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THE LH RECEPTOR : STRUCTURE / ACTIVITY RELATIONSHIPS USING ANTI-PEPTIDE ANTIBODIES

BY

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A thesis submitted in fulfilment of the conditions

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of the University of London

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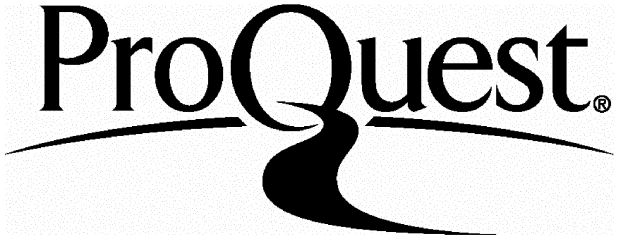

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DEDICATION

To

My Mother and Father

Nondas and Roulla

ABSTRACT

THE LH RECEPTOR : STRUCTURE / ACTIVITY RELATIONSHIPS USING ANTI-PEPTIDE ANTIBODIES

BY

ZAKOS PALLIKAROS

Site-directed antibodies were used to investigate the structure / activity relationships of the LH/CG receptor. Polyclonal antibodies were raised against synthetic peptides corresponding to regions within the extracellular N-terminal domain (antibody 1: Arg^{48}-Glu^{65}; antibody 2: Tyr^{187}-Asp^{206}) and the cytoplasmic C-terminal domain (antibody 3: Cys^{622}-Ala^{636}) of the receptor. Affinity purified antibodies proved to be peptide-specific by both ELISA and dot-blotting assays. On Western blots of membrane proteins prepared from superovulated rat ovaries, mouse Leydig tumour (MA10) cells and rat testes, all three antibodies recognised a single broad band (95-100 kDa) corresponding to the putative LH/CG receptor. The 95-100 kDa protein also bound ^{125}I-hCG on ligand blots and this binding was inhibited by the two N-terminal antibodies. Antibody 1 significantly inhibited ^{125}I-hCG binding to intact MA10 cells to a greater extent than did antibody 2, whereas antibody 3 and pre-immune IgG were without effect. Similarly both N-terminal antibodies significantly inhibited the cAMP and progesterone response of MA10 cells to LH (antibody 1 being more potent than antibody 2), whereas antibody 3 and pre-immune IgG had no significant effect. Both the cAMP and progesterone responses of MA10 cells to dbcAMP, cholera-toxin and forskolin were unaffected by any of the antibodies, as was the stimulation of membrane adenylyl cyclase activity by NaF and a GTP analogue. These results

indicate that the antibodies inhibit LH action at the level of the LH/CG receptor and that residues Arg^{48}-Glu^{65} (and to a lesser extent Tyr^{187}-Asp^{206}) are close to the binding site for LH/CG. Data obtained with antibody 3 suggest that antibody binding to the C-terminal residues Cys^{622}-Ala^{636} does not interfere with hormone binding or actions. The differential effects of these antibodies on LH-induced signal transduction provide important information on the structure / activity relationships of the LH receptor.

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LIST OF ABBREVIATIONS

AA	=	arachidonic acid
aa	=	amino acid
Ab	=	antibody
AC	=	adenylyl cyclase
Acetyl CoA	=	acetyl-coenzyme A
ACTH	=	adrenocorticotrophic hormone
AVP	=	arginine vasopressin
α_s	=	α-subunit of G_s
ADP	=	adenosine diphosphate
ATP	=	adenosine triphosphate
$\beta\gamma$	=	G-protein $\beta\gamma$-subunit
3βHSD	=	3β-hydroxysteroid dehydrogenase
17βHSD	=	17β-hydroxysteroid dehydrogenase
BSA	=	bovine serum albumin
Ca^{2+}	=	calcium ions
cAMP	=	cyclic adenosine-3',5'-monophosphate
cDNA	=	complementary DNA
CG	=	chorionic gonadotrophin
CHO	=	Chinese hamster ovary
CNS	=	central nervous system
CR-127	=	batch number of hCG
CSCC	=	cholesterol side-chain cleavage
CSF	=	cerebrospinal fluid
C-terminal	=	carboxy-terminal
CTX	=	cholera toxin
DAG	=	diacylglycerol
dbcAMP	=	dibutyryl-cAMP
DBI	=	diazepam binding inhibitor
DHEA	=	dehydroepiandrostenedione
DMEM	=	Dulbecco's modified Eagle's medium
DMF	=	dimethylformamide
DMSO	=	dimethylsulphoxide
DNA	=	deoxyribonucleic acid
DPM	=	disintegration per min
DTT	=	dithiothreitol
ECL	=	enhanced chemiluminescence
EDT	=	1,2-ethanedithiol
EDTA	=	ethylene-diaminetetra-acetic acid
EGF	=	epidermal growth factor
EIA	=	enzyme immunoassay
ELISA	=	enzyme-linked immunosorbent assay
Endo F	=	endoglycosidase F (N-glycosidase F-free)
ETOH	=	ethanol
FSH	=	follicle-stimulating hormone
ETSHr	=	extracellular TSH receptor
ExG2	=	human TSH receptor recombinant extracellular protein

