactive antigen are fixed. Unlabelled antigen competes with radioactive antigen for binding sites on the antibody. This reduces the amount of radioactivity in the antibody-antigen precipitate. The amount of the unlabelled antigen is either known (various concentrations of the unlabelled antigen serve as standard) or unknown (unknown samples). The amount by which the precipitated radioactivity is reduced compared to the standard indicates the concentration of antigen in the unknown sample.

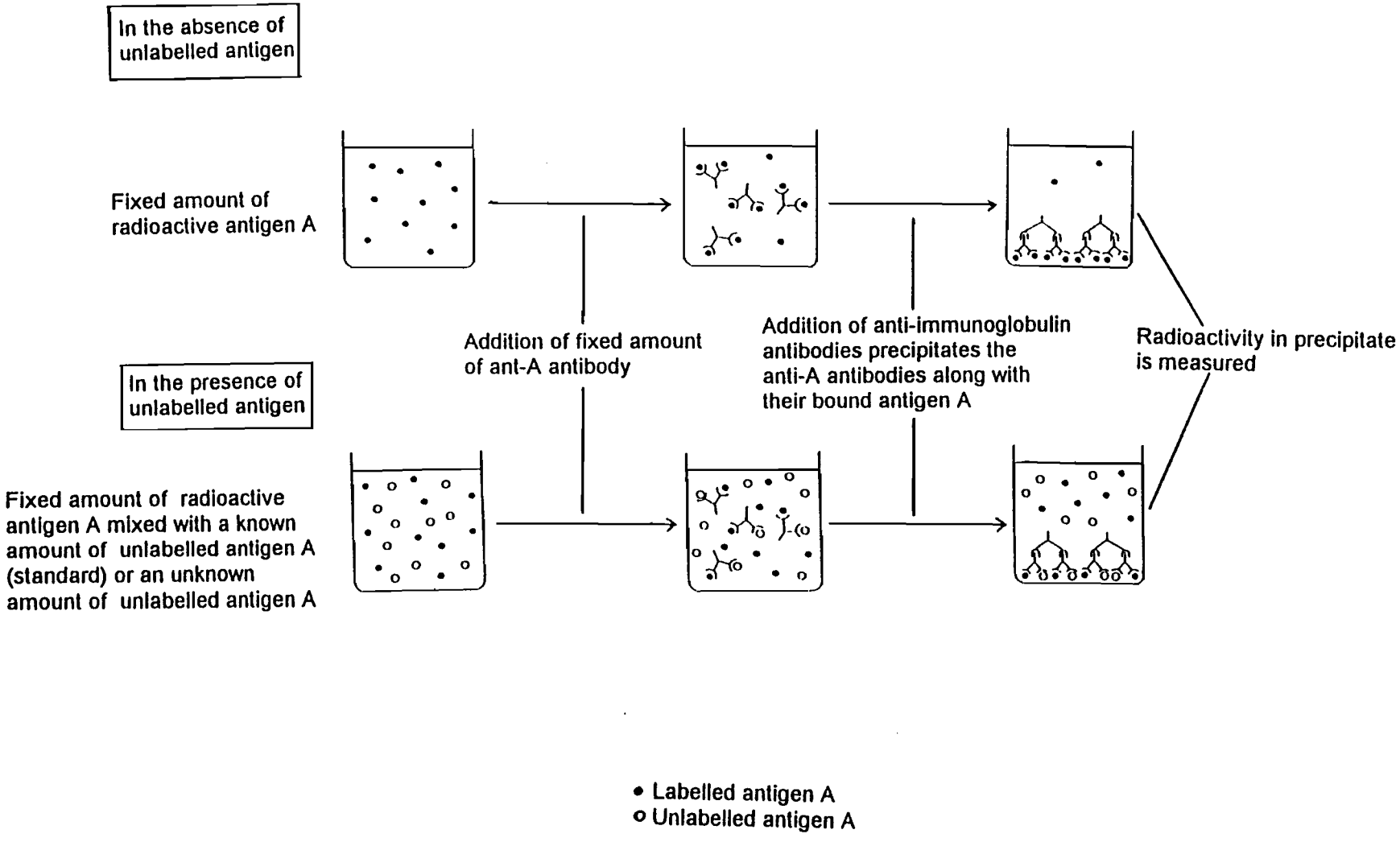

In the absence of unlabelled antigen
Fixed amount of radioactive antigen A
Addition of fixed amount of ant-A antibody
Addition of anti-immunoglobulin antibodies precipitates the anti-A antibodies along with their bound antigen A
Radioactivity in precipitate is measured
In the presence of unlabelled antigen
Fixed amount of radioactive antigen A mixed with a known amount of unlabelled antigen A (standard) or an unknown amount of unlabelled antigen A
• Labelled antigen A
o Unlabelled antigen A

Figure 5. Characterization of $14B_2$. (A). Fluorogram from cultured pituitary cell extracts affinity-labelled with [^{3}H] dexamethasone mesylate, then immunoreacted with $14B_2$, immunoprecipitated with protein A-Sepharose, and finally electrophoresed on SDS-PAGE. (B). Radioautogram of HTC cell extracts electrophoresed on SDS-PAGE then transferred onto nitrocellulose paper. After incubation with $14B_2$, immunoreactivity was detected by ^{125}I-protein A. The molecular weight markers shown are: myosin (200 Kda), phosphorylase B (97 Kda), BSA (68 Kda).

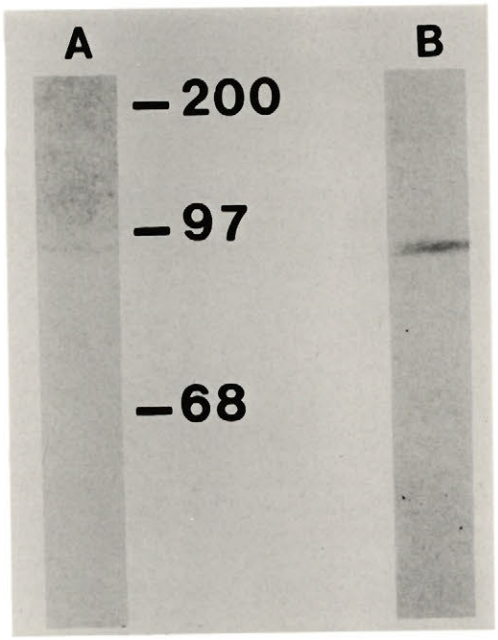

A
B
200
97
68

Figure 6. Characterization of iodinated 14-mer by HPLC. The 14-mer was iodinated by Chloramine T method. The ^{125}I 14-mer was purified by Sep-Pak cartridge and its purity was examined by HPLC using a Zorbax RP300-C18 reverse-phase column. The HPLC eluted peak was revealed by a radioactivity detector. Panel A shows the peak of the ^{125}I 14-mer and panel B shows the elution gradient. mV, millivolt.

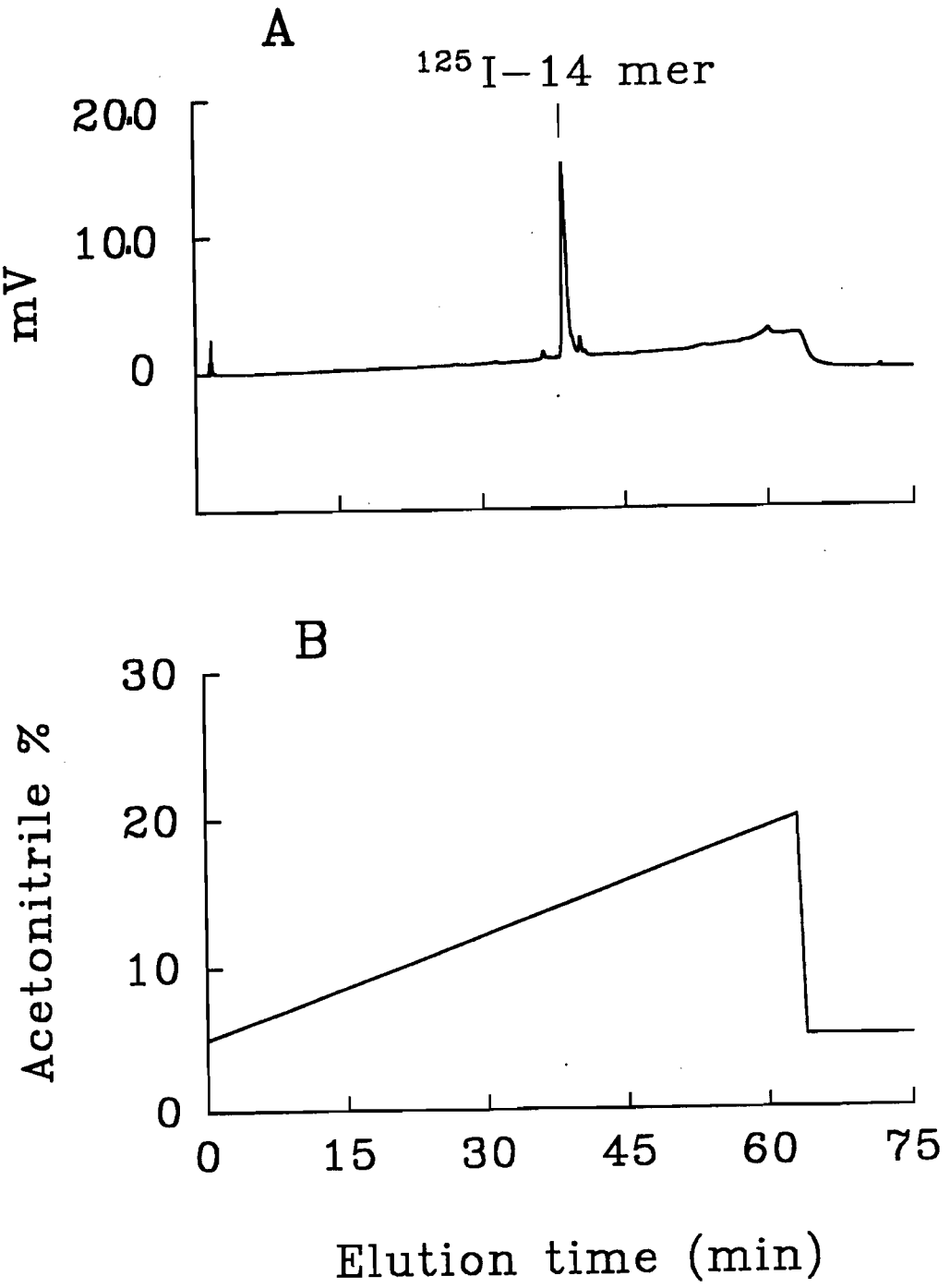

A
^{125}I–14 mer
20.0
10.0
0
mV
B
30
20
10
0
Acetonitrile %
0
15
30
45
60
75
Elution time (min)

Figure 7. A standard curve of the GR RIA. The synthetic peptide (14-mer) of the human GR was labelled with ^{125}I by Chloramine T method. Various concentrations of the unlabelled 14-mer were incubated with the fixed amount of labelled 14-mer and the antibody $14B_2$. A precipitate was formed in the antibody and antigen reaction. The antibody bound (B, in the precipitate) and the free (in the supernatant) ^{125}I-14-mer were separated from each other by centrifugation. The radioactivity in the precipitate was counted. The ratio of the bound to the total radioactivity (T, the total radioactivity added to the tube) versus the concentration (at the logarithmic scale) of the unlabelled 14-mer was plotted. The GR concentrations in the unknown samples were calculated from the linear portion of the standard curve (B/T of 0.07-0.31). The affinity of the $14B_2$ with the 14-mer was calculated as the concentration of the unlabelled 14-mer at half-saturation of the antibody and was 1.8 fmoles/tube at the logarithmic scale. This was equivalent to 63 fmoles/tube of the 14-mer. As the total reaction volume in the RIA was 400 μl, 63 fmoles/tube of the 14-mer was equal to 157 fmoles/ml. $Ka = 1 \div (157 \text{ fmoles/tube}) = 1 \div (1.57 \times 10^{-10} \text{ Moles/L}) = 6 \times 10^{9}$ L/mole.

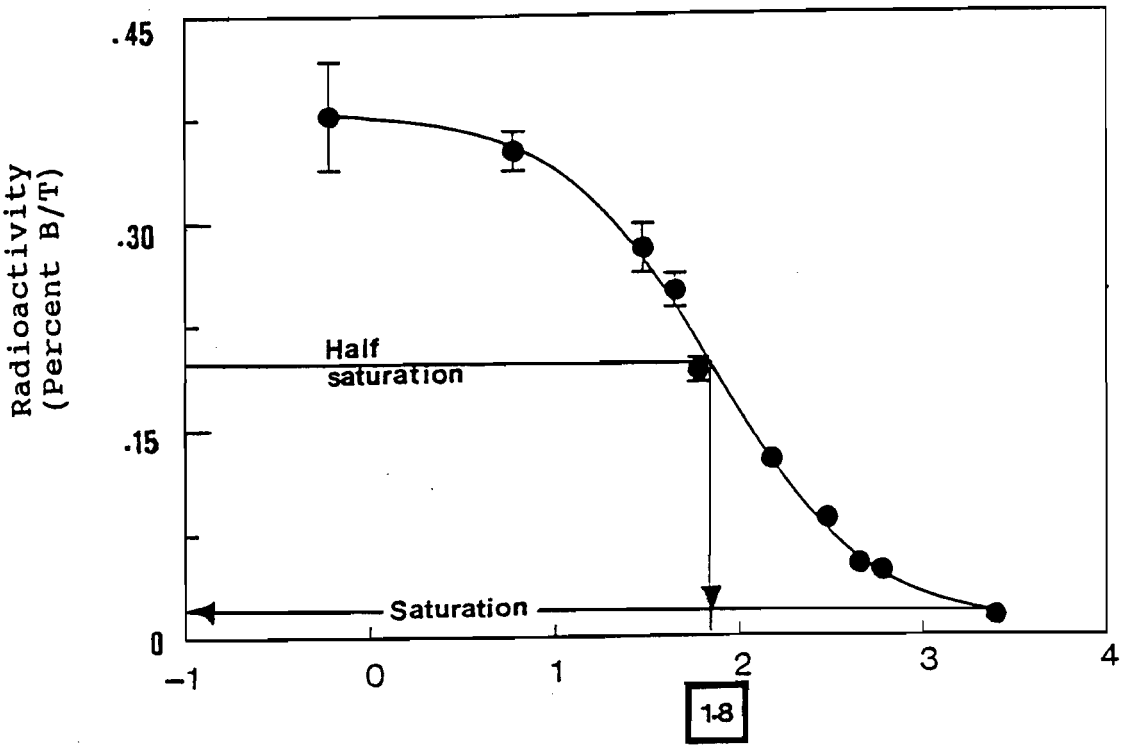

.45
.30
.15
0
Radioactivity
(Percent B/T)
Half
saturation
Saturation
−1
0
1
2
3
4
1-8
[14-mer] fmoles/tube
(Log dose)

Figure 8. Comparison of immunoreactivity of serum from rabbit B before (preimmune B) or following the first ($14B_1$) and second ($14B_2$) immunization booster injections and rabbit C after the first booster injection (14C1). These RIA standard curves were generated using antibody dilution of 1:20,000. Assays were done in triplicate and the error bars indicate standard deviations.

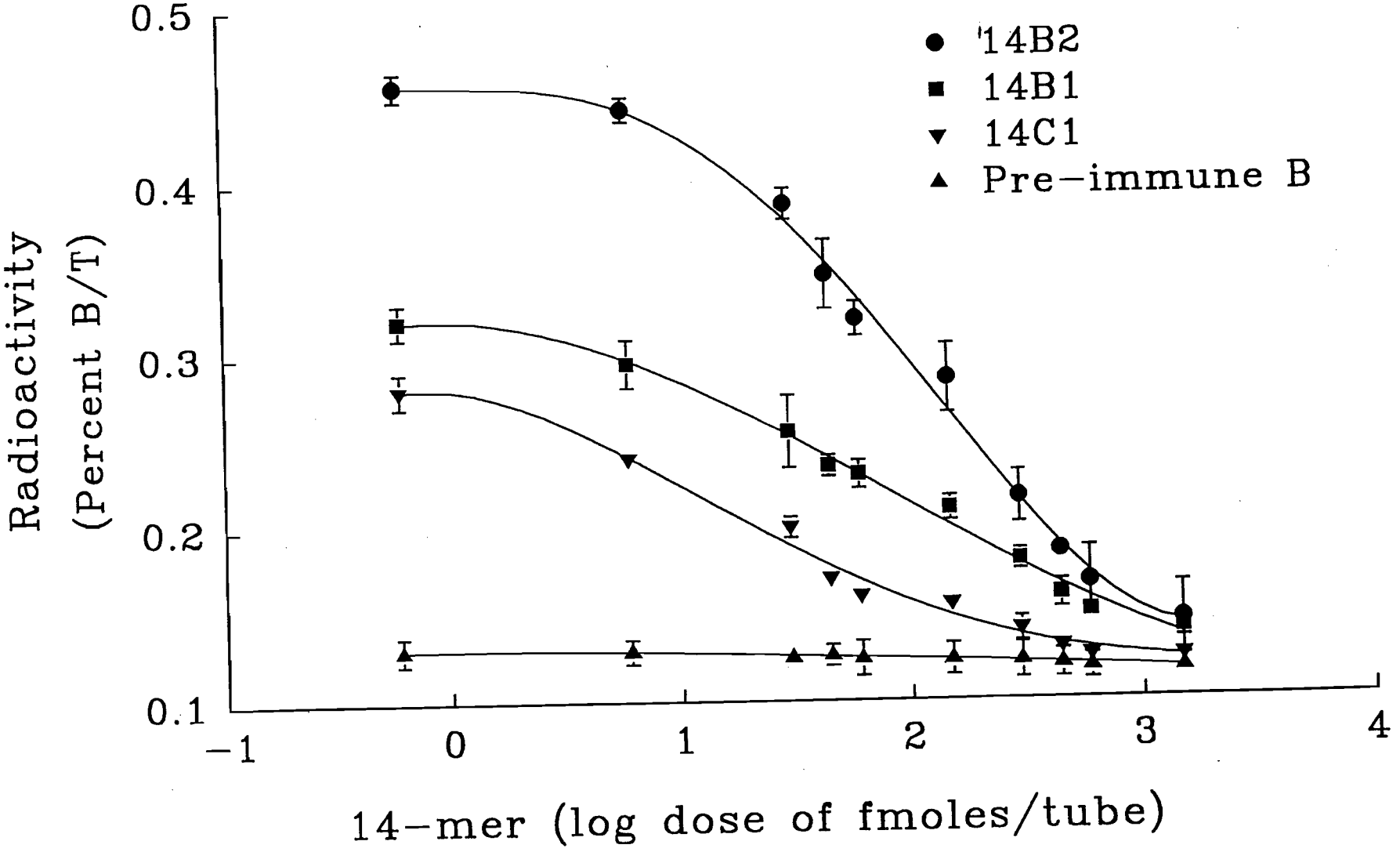

0.5
0.4
0.3
0.2
0.1
Radioactivity
(Percent B/T)
14B2
14B1
14C1
Pre-immune B
−1
0
1
2
3
4
14-mer (log dose of fmoles/tube)

Figure 9. Affinity of $14B_2$ with liver cytosol GR. The standard curve was plotted as the ratio of the antibody bound (B) to the total (T) radioactivity against the concentrations of the unlabelled 14-mer (the top abscissa, at the logarithmic scale). In the same RIA run, various concentrations of a liver cytosol were used to compete for binding sites with the ^{125}I-14-mer. The liver cytosol dilution curve was plotted as the ratio of the antibody bound (B) to the total (T) radioactive 14-mer against the concentrations of liver cytosol (bottom abscissa). At the protein concentration of 0.8 mg/ml (logarithmic scale) in the liver cytosol, the antibody was half-saturated. The affinity of the $14B_2$ with the liver cytosol GR, calculated as the GR concentration in the liver cytosol at half-saturation of the antibody, was 1.9 fmoles/tube (calculated from the standard curve) at the logarithmic scale. This was equivalent to 79 fmoles/tube of the GR. As the total reaction volume in the RIA was 400 μl, 79 fmoles/tube of the GR was equal to 197.5 fmoles/ml. $Ka = 1 \div (197.5 \text{ fmoles/ml}) = 1 \div (1.975 \times 10^{-10} \text{ moles/L}) = 5 \times 10^9$ L/mole.

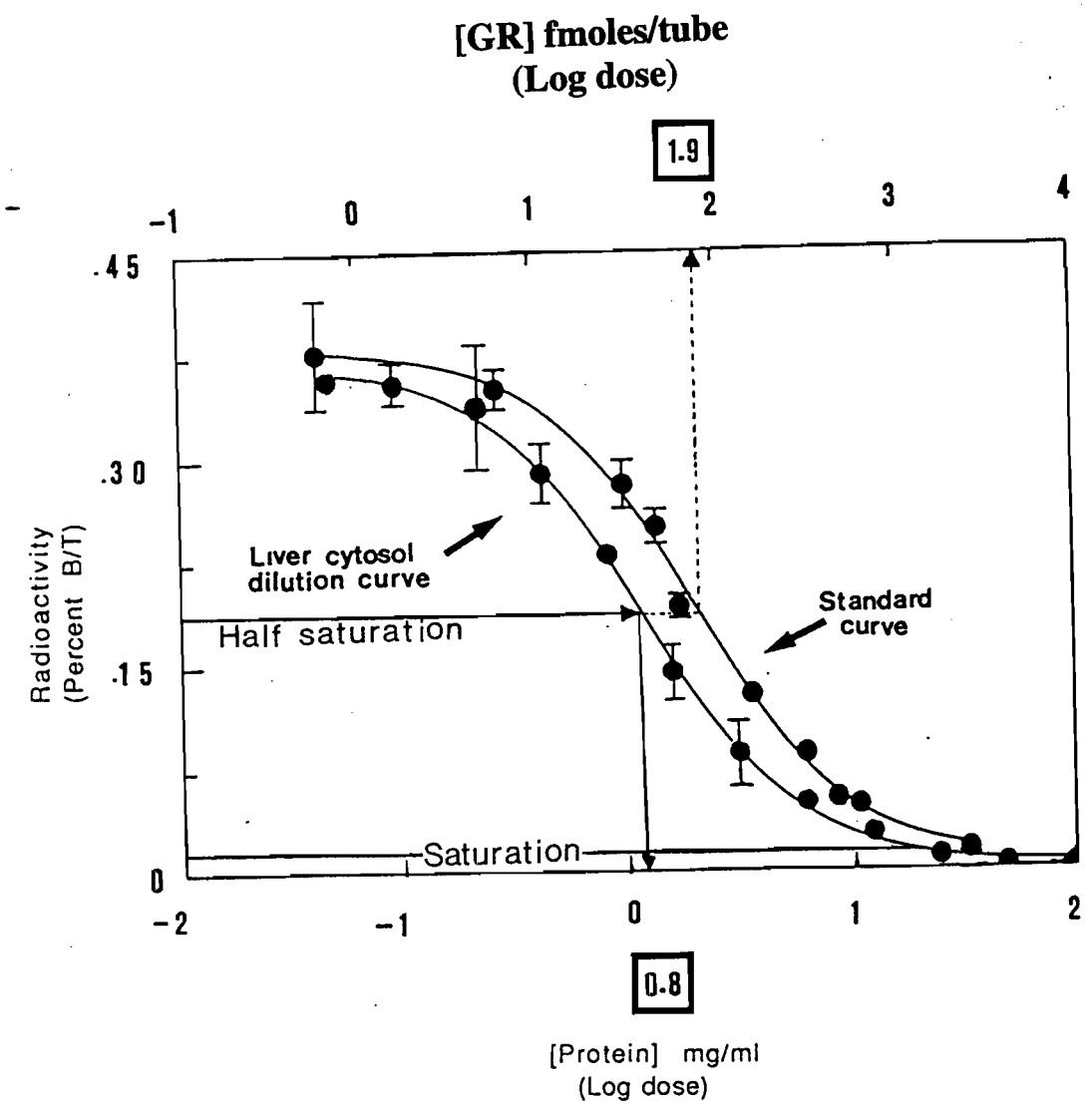

[GR] fmoles/tube
(Log dose)
1.9
-1
0
1
2
3
4
.45
.30
.15
0
Radioactivity
(Percent B/T)
Liver cytosol
dilution curve
Half saturation
Standard
curve
Saturation
-2
-1
0
1
2
0.8
[Protein] mg/ml
(Log dose)

Figure 10. Tissue cytosol dilution curves. The antibody bound ^{125}I-14-mer was displaced by various concentrations of tissue cytosol of either the anterior lobe of the pituitary, spleen or liver. The standard curve is also shown. The GR levels of the tissue cytosols were calculated from the standard curve. Assays were done in triplicate and the error bars indicate standard deviations.

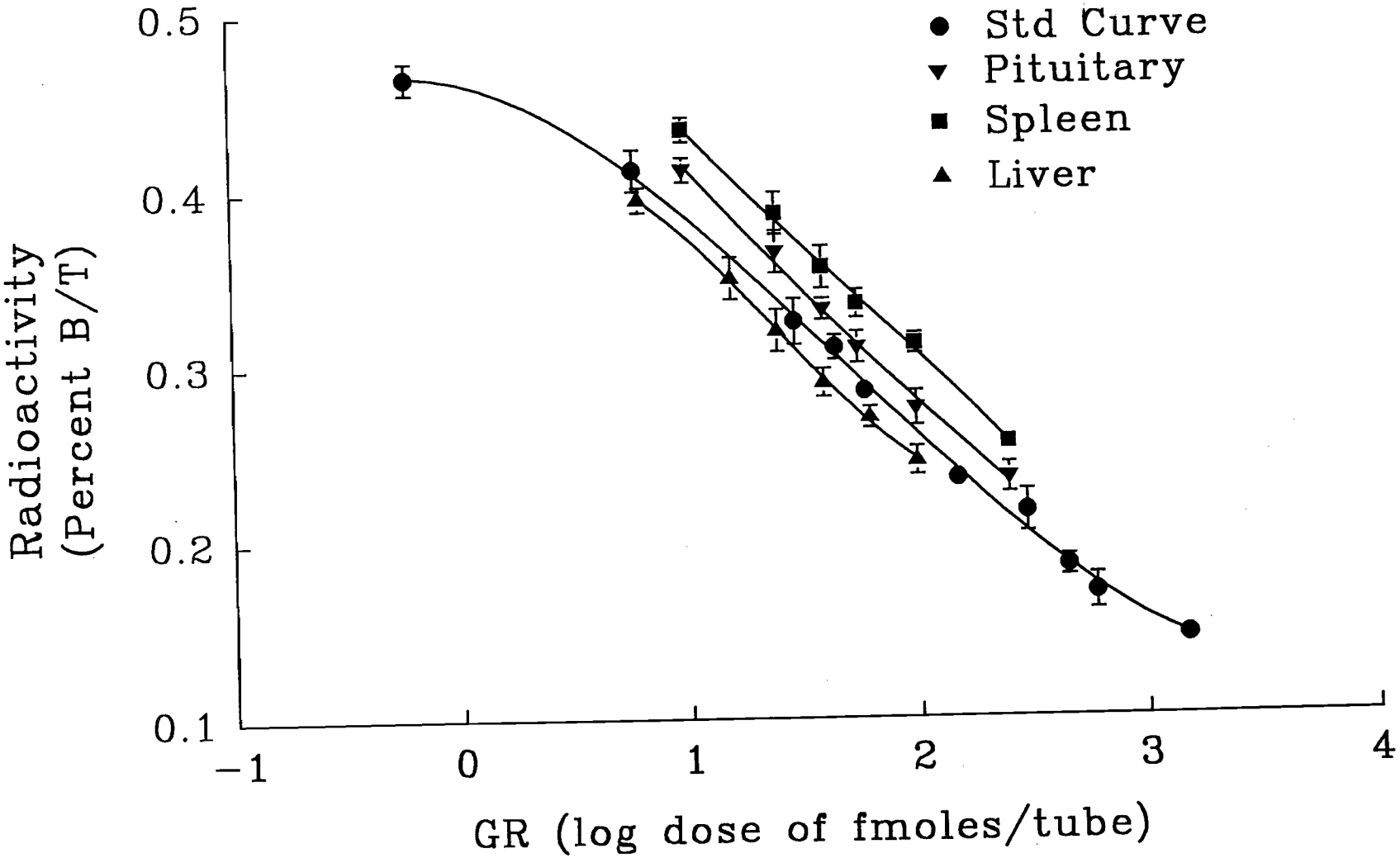

Std Curve
Pituitary
Spleen
Liver
Radioactivity
(Percent B/T)
0.5
0.4
0.3
0.2
0.1
−1
0
1
2
3
4
GR (log dose of fmoles/tube)

Figure 11. Dose response analysis of Dex on GR levels. Normal male Sprague Dawley rats were injected intraperitoneally with various doses of Dex for 4 days. GR levels in the liver cytosols were measured by RIA. The error bars indicate standard deviations. n=6 rats.

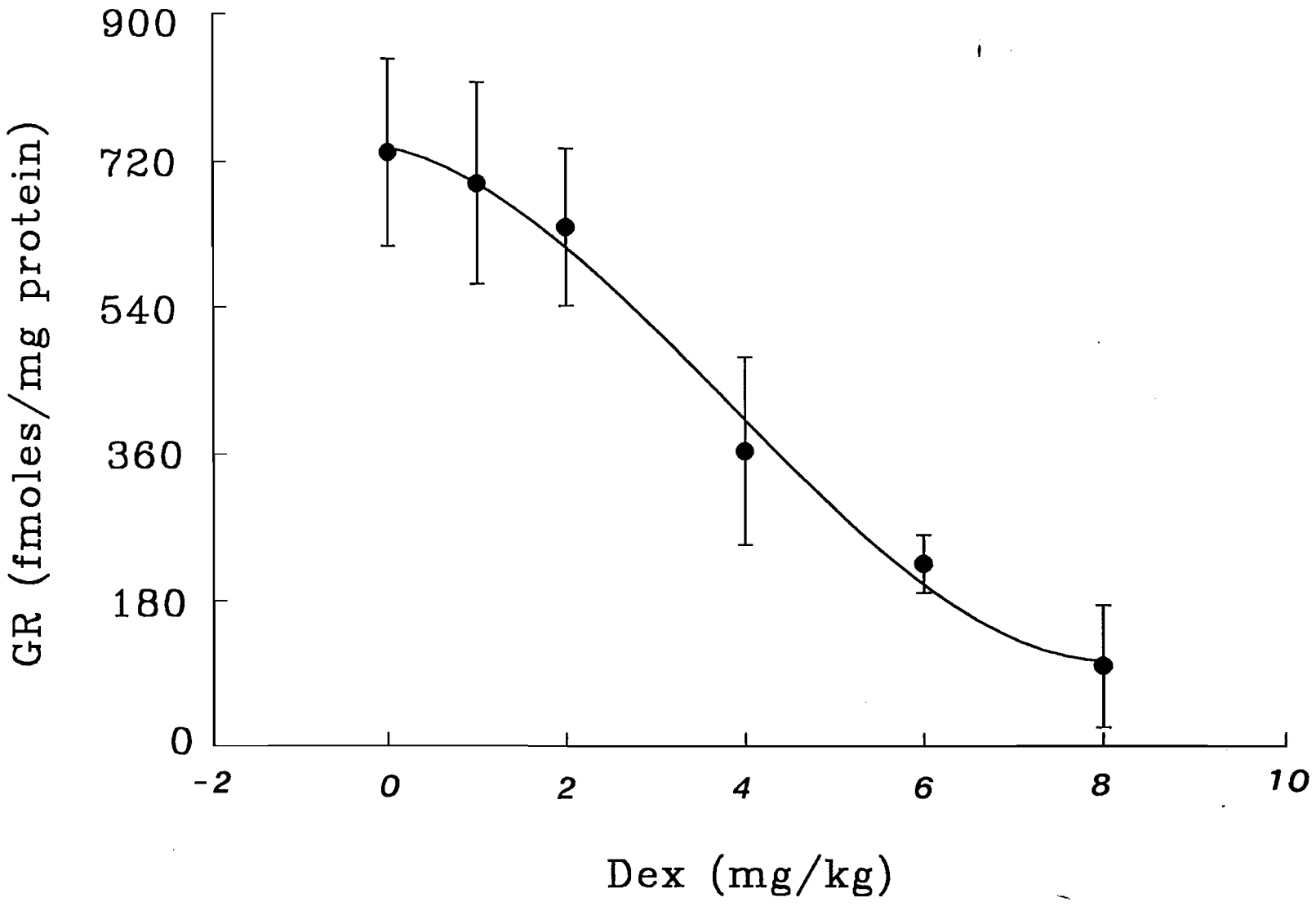

900
720
540
360
180
0
GR (fmoles/mg protein)
-2
0
2
4
6
8
10
Dex (mg/kg)

Figure 12. Time course of Dex effects on GR levels. Normal male Sprague Dawley rats were injected intraperitoneally with Dex (4 mg/kg) for various time lengths. GR levels in the liver cytosols were measured by RIA. The error bars indicate standard deviations. * indicates that the GR level is significantly different between Dex treatment and control ($p < 0.05$) by analysis of variance and subsequent Duncan's test. $n=6$ rats.