Fmoc	=	N^{α}-fluorenylmethoxycarbonyl
GABA	=	γ-aminobutyric acid
GDP	=	guanosine diphosphate
GH	=	growth hormone
GnRH	=	gonadotrophin releasing-hormone
p(NH)ppG	=	guanosine 5'-[β,γ-imido]triphosphate
G-protein	=	GTP-binding protein
G_i	=	G-protein which mediates inhibition of adenylyl cyclase
G_s	=	G-protein which mediates stimulation of adenylyl cyclase
GTP	=	guanosine triphosphate
hCG	=	human chorionic gonadotrophin
HCl	=	hydrochloric acid
$HClO_4$	=	perchloric acid
HEPES	=	N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonicacid
hLH	=	human luteinizing hormone
HOBT	=	1-hydroxybenzotriazole
HPLC	=	high performance liquid chromatography
HPG	=	hypothalamic-pituitary-gonadal axis
^{125}I	=	iodine-125 radioisotope
IBMX	=	iso-3'-butyl-5'-methyl xanthine
IgG	=	immunoglobulin G
IP_3	=	inositol-1,4,5-trisphosphate
$K_{a/D}$	=	affinity/dissociation constant
K_3PO_4	=	potassium phosphate
LH	=	luteinizing hormone
LH/CG	=	luteinizing hormone/chorionic gonadotrophin
LHRH	=	luteinizing hormone releasing-hormone
MA10 cells	=	mouse tumour Leydig cells
MBS	=	maleimidobenzoyl-*N*-hydroxysuccinimide ester
Mg^{2+}	=	magnesium ions
$MgCl_2$	=	magnesium chloride
MLTC-1	=	mouse tumour Leydig cells
M_r	=	apparent molecular mass
mRNA	=	messenger RNA
Mtr	=	4-Methoxy-2,3,6-trimethylbenzene
n	=	number of repetitions of each experiment using cells/tissue from different animals on each occasion
NaCl	=	sodium chloride
NAD^+	=	nicotinamide adenine dinucleotide (oxidized form)
NADH	=	nicotinamide adenine dinucleotide (reduced form)
NADPH	=	nicotinamide adenine dinucleotide phosphate (reduced form)
Na_2-EDTA	=	sodium ethylene diaminotetraacetate
NaF	=	sodium fluoride
NaN_3	=	sodium azide
NaOH	=	sodium hydroxide
N-linked	=	asparagine-linked

NSB	=	non-specific binding
N-terminal	=	amino-terminal
O_2	=	oxygen
O-linked	=	serine or threonine-linked
$P450_{arom}$	=	cytochrome P450: aromatase enzyme
$P450_{C17}$	=	cytochrome P450: 17α-hydroxylase/17,20-lyase enzyme
$P450_{SCC}$	=	cytochrome P450: cholesterol side-chain cleavage enzyme
PAGE	=	polyacrylamide gel electrophoresis
PBS	=	phosphate-buffered saline
PBSA-T	=	PBS (containing 0.02% (w/v) NaN_3 and 0.05% (v/v) Tween-20)
PEG	=	polyethelene glycol
pfp	=	pentafluorophenyl
PGB	=	pas-gel buffer
P.I.	=	pre-immune
PI	=	phosphatidylinositol
PKA	=	protein kinase A
PKC	=	protein kinase C
PLA_2	=	phospholipase A_2
PLC	=	phospholipase C
PMSF	=	phenylmethylsulfonyl fluoride
PMSG	=	pregnant mare's serum gonadotrophin
PRL	=	prolactin
PTFE	=	polytetrafluoroethylene
PTX	=	pertussis toxin
R14	=	anti-ExG2 antibody
R2C	=	rat tumour Leydig cells
RIA	=	radioimmunoassay
RME	=	receptor mediated endocytosis
RNA	=	ribonucleic acid
RT-PCR	=	reverse transcription polymerase chain reaction
SAP	=	steroidogenesis activator polypeptide
ScAMP-TME	=	2'-monosuccinyl adenosine-3',5'-cyclic monophosphate tyrosyl methyl ester
SCC	=	side-chain cleavage
SCP_2	=	sterol carrier protein 2
SDS	=	sodium dodecyl sulphate
SD	=	standard deviation
S.E.M.	=	standard error of the mean
SKR	=	substance K receptor
StAR	=	steroidogenic acute regulatory protein
T3	=	triiodothyronine
T4	=	thyroxine
TBS	=	Tris buffered saline
TCA	=	trichloroacetic acid
TEMED	=	N,N,N',N',tetramethylethylenediamine
TFA	=	trifluoroacetic acid