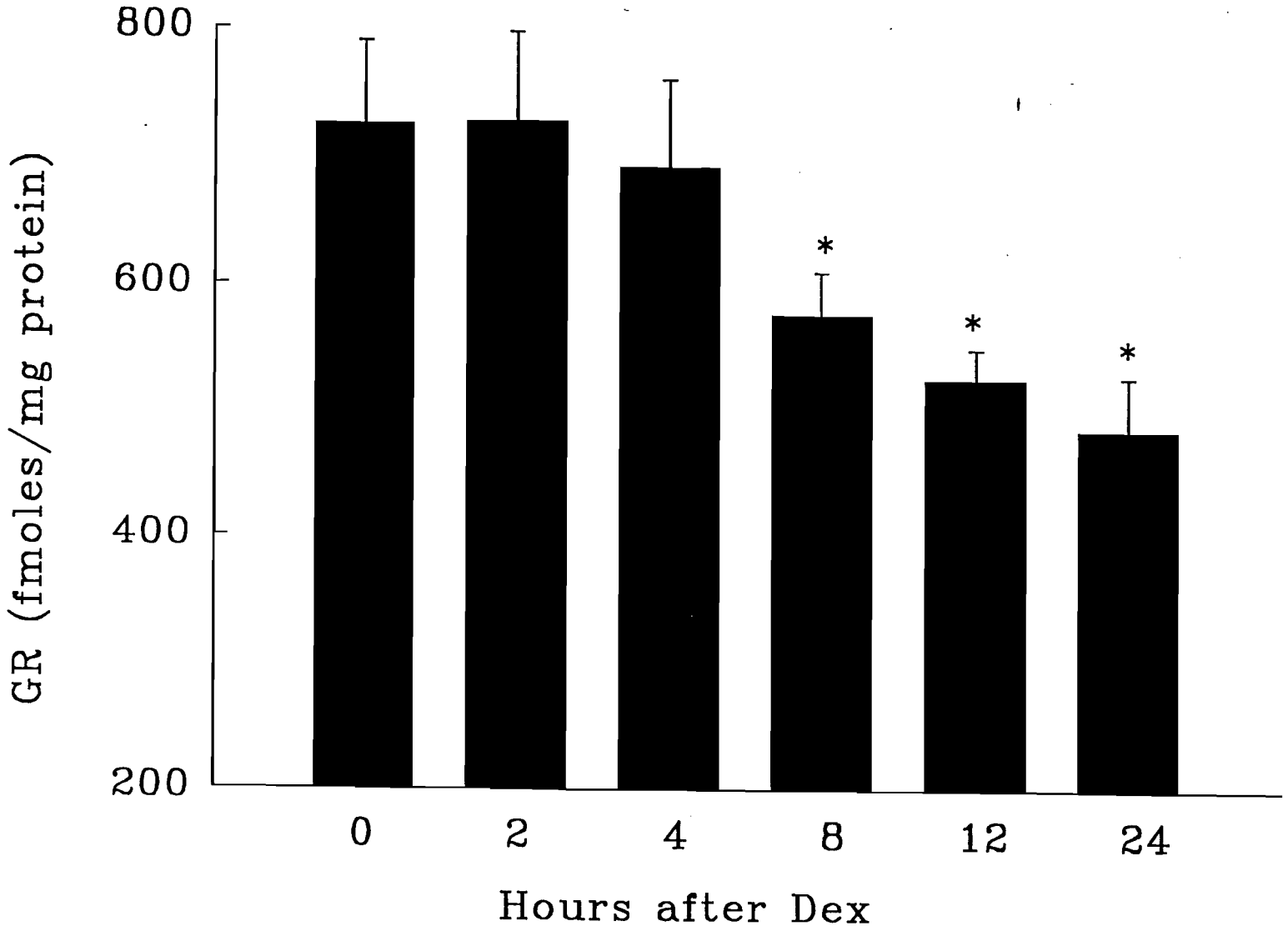

800
600
400
200
GR (fmoles/mg protein)
*
*
*
0
2
4
8
12
24
Hours after Dex

Figure 13. PBS-injection and GR levels. Male Sprague-Dawley rats were either uninjected or injected intraperitoneally with PBS daily for 1, 4 or 8 day(s). The volume of PBS injected was 0.25 ml which was the volume used for Dex injection in the other experiments. GR levels were quantified by RIA. Using analysis of variance, no significant difference was found in GR levels among different groups for all the tissues examined. Panels A, B, C, D, E, and F show the effect of PBS-injection on the GR levels in liver, spleen, the left cardiac ventricle, the right cardiac ventricle, the left cardiac atrium, and the right cardiac atrium, respectively. The error bars indicate the standard deviation. n=7 rats.

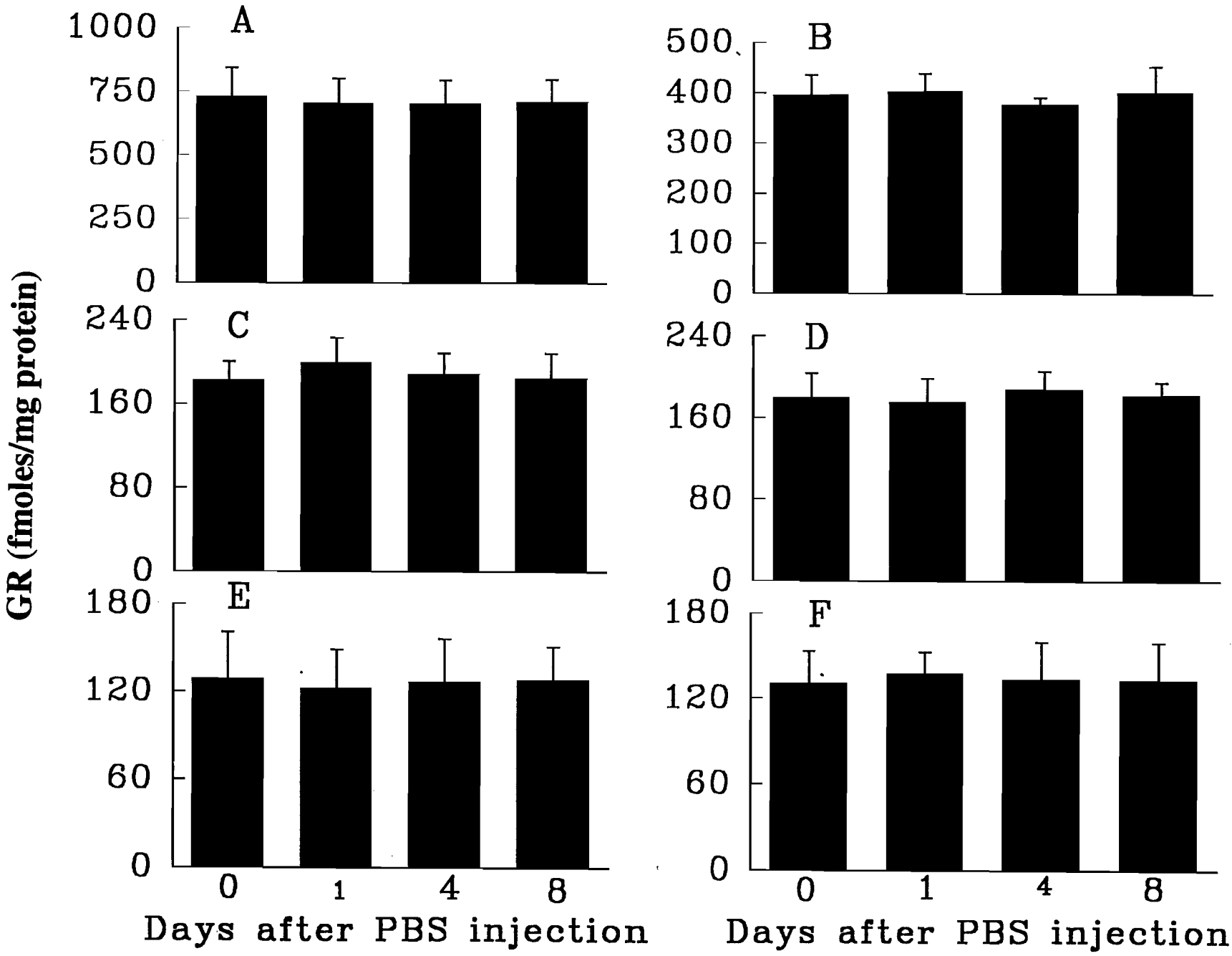

A
B
C
D
E
F
1000
750
500
250
0
500
400
300
200
100
0
240
160
80
0
240
160
80
0
180
120
60
0
180
120
60
0
0
1
4
8
0
1
4
8
GR (fmoles/mg protein)
Days after PBS injection
Days after PBS injection

Figure 14. Time course of adrenalectomy on GR levels. Male Sprague-Dawley rats were either unoperated (0 week as controls) or bilaterally adrenalectomized for 1 week, 2 weeks, and 3 weeks before being sacrificed. GR levels were measured by RIA. Analysis of variance and subsequent Duncan's test for multiple comparisons were used to analyze the data. * indicates that the GR level is significantly different at the 0.05 level from the respective control. Panels A, B, C, D, E, F, and G show the GR level changes in the left ventricle, the right ventricle, the left atria, the right atria, liver, spleen, and the anterior lobe of the pituitary, respectively. The error bars indicate the standard deviation. n= 8 rats.

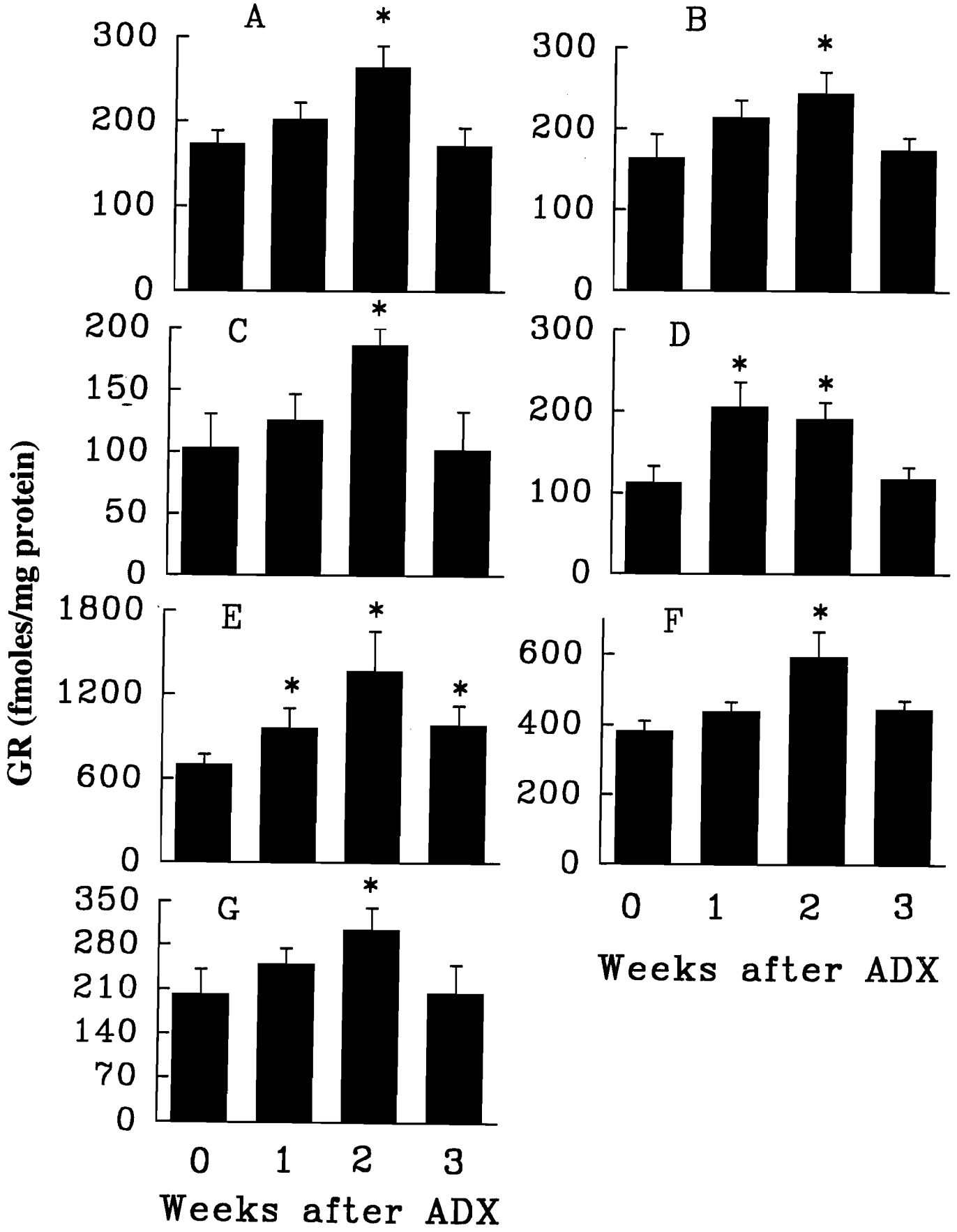

A
B
C
D
E
F
G
*
300
200
100
0
150
50
1800
1200
600
400
350
280
210
140
70
GR (fmoles/mg protein)
0 1 2 3
Weeks after ADX

Figure 15. Effect of Dex on GR levels in liver. Rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). GR levels were measured by RIA. Panel A and panel B show intact and ADX rats, respectively. Analysis of variance and subsequent Duncan's test for multiple comparisons were used to analyze the data. * indicates that the GR level is significantly different (at the 0.05 level) from the respective control. The error bars indicate the standard deviation. n=7 rats.

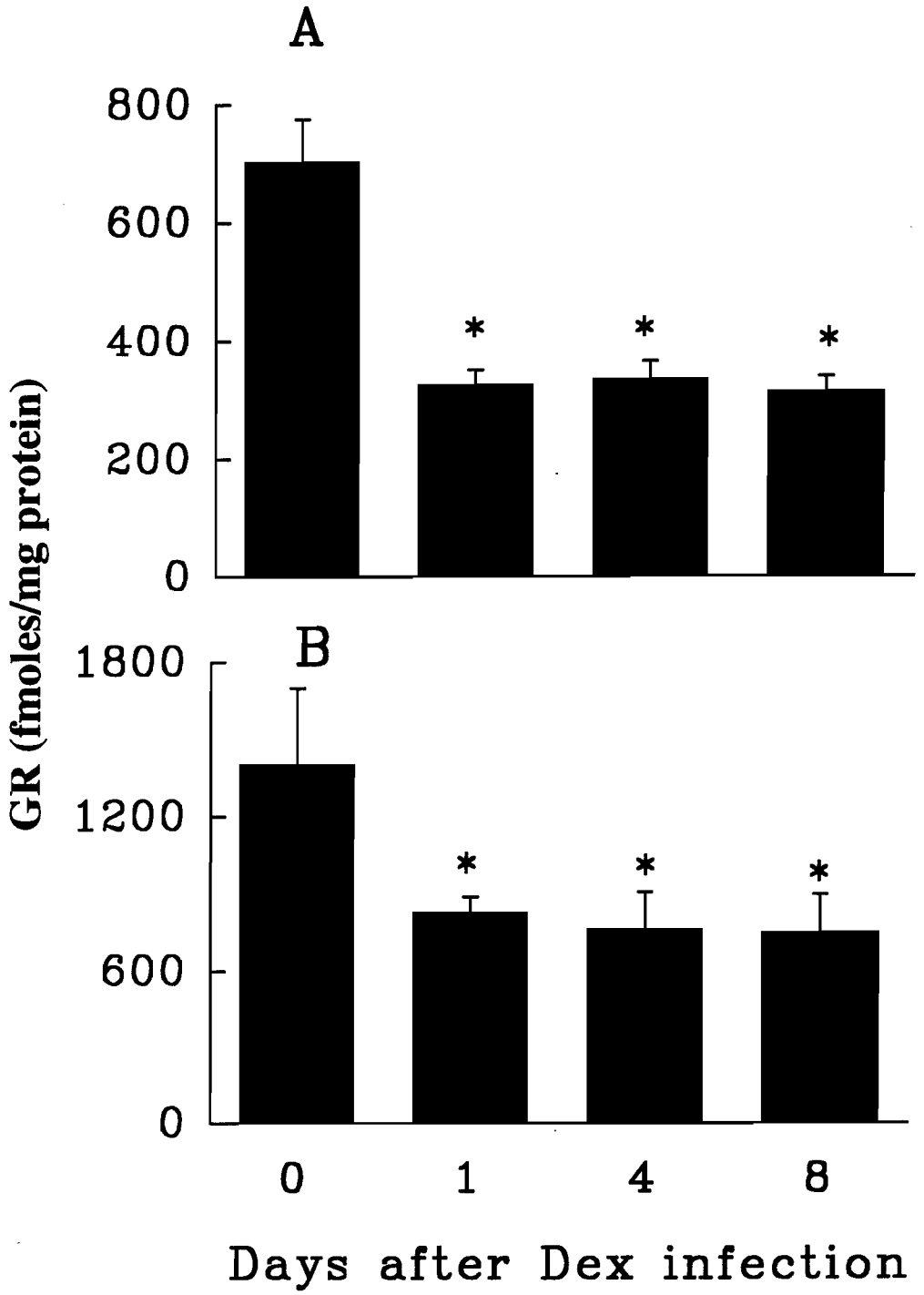

A
800
600
400
200
0
*
*
*
B
1800
1200
600
0
*
*
*
GR (fmoles/mg protein)
0
1
4
8
Days after Dex infection

Figure 16. Effect of Dex on GR levels in the cardiac ventricles. The rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). GR levels were measure by RIA. Panels A, B, C, and D show the GR level in the left ventricles of intact rats, the right ventricles of intact rats, the left ventricles of ADX rats, and the right ventricles of ADX rats, respectively. Analysis of variance and subsequent Duncan's test for multiple comparisons were used to analyze the data. * indicates that the GR level is significantly different (at the 0.05 level) from the respective control. The error bars indicate the standard deviation. n=7 rats.

Figure 17. Effect of Dex on GR levels in the anterior pituitary. Male Sprague-Dawley rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). GR levels were measured by RIA. Panel A and B show GR levels in intact and ADX rats, respectively. Analysis of variance and subsequent Duncan's test for multiple comparisons were used to analyze the data. * indicates that the GR level is significantly different (at the 0.05 level) from the respective control. The error bars indicate the standard deviation. n=7 rats.

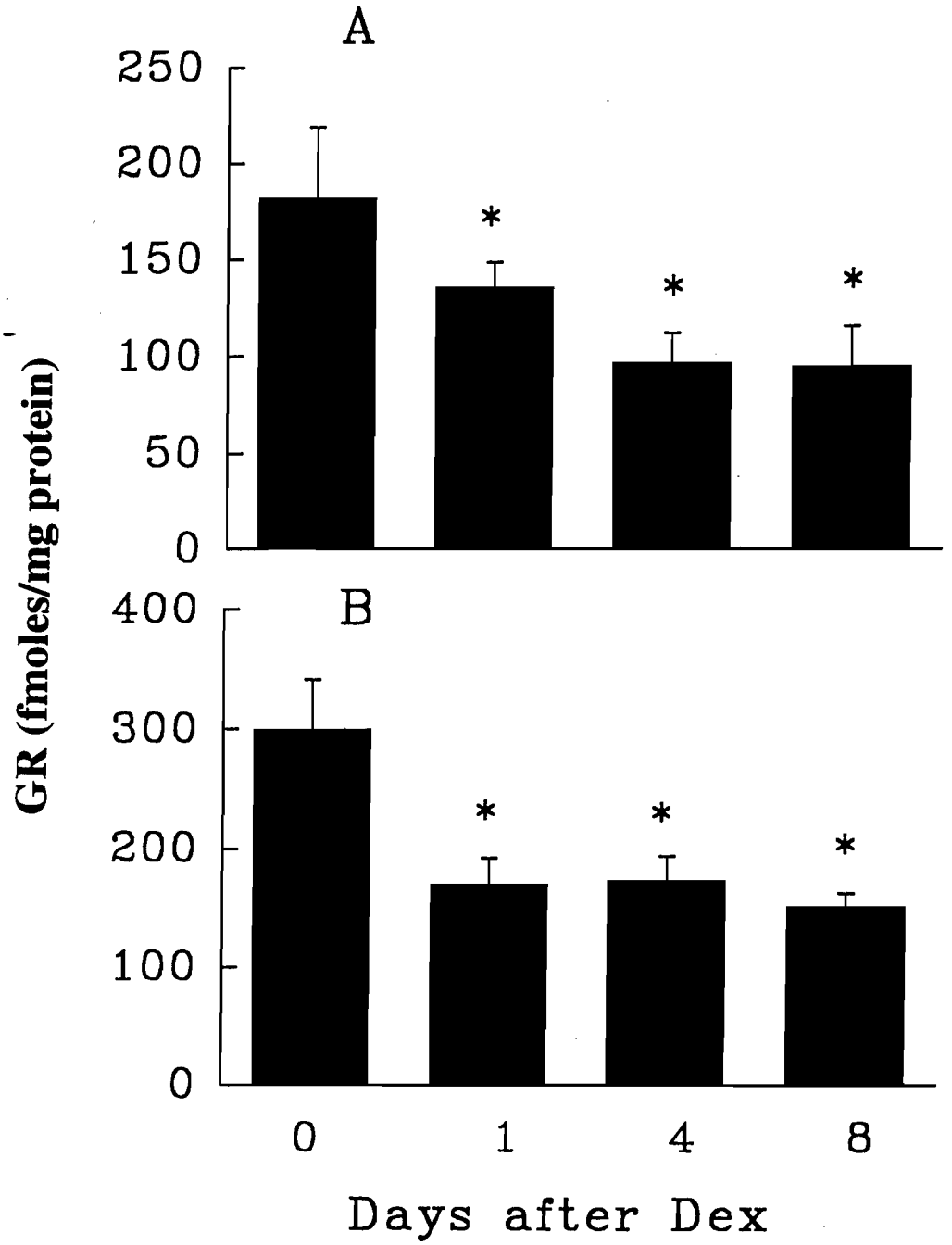

A
250
200
150
100
50
0
*
*
*
B
400
300
200
100
0
*
*
*
GR (fmoles/mg protein)
0
1
4
8
Days after Dex

Figure 18. Effect of Dex on GR levels in hypothalamus. Male Sprague-Dawley rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). GR levels were measured by RIA. Panel A and B show GR levels in intact and ADX rats, respectively. Analysis of variance and subsequent Duncan's test for multiple comparisons were used to analyze the data. * indicates that the GR level is significantly different (at the 0.05 level) from the respective control. The error bars indicate the standard deviation. n=7 rats.

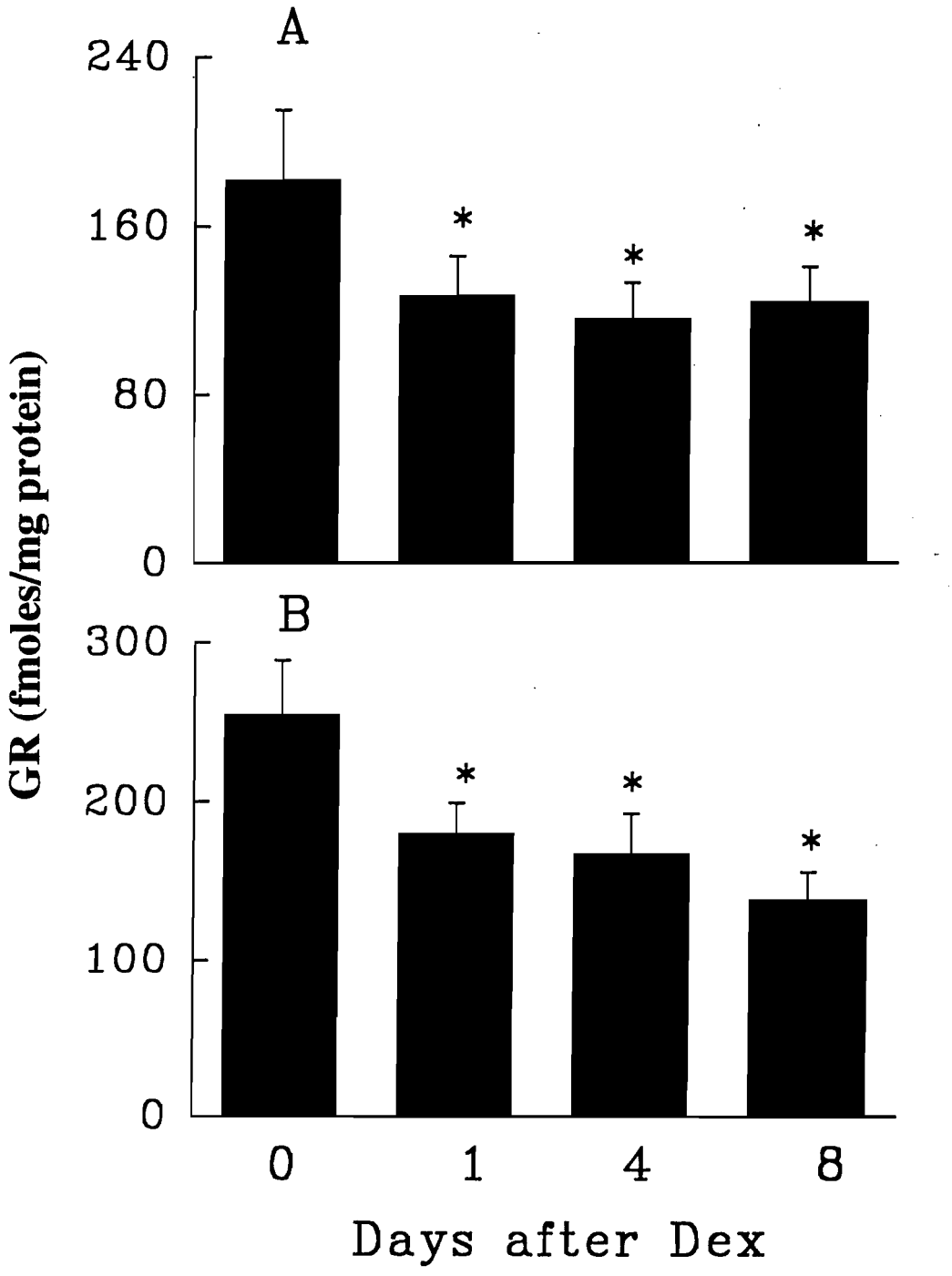

A
240
160
80
0
B
300
200
100
0
*
*
*
*
*
*
GR (fmoles/mg protein)
0
1
4
8
Days after Dex

Figure 19. Effect of Dex on GR levels in hippocampus. Male Sprague-Dawley rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). GR levels were measured by RIA. Panel A and B show GR levels in intact and ADX rats, respectively. Analysis of variance and subsequent Duncan's test for multiple comparisons were used to analyze the data. * indicates that the GR level is significantly different (at the 0.05 level) from the respective control. The error bars indicate the standard deviation. n=7 rats.

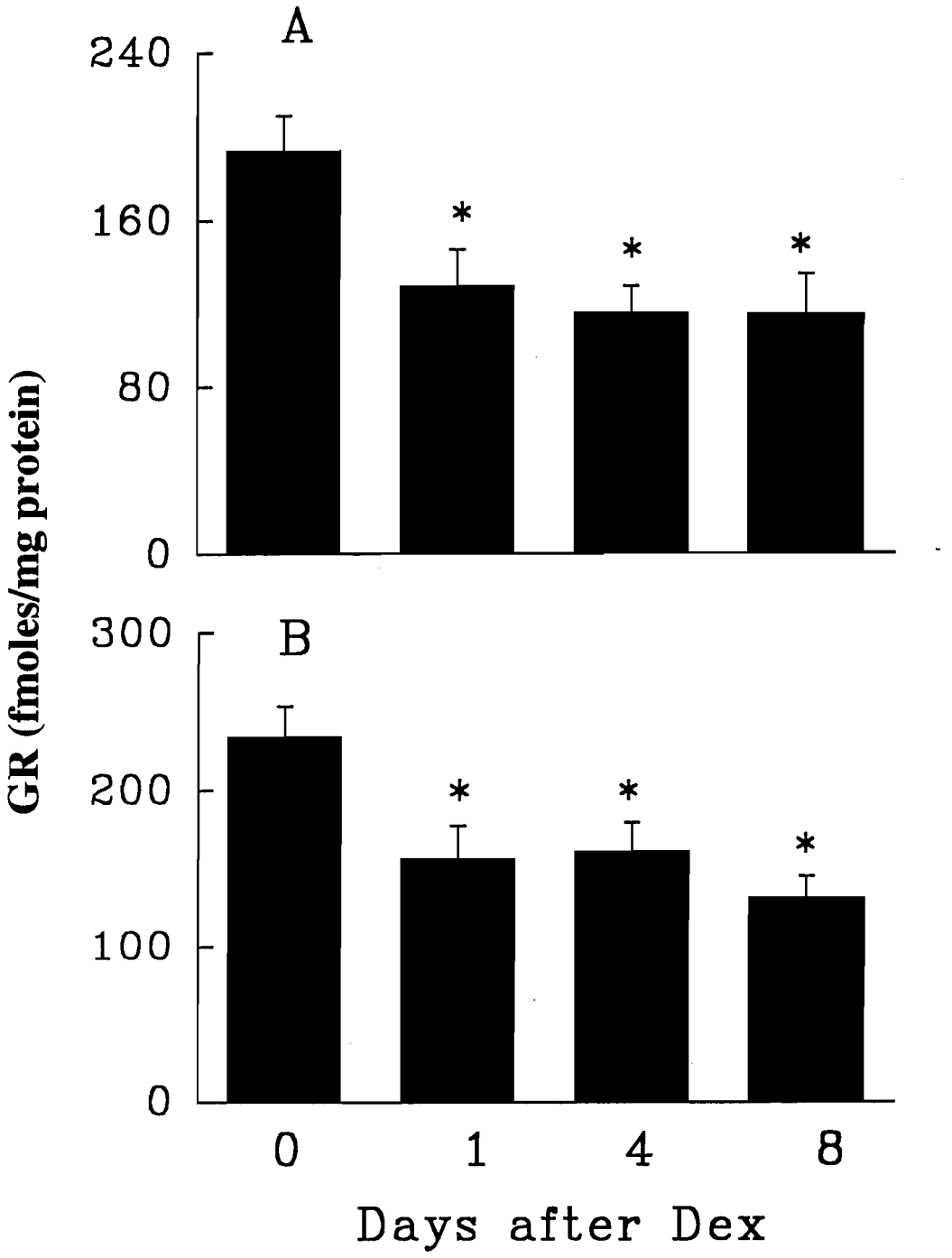

A
240
160
80
0
*
*
*
B
300
200
100
0
*
*
*
0
1
4
8
Days after Dex
GR (fmoles/mg protein)

Figure 20. Effect of Dex on GR levels in spleen. Male Sprague-Dawley rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). GR levels were measured by RIA. Panel A and B show GR levels in intact and ADX rats, respectively. Analysis of variance and subsequent Duncan's test for multiple comparisons were used to analyze the data. * indicates that the GR level is significantly different (at the 0.05 level) from the respective control. The error bars indicate the standard deviation. n=7 rats.

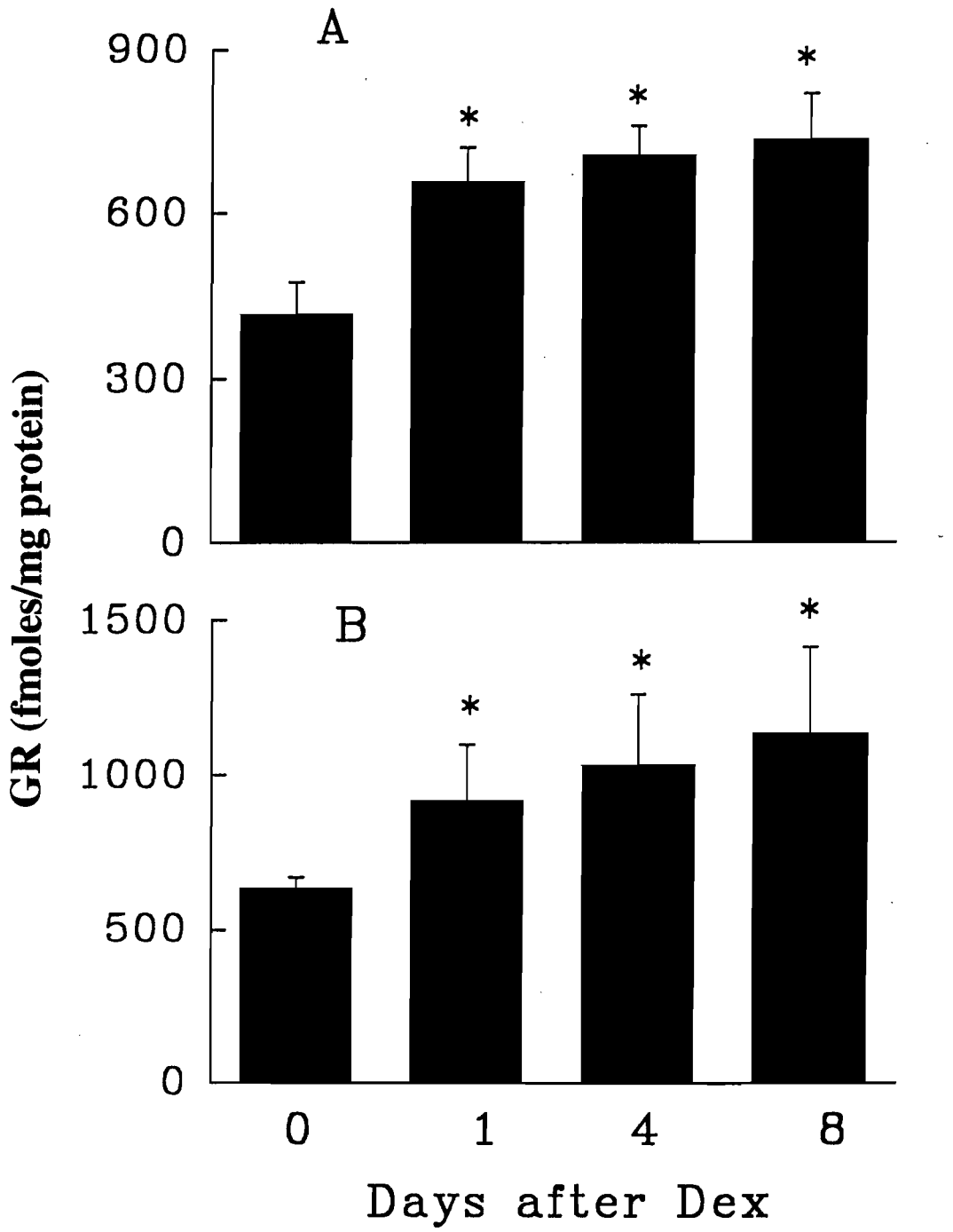

A
900
600
300
0
*
*
*
B
1500
1000
500
0
*
*
*
GR (fmoles/mg protein)
0
1
4
8
Days after Dex

Figure 21. Effect of Dex on GR levels in thymus. Male Sprague-Dawley rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). GR levels were measured by RIA. Panel A and B show GR levels in intact and ADX rats, respectively. Analysis of variance and subsequent Duncan's test for multiple comparisons were used to analyze the data. * indicates that the GR level is significantly different (at the 0.05 level) from the respective control. The error bars indicate the standard deviation. n=7 rats.