Tris	=	tris(hydroxymethyl)aminomethane
TSAb	=	thyroid stimulating antibodies
TSBAb	=	thyroid stimulating blocking antibodies
TSH	=	thyroid stimulating hormone
TTBS	=	Tris buffered saline (containing 0.02% (v/v) Tween 20)
v/v	=	volume per volume
wt	=	weight
w/v	=	weight per volume

UNITS AND MEASURES

Centrifugal force	:	*g*	=	gravitational force
Concentrations	:	IU	=	international units
		U	=	units
		mol	=	moles
		M	=	molar
			=	mol/l
Mass	:	Da	=	daltons
		g	=	grams
Radioactivity	:	Bq	=	becquerel
		cpm	=	counts per minute
			=	1 disintegration per second
		Ci	=	curie
			=	3.7×10^{10} Bq
			=	2.22×10^{12} cpm
Sedimentation Velocity	:	*g*	=	centrifugal force
		rpm	=	revolutions per min
Standard prefixes	:	giga	=	G $\times 10^{9}$
		mega	=	M $\times 10^{6}$
		kilo	=	k $\times 10^{3}$
		milli	=	m $\times 10^{-3}$
		micro	=	μ $\times 10^{-6}$
		nano	=	n $\times 10^{-9}$
		pico	=	p $\times 10^{-12}$
		femto	=	f $\times 10^{-15}$
Temperature	:	^{o}C	=	degrees celsius
Time	:	h	=	hours
		min	=	minutes
Volume	:	l	=	litres

CHAPTER 1

GENERAL INTRODUCTION

Luteinizing hormone (LH), together with follicle-stimulating hormone (FSH), has very important roles in reproductive physiology. The receptor for LH is found on ovarian theca-interstitial cells, mature granulosa cells and cells of the corpus luteum. In the testis it is present in the Leydig cells. In both the testis and the ovary, the LH receptor in addition to recognizing the pituitary glycoprotein LH, also has the capacity to bind the related glycoprotein human chorionic gonadotropin (hCG). Binding of LH to the LH receptor in the plasma membrane of gonadal cells results in the stimulation of adenylyl cyclase to form cAMP and subsequent stimulation of cAMP-dependent protein kinase and steroidogenesis.

1.1 Essentials of gonadal physiology

1.1.1 The Testis: structure and function

The testis is a compound organ which consists of the seminiferous tubules and interstitial tissue. These two compartments are closely interlinked, thereby enabling the testis to fulfil its dual function of spermatogenesis and steroidogenesis. The testis also contains connective tissue, blood vessels and nerve endings. It is surrounded by a thick connective tissue capsule, known as the tunica albuginea (fig. 1.1).

The seminiferous tubules are the compartments for sperm production. The number, length and diameter of the tubules differ between animal species. In most mammals, the seminiferous tubules account for over 90% of the volume of the testis. In the rat testis, 82.4% of the volume is occupied by seminiferous tubules, 15.7% by the interstitial tissue and 1.9% by the capsule (Mori and Christensen, 1980). The

seminiferous tubules contain germ cells at different stages of spermatogenesis and the non-proliferating somatic cells, the Sertoli cells. The germ cells undergo successive mitotic and meiotic divisions and so convert the spermatogonia through the spermatocyte and spermatid stages into mature spermatozoa. Within each tubule the developing germ cells mature towards the lumen and are always in close association with Sertoli cells throughout development. It has been shown from three-dimensional studies that each Sertoli cell is in contact with up to 5 other Sertoli cells at its base and up to 47 germ cells at different stages of development (see review Skinner, 1991). The Sertoli cells form specialized "tight junctions" between them so that in essence they make a continuous barrier within every seminiferous