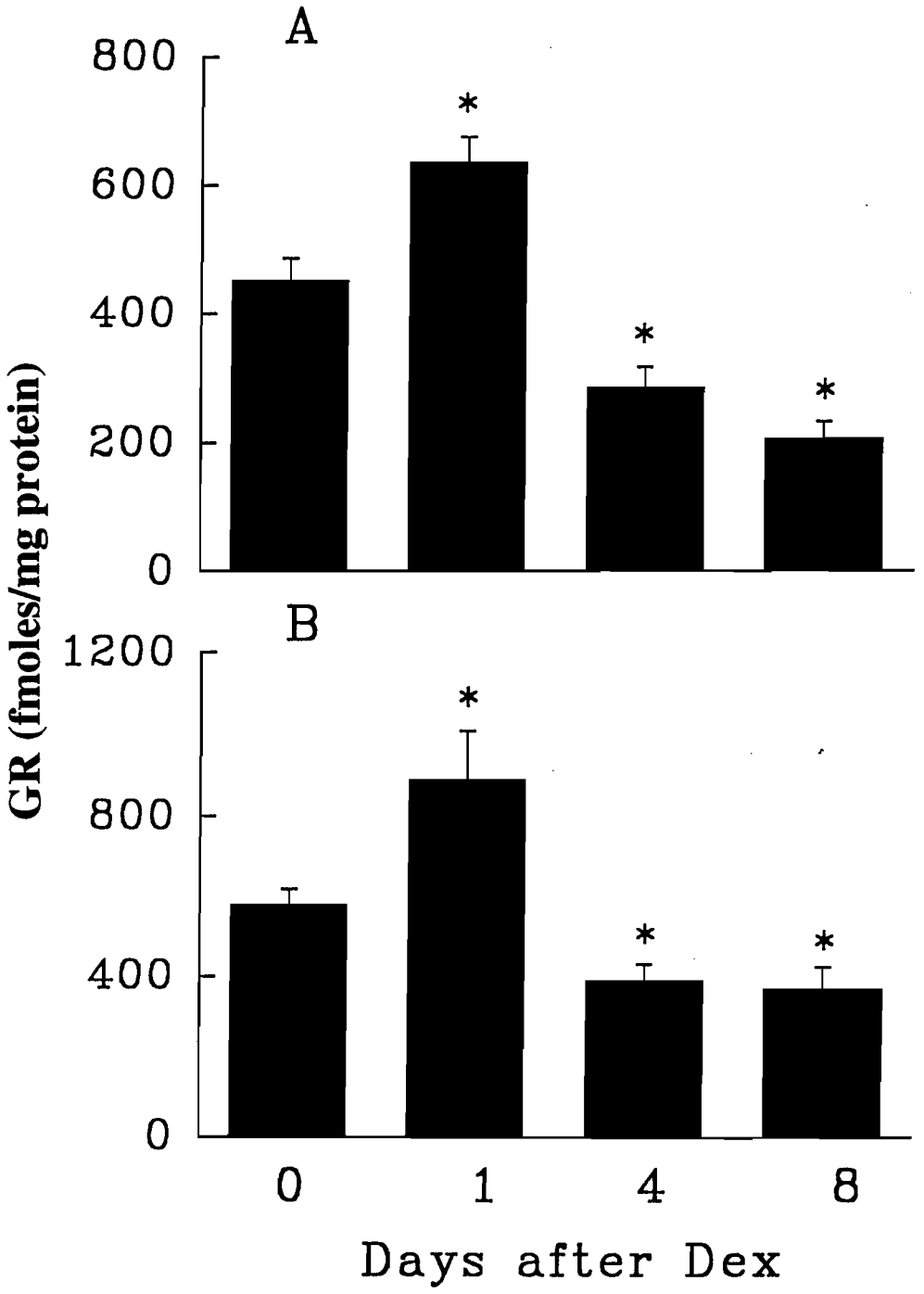

A
800
600
400
200
0
*
*
*
B
1200
800
400
0
*
*
*
GR (fmoles/mg protein)
0
1
4
8
Days after Dex

Figure 22. Effect of adrenalectomy or Dex treatment on thymus weight. Male Sprague-Dawley rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). Thymi were weighed immediately after the rats were sacrificed. Panel A and B show intact and ADX rats, respectively. Analysis of variance and subsequent Duncan's test for multiple comparisons were used to analyze the data. * indicates that the GR level is significantly different (at the 0.05 level) from the respective control. The error bars indicate the standard deviation. n=7 rats.

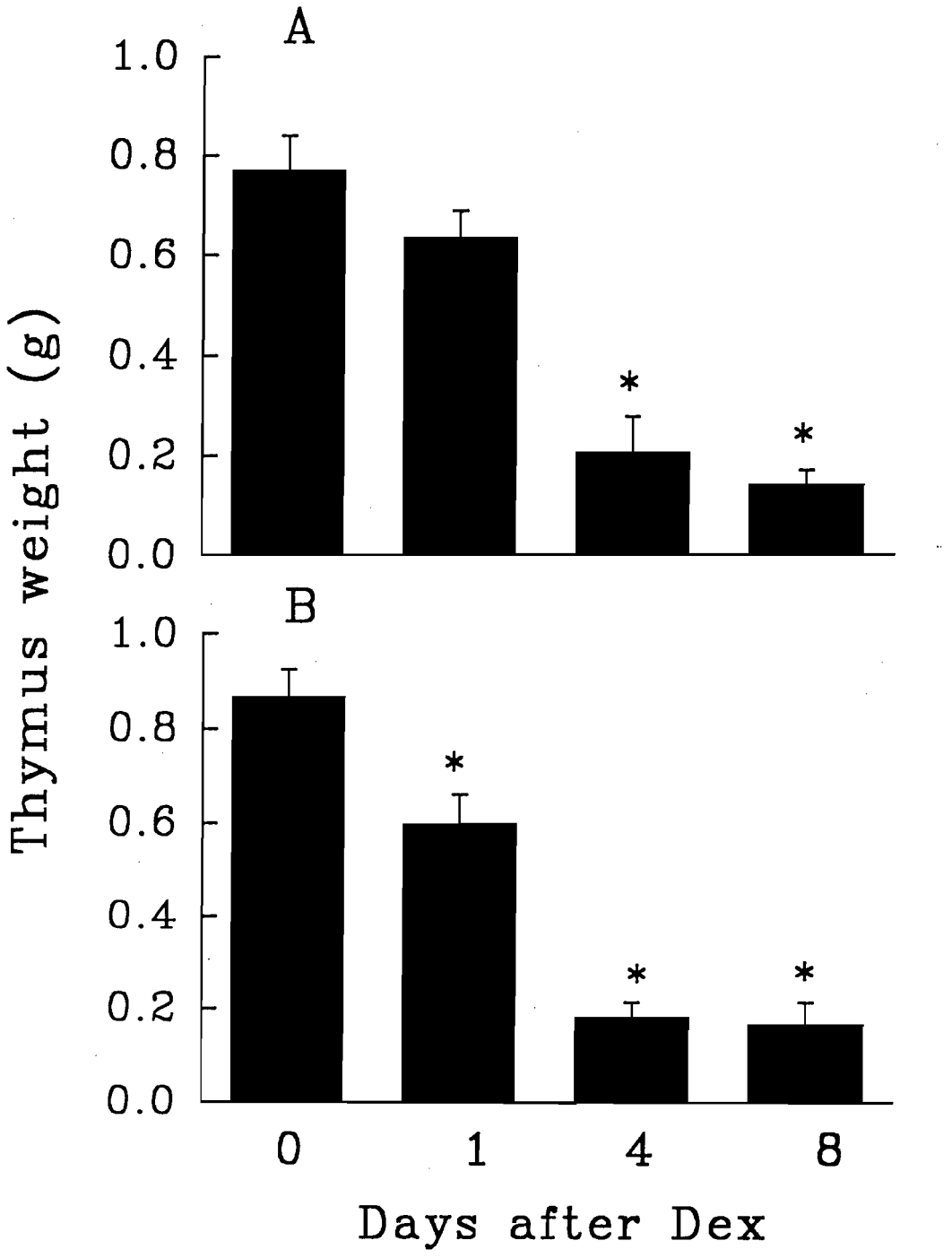

A
B
1.0
0.8
0.6
0.4
0.2
0.0
Thymus weight (g)
*
0
1
4
8
Days after Dex

Figure 23. Effect of Dex on GR levels in cardiac atria. Male Sprague-Dawley rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). GR levels were measured by RIA. Panels A, B, C and D show GR levels in the left atrium of the intact rats, the right atrium of the intact rats, the left atrium of the ADX rats and the right atrium of the ADX rats, respectively. Analysis of variance and subsequent Duncan's test for multiple comparisons were used to analyze the data. * indicates that the GR level is significantly different (at the 0.05 level) from the respective control. The error bars indicate the standard deviation. n=7 rats.

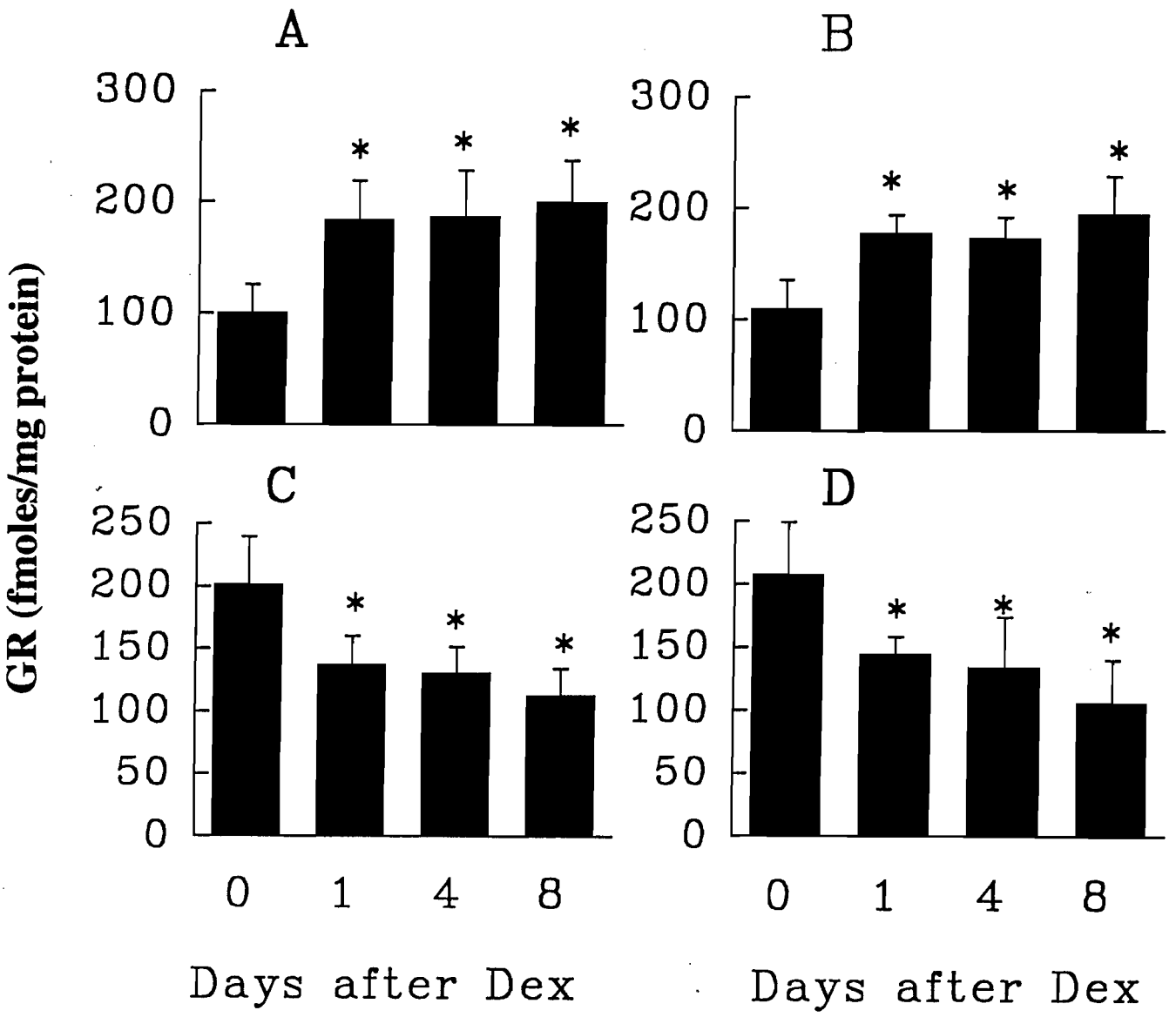

A
B
C
D
300
200
100
0
250
150
50
0
*
0
1
4
8
GR (fmoles/mg protein)
Days after Dex
Days after Dex

Figure 24. Effect of adrenalectomy on GR levels in the "cytosols" and the "particulate extracts" of liver and spleen. Male Sprague-Dawley rats were either unoperated (control) or adrenalectomized for 2 weeks (ADX). GR levels were measured by RIA in "Cytosols" and "particulate extracts". Panels A, B, C, and D show the GR level in the "cytosols" of liver, the "cytosols" of spleen, the "particulate extracts" of liver, and the "particulate extracts" of spleen, respectively. The error bars indicate the standard deviation. n=7 rats.

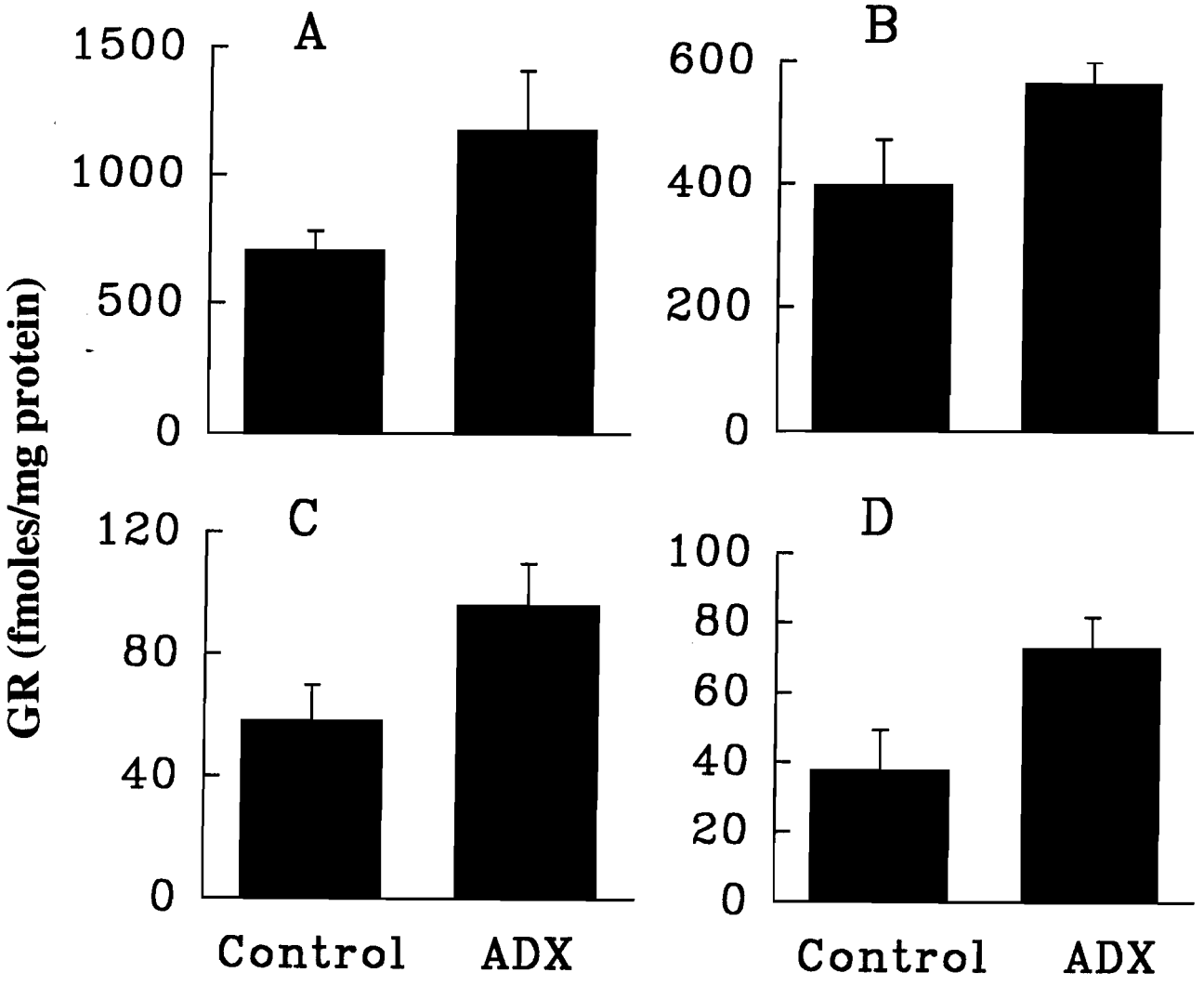

A
B
C
D
1500
1000
500
0
600
400
200
0
120
80
40
0
100
80
60
40
20
0
GR (fmoles/mg protein)
Control
ADX
Control
ADX

Figure 25. Effect of Dex on GR levels in the "cytosols" and the "particulate extracts" of liver. Male Sprague-Dawley rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). GR levels were measured in the "cytosols" and the "particulate extracts" of liver by RIA. Panels A, B, C, and D show the GR level in the "cytosols" of intact rats, the "cytosols" of ADX rats, the "particulate extracts" of intact rats, and the "particulate extracts" of ADX rats, respectively. The error bars indicate the standard deviation. n=7 rats.

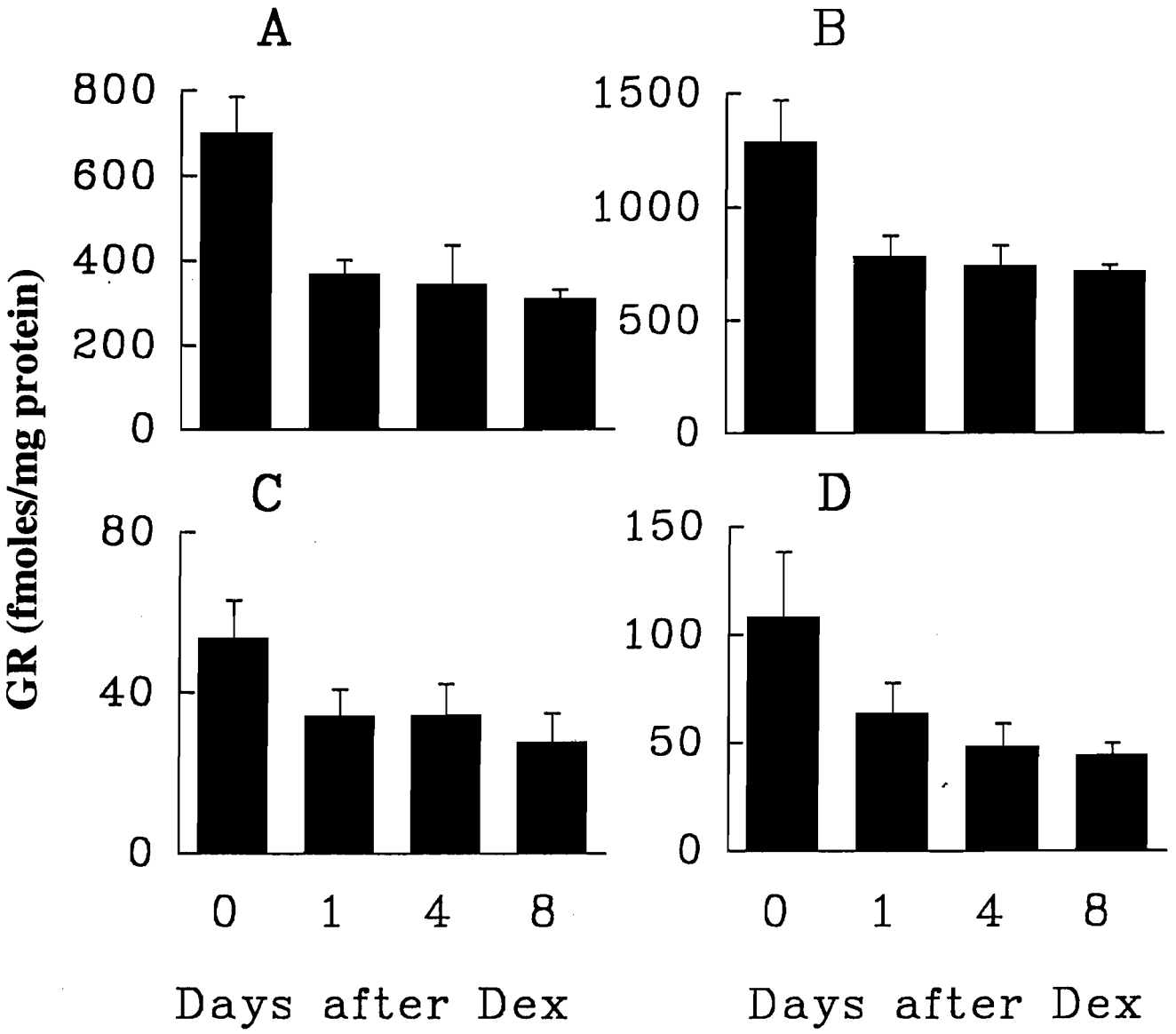

A
B
C
D
800
600
400
200
0
1500
1000
500
0
80
40
0
150
100
50
0
0 1 4 8
0 1 4 8
GR (fmoles/mg protein)
Days after Dex
Days after Dex

Figure 26. Effect of Dex on GR levels in the "cytosols" and the "particulate extracts" of spleen. Male Sprague-Dawley rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). GR levels were measured in the "cytosols" and the "particulate extracts" of liver by RIA. Panel A, B, C, and D show the GR level in the "cytosols" of intact rats, the "cytosols" of ADX rats, the "particulate extracts" of intact rats, and the "particulate extracts" of ADX rats, respectively. The error bars indicate the standard deviation. n=7 rats.

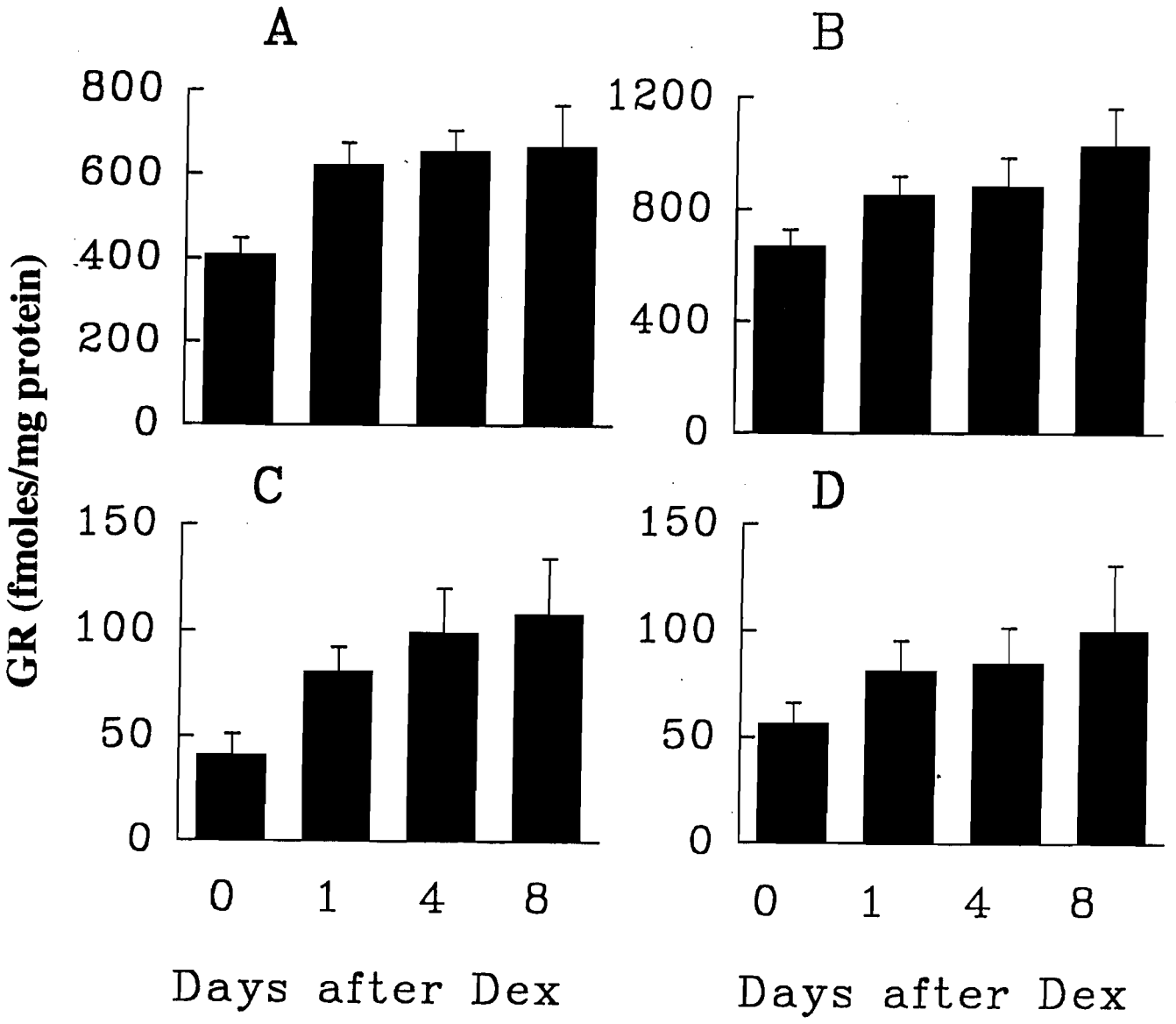

A
B
C
D
800
600
400
200
0
1200
800
400
0
150
100
50
0
0 1 4 8
0 1 4 8
GR (fmoles/mg protein)
Days after Dex
Days after Dex

Figure 27. A standard curve of ANF RIA . A synthetic ANF-(99-126) was labelled with ^{125}I (Ong et al., 1986). Increasing concentrations of the unlabelled ANF were incubated with a fixed amount of labelled ANF and the antibodies (a rabbit antibody against rat ANF and a goat anti-rabbit antibody). A precipitate was formed in the antibody and antigen reaction. The antibody bound (B, in the precipitate) and the free (in the supernatant) ^{125}I-ANF were separated from each other by centrifugation. The radioactivity in the precipitate was counted. The ratio of the bound radioactivity to the total (T, the total radioactivity added to the tube) radioactivity versus the concentration (log dose) of the unlabelled ANF was plotted. The ANF concentrations in the unknown samples were calculated from the linear portion of the standard curve.

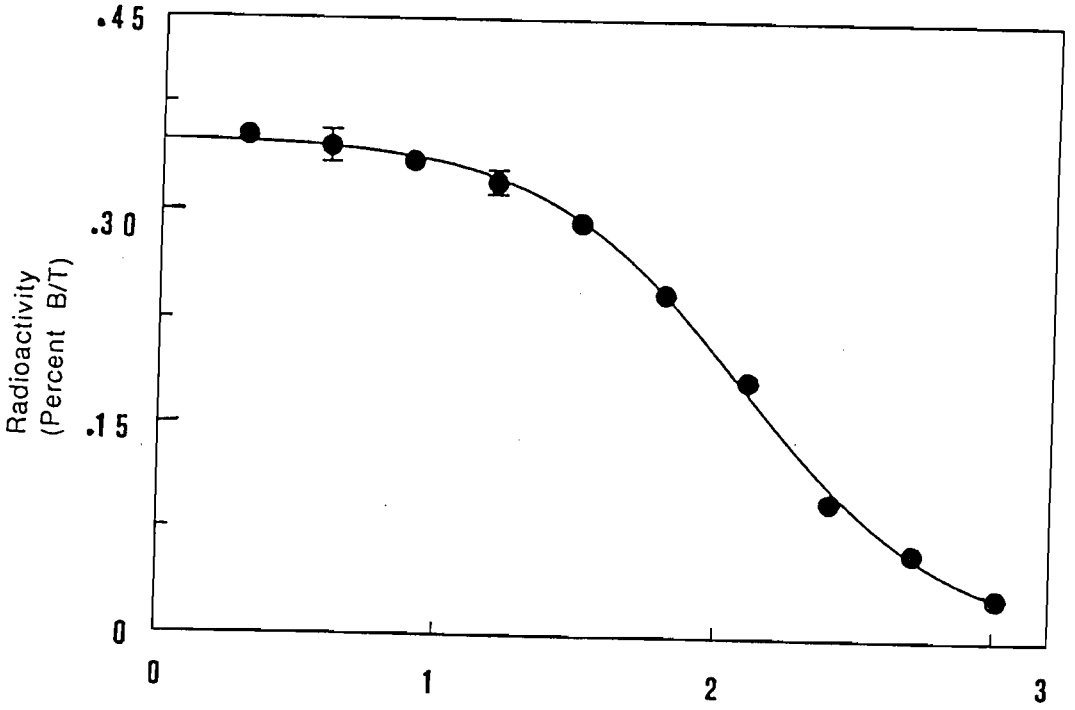
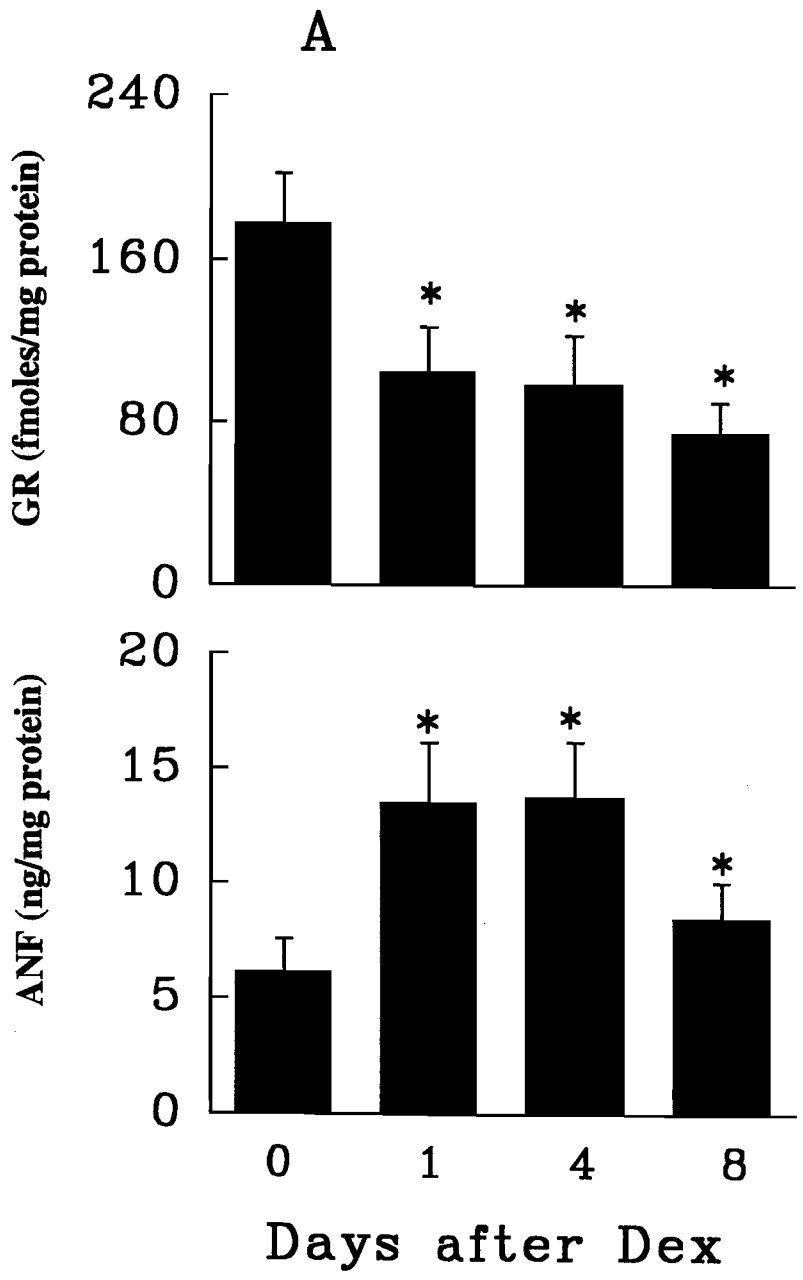

.45
.30
.15
0
Radioactivity
(Percent B/T)
0
1
2
3
[ANF] pg/tube
(Log dose)

Figure 28. GR levels and ANF responses to Dex in the left cardiac ventricle. Intact male Sprague-Dawley rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). The GR and ANF levels were measured, by RIA, in the same "cytosol" preparations. Panel A shows the GR level (top) and ANF level (bottom). Panel B shows the relationship between the GR levels and the ANF responses to Dex treatment. * indicates that the levels were significantly different (at the 0.05 level by analysis of variance and subsequent Duncan's test for multiple comparisons) from the respective controls. The error bars indicate standard deviation. r indicates correlation coefficient. n=7.


A
240
160
80
0
GR (fmoles/mg protein)
*
*
*
20
15
10
5
0
ANF (ng/mg protein)
*
*
*
0
1
4
8
Days after Dex

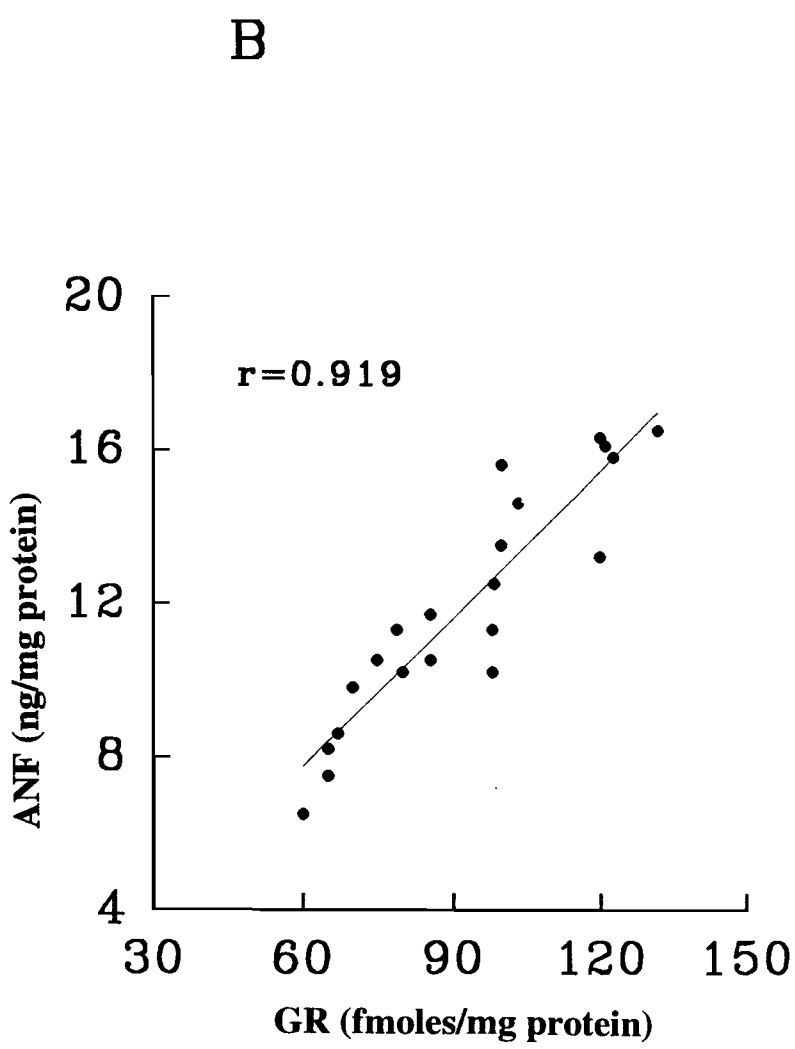

B
20
16
12
8
4
ANF (ng/mg protein)
r=0.919
30
60
90
120
150
GR (fmoles/mg protein)

Figure 29. GR levels and ANF responses to Dex in the right cardiac ventricle. Intact male Sprague-Dawley rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). The GR and ANF levels were measured, by RIA, in the same "cytosol" preparations. Panel A shows the GR level (top) and ANF level (bottom). Panel B shows the relationship between the GR levels and the ANF responses to Dex treatment. * indicates that the levels were significantly different (at the 0.05 level by analysis of variance and subsequent Duncan's test for multiple comparisons) from the respective controls. The error bars indicate standard deviation. r indicates correlation coefficient. n=7.

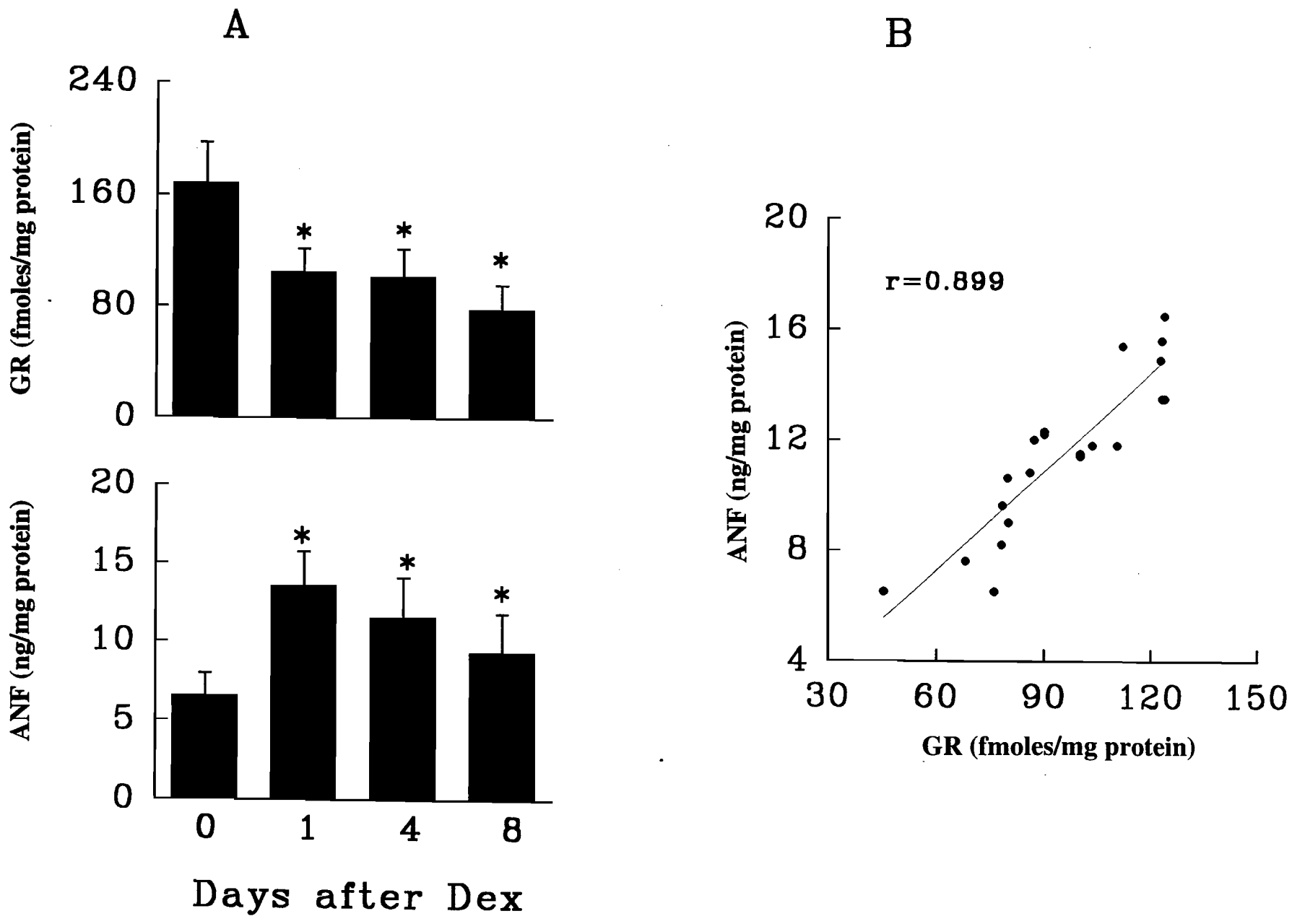

A
240
160
80
0
GR (fmoles/mg protein)
*
*
*
20
15
10
5
0
ANF (ng/mg protein)
*
*
*
0
1
4
8
Days after Dex
B
20
16
12
8
4
ANF (ng/mg protein)
r=0.899
30
60
90
120
150
GR (fmoles/mg protein)

Figure 30. GR levels and ANF responses to Dex in the left cardiac atrium. Intact male Sprague-Dawley rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). The GR and ANF levels were measured, by RIA, in the same "cytosol" preparations. Panel A shows the GR level (top) and ANF level (bottom). Panel B shows the relationship between the GR levels and the ANF responses to Dex treatment. * indicates that the levels were significantly different (at the 0.05 level by analysis of variance and subsequent Duncan's test for multiple comparisons) from the respective controls. The error bars indicate standard deviation. r indicates correlation coefficient. n=7.

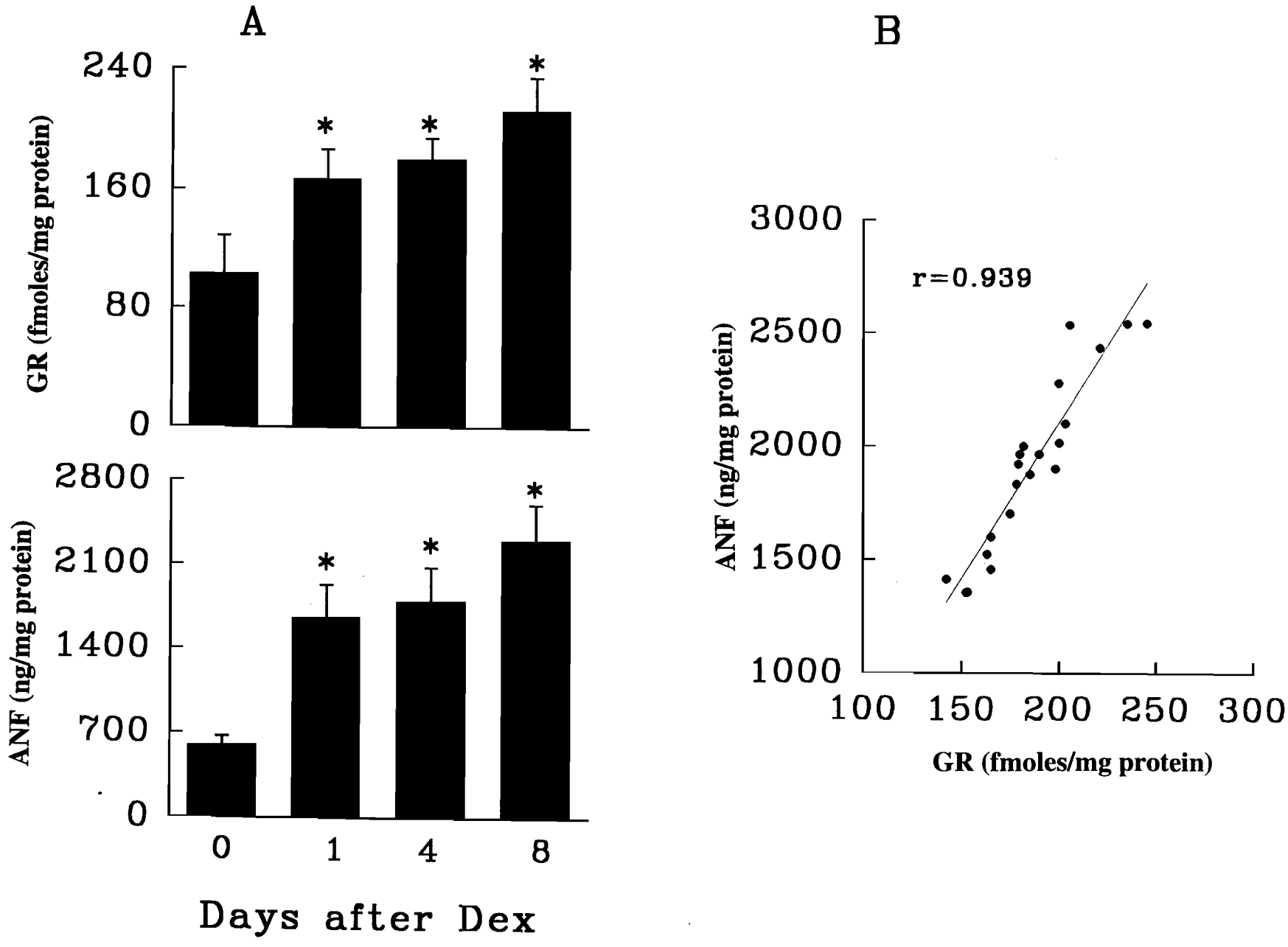

A
240
160
80
0
GR (fmoles/mg protein)
2800
2100
1400
700
0
ANF (ng/mg protein)
0
1
4
8
Days after Dex
B
3000
2500
2000
1500
1000
r=0.939
ANF (ng/mg protein)
100
150
200
250
300
GR (fmoles/mg protein)

Figure 31. GR levels and ANF responses to Dex in the right cardiac atrium. Intact male Sprague-Dawley rats were either uninjected (0 day as controls) or injected with Dex for 1, 4 and 8 day(s). The GR and ANF levels were measured, by RIA, in the same "cytosol" preparations. Panel A shows the GR level (top) and ANF level (bottom). Panel B shows the relationship between the GR levels and the ANF responses to Dex treatment. * indicates that the levels were significantly different (at the 0.05 level by analysis of variance and subsequent Duncan's test for multiple comparisons) from the respective controls. The error bars indicate standard deviation. r indicates correlation coefficient. n=7.

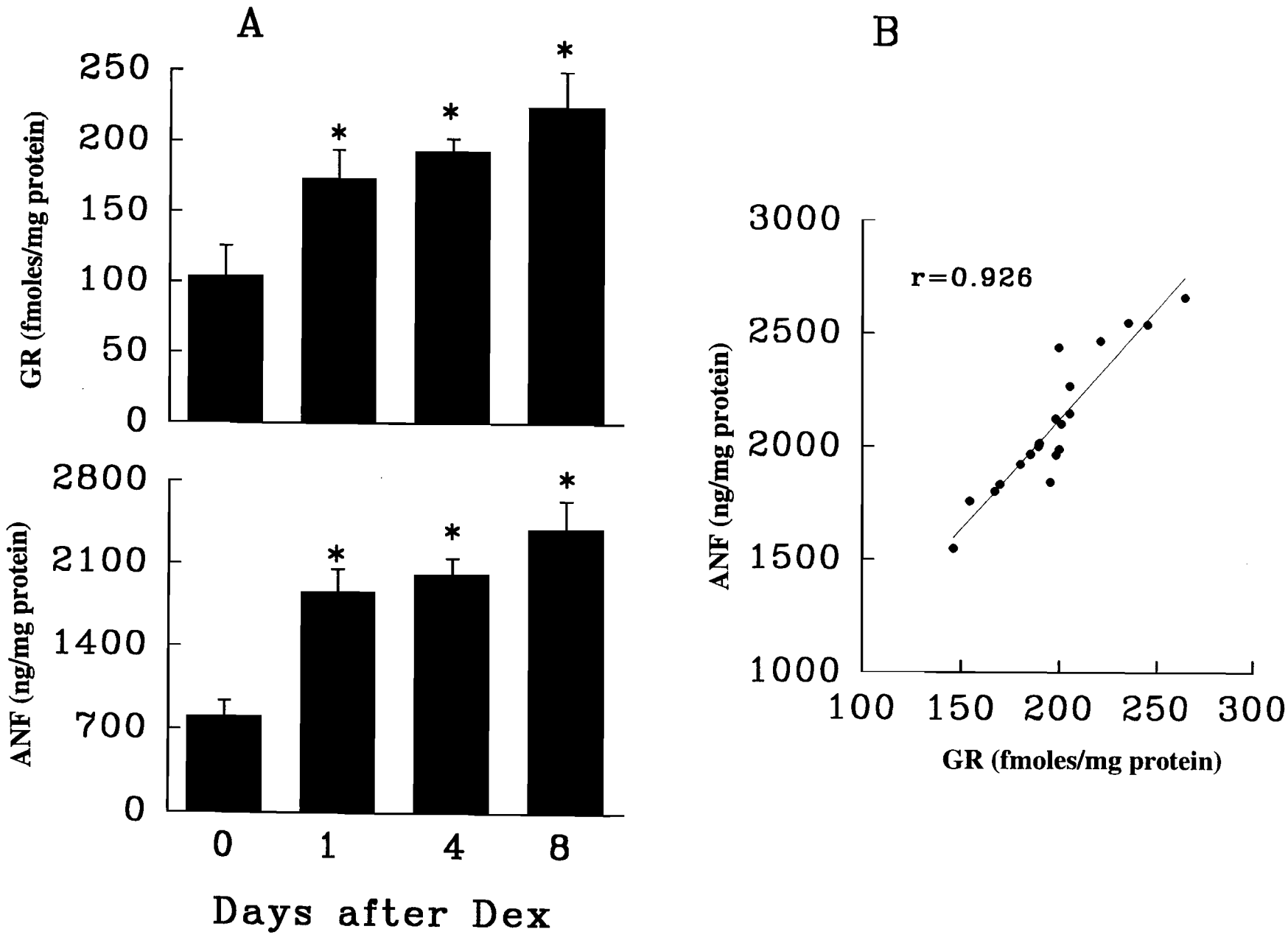

A
GR (fmoles/mg protein)
250
200
150
100
50
0
ANF (ng/mg protein)
2800
2100
1400
700
0
0
1
4
8
Days after Dex
B
ANF (ng/mg protein)
3000
2500
2000
1500
1000
r=0.926
100
150
200
250
300
GR (fmoles/mg protein)

Figure 32. Immunocytochemical localization of GR and ANF in cardiac tissue. Atrial sections from adrenalectomized rats (2 weeks post-ADX) were shown. The section shown in panel A was first stained with BuGR1 (a mouse monoclonal antibody against purified rat liver GR) and the antigen-antibody reactions were visualized by the immunoperoxidase method. The same section was washed and re-incubated with ANF antibody (made in rabbit), and this antigen-antibody reaction was revealed by the immunofluorescence method (panel C). Note that cardiac muscle cells (cardiac) were stained for both GR (panel A) and ANF (panel C), and the same cells contained GR and ANF (curved thick arrows). GR was localized predorminantly in the cytoplasm and, to a lesser extent, in the nucleus of the cardiac cells. ANF was localized in the cytoplasm of the cardiac cells. The straight thin arrows indicates a mesothelian cell. Panel B shows another atrial section stained with $14B_2$ and the antibody-antigen reaction was revealed by the immunoperoxidase procedure. Note that similar staining was observed in panel A and panel B. Panel D shows a control experiment in which the atrial section was incubated with non-immune rabbit serum to substitute the primary antibody and the section was processed for subsequent immunofluorescence staining. Only background staining was observed in the section shown in panel D. (Bar = 10 μm).

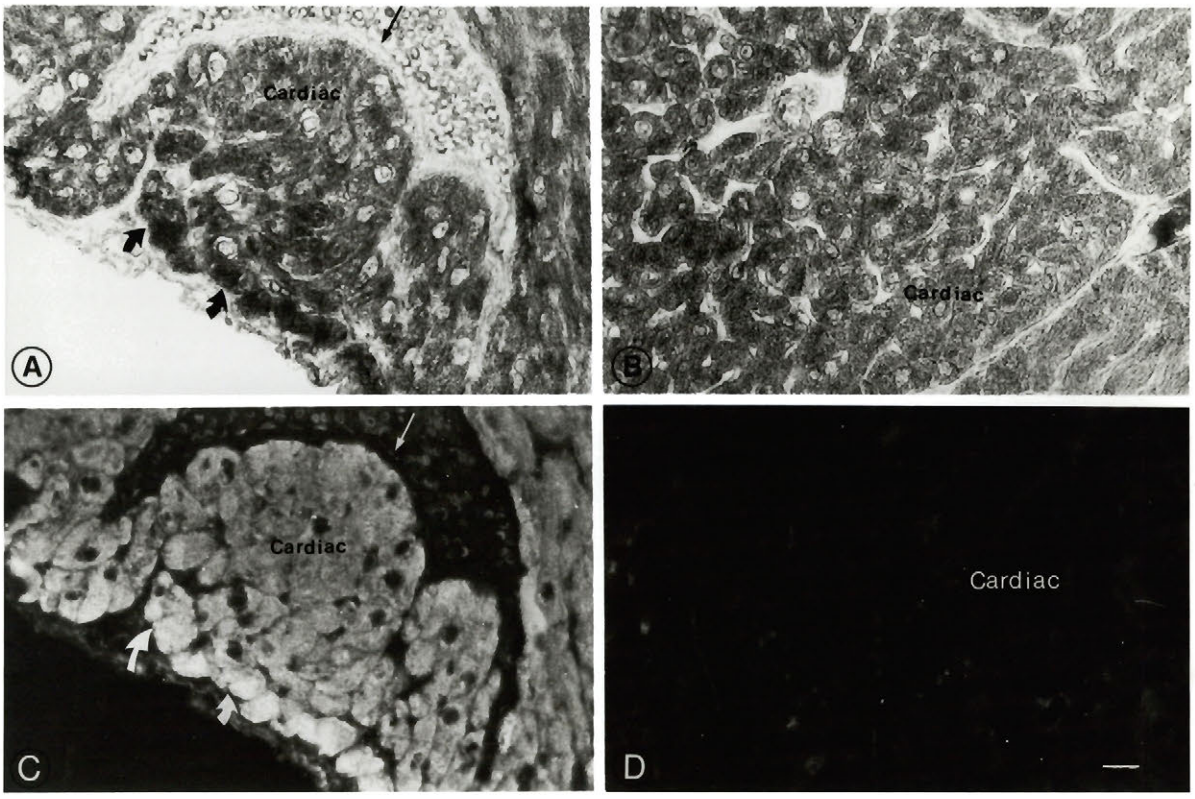

Cardiac
A
Cardiac
B
Cardiac
C
Cardiac
D

Figure 33. Immunocytochemical localization of GR and EGF in the submaxillary gland. The sections shown are from intact rats. The sections shown in panel A and panel B are two consecutive sections from the paraffin embedded submaxillary gland block. The section in panel A was stained with $14B_2$ and the section in panel B was stained with EGF antibody by the immunoperoxidase procedure. The GCT cells (G) but not the acinar cells (A) immunoreacted with both antibodies. The same GCT cells contained both GR and EGF (arrows). The GR was localized predominantly to the cytoplasm and to a lesser extent to the nucleus of the GCT cells; EGF was localized to the cytoplasm of the GCT cells. Panel C shows a control experiment in which the submaxillary gland section was incubated with $14B_2$ previously adsorbed with purified rat liver GR; only a background staining was observed. (Bar = 10 μm).

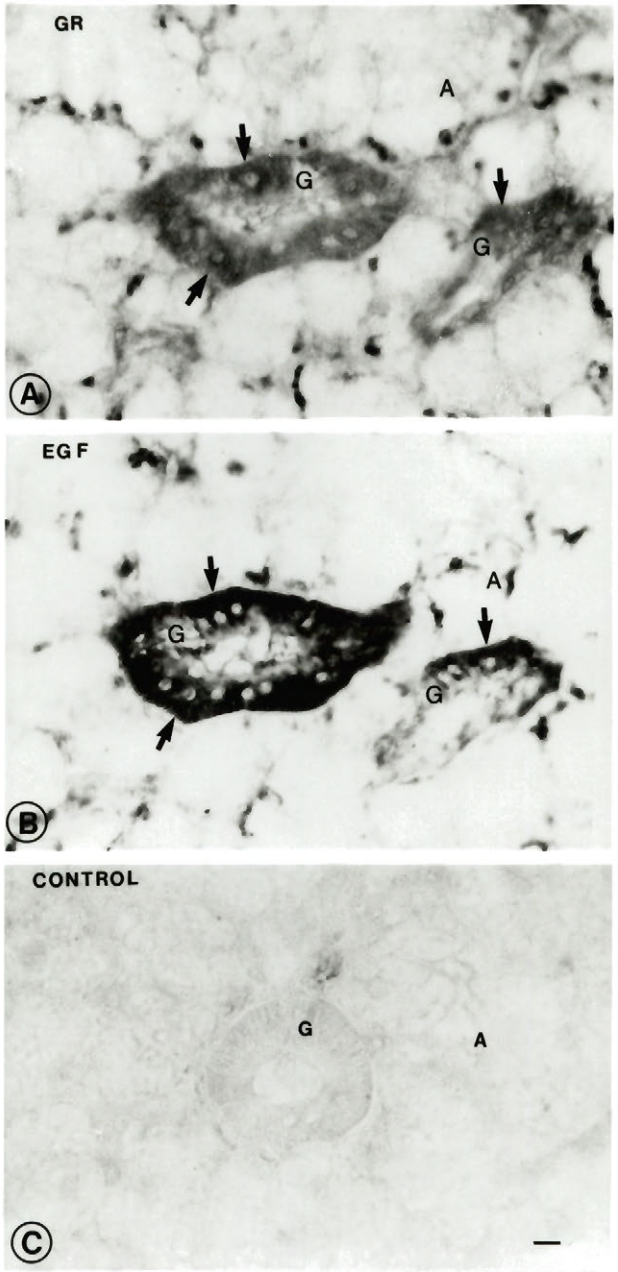

GR
A
G
G
A
EGF
A
G
G
B
CONTROL
G
A
C

Figure 34. Immunocytochemical localization of GR and α 2u globulin in the submaxillary gland. The sections shown are from intact rats. The sections shown in panel A and panel B are two consecutive sections from a paraffin embedded submaxillary gland block. The section shown in panel A was processed for GR localization using BuGR1 and the section shown in panel B was processed for the localization of α 2u globulin using an antibody to α 2u globulin by the immunoperoxidase procedure. GCT cells (G) reacted with both antibodies. However, acinar cells (A) were devoid of staining. The same GCT cells contained both GR and α 2u globulin (arrows). The GR was localized predominantly to the cytoplasm and to a lesser extent to the nucleus of the GCT cells; and α 2u globulin was localized to the cytoplasm of the GCT cells. Panel C shows a control experiment in which the primary antibody was substituted with normal rabbit serum; only background staining was observed. (Bar=10 μm).

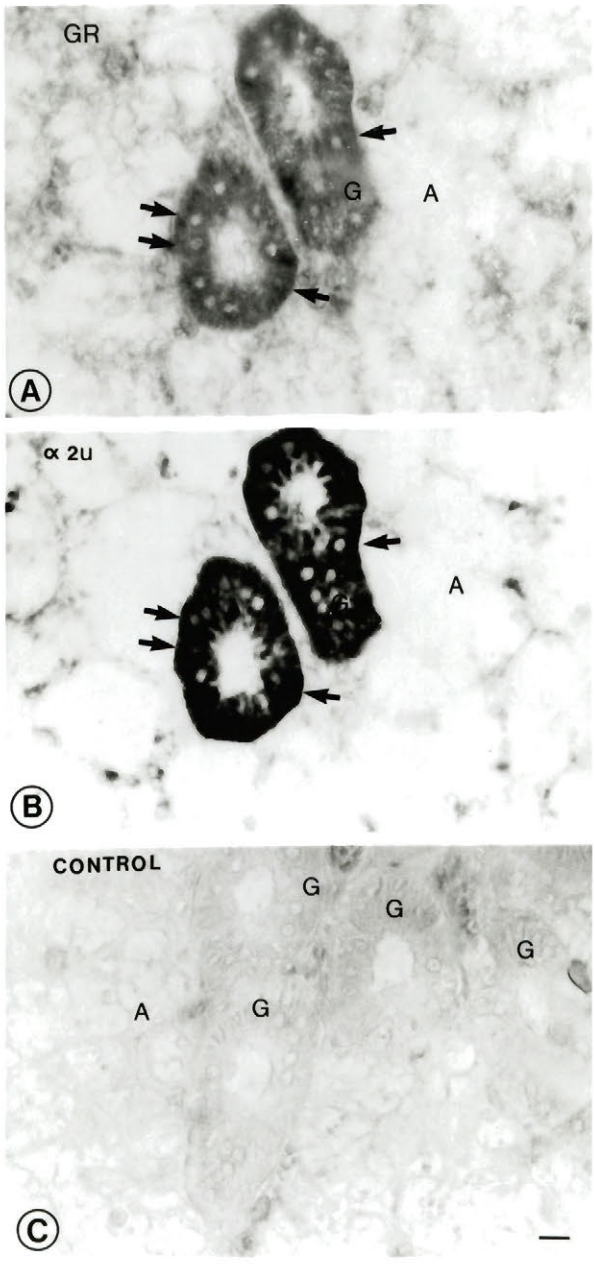

GR
G
A
A
α 2u
A
B
CONTROL
G
G
G
A
G
C

Figure 35. Immunocytochemical localization of GR and ANF in the submaxillary gland. The sections shown are from intact rats. Panel A shows a section processed for GR localization using BuGR1 (a mouse antibody to GR) by the immunoperoxidase procedure. The same section was thoroughly washed with PBS and subsequently processed for ANF localization using a rabbit antibody to ANF by the immunofluorescence procedure (panel B). The GCT cells (G) but not the acinar cells (A) reacted with both GR and ANF antibodies. The same GCT cells contained GR and ANF (arrows). The GR was localized predominantly to the cytoplasm and to a lesser extent to the nucleus of the GCT cells; ANF was localized to the cytoplasm of the GCT cells. Panel C shows a control experiment in which the primary antibody was substituted with normal rabbit serum; only background staining was observed. (Bar = 10 μm).

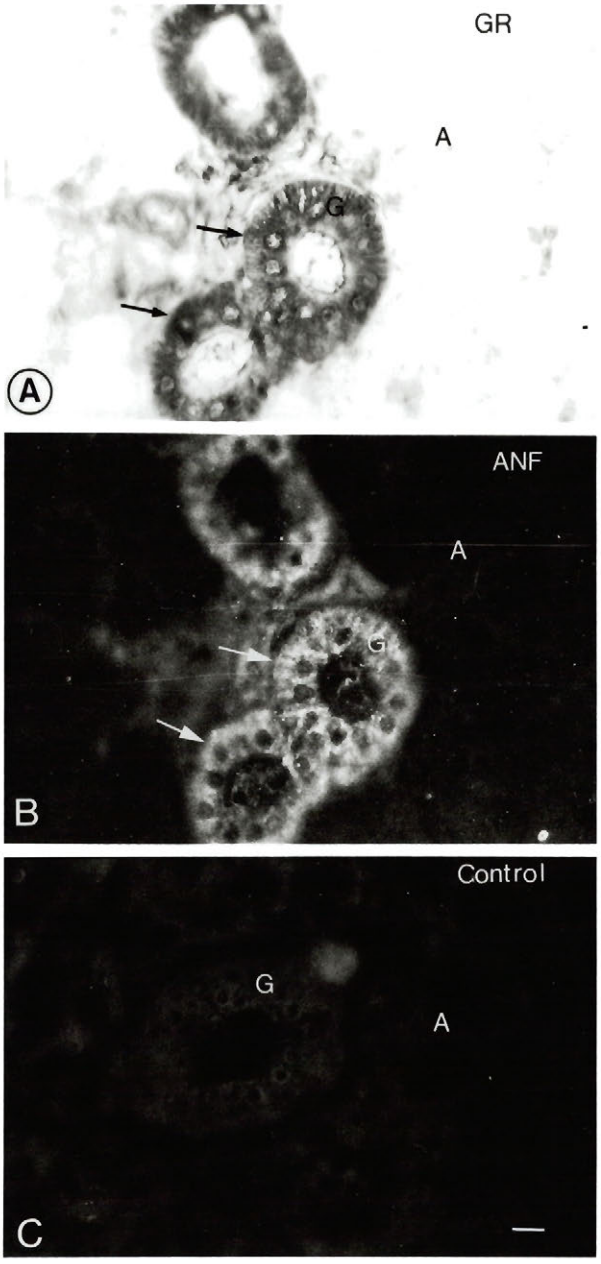

GR
A
G
A
ANF
A
G
B
Control
G
A
C

Figure 36. Immunocytochemical localization of GR and ACTH in the testis. The sections shown are from intact rats. The section shown in panel A was first processed for GR localization using BuGR1 (a mouse antibody against GR) by the immunoperoxidase procedure. The same section was then washed thoroughly with PBS and subsequently processed for the localization of ACTH using an rabbit antibody against ACTH by immunofluorescence procedure (result shown in panel B). Leydig cells (L) were labelled with both antibodies. The seminiferous tubular cells (T) were not significantly labelled with either of the antibodies. In Leydig cells, GR was localized largely to the cytoplasm and to a lesser extent to the nucleus. ACTH was observed in the cytoplasm of the Leydig cells. The same Leydig cells contained both GR and ACTH (arrows). Panel C shows a control experiment in which the primary antibody was substituted with non-immunized cell culture medium; only background staining was seen. V indicates blood vessel. (Bar=5 μm).

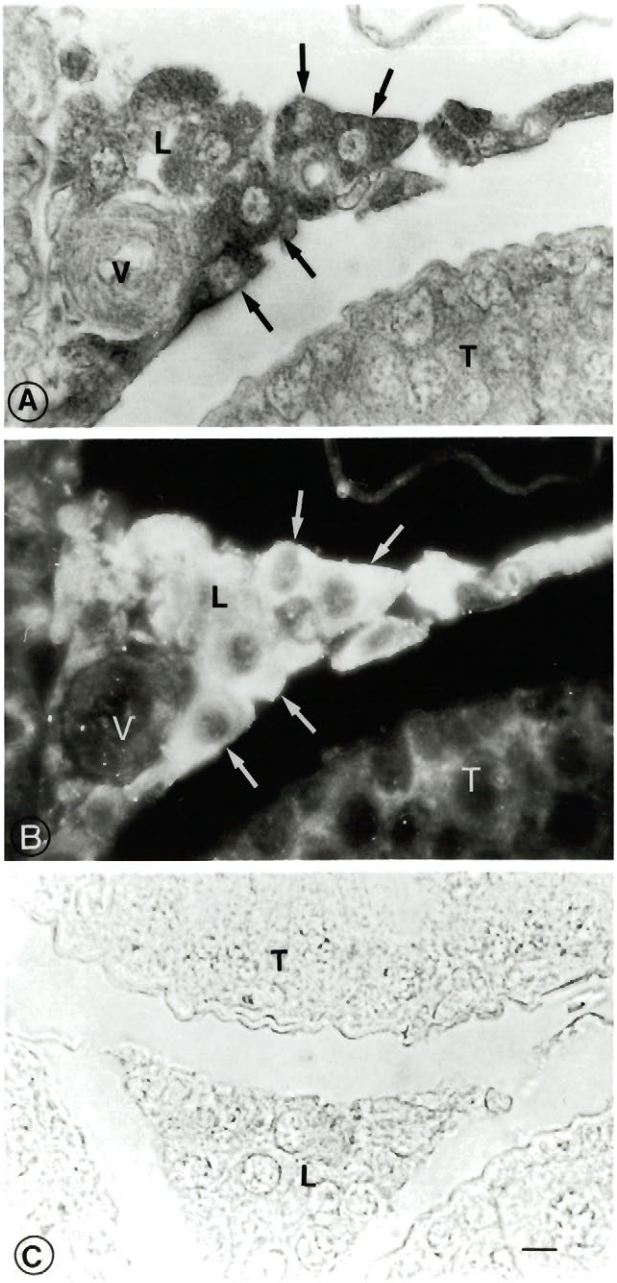
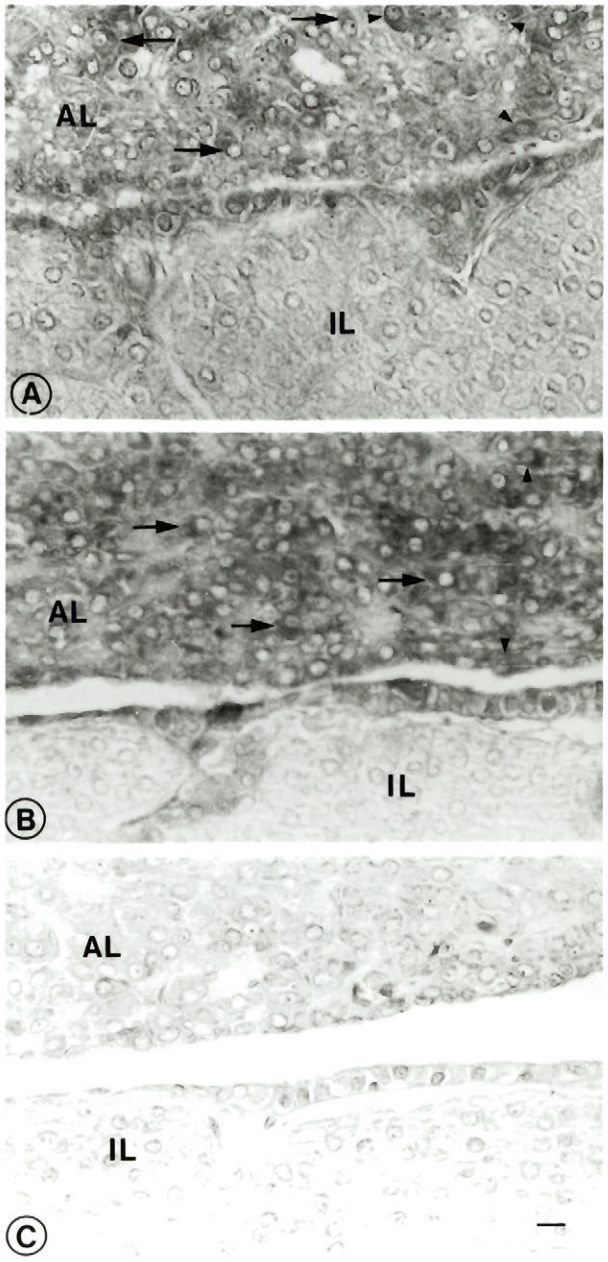

L
V
T
A
L
V
T
B
T
L
C

Figure 37. Immunocytochemical localization of GR in the pituitary gland. Panel A shows a section stained with BuGR1 and panel B shows a section stained with $14B_2$. Note that the staining patterns in panel A and panel B are similar. The anterior lobe (AL) but not the intermediate lobe (IL) of the pituitary was stained. In the anterior lobe, approximately 70% of the cells were labelled; these cells were stained with their cytoplasm (arrows) or both the cytoplasm and nucleus (arrow heads). Panel C shows a control experiment in which the primary antibody was substituted with normal rabbit serum; only background labelling was observed. (Bar = 10 μm).


AL
IL
A
AL
IL
B
AL
IL
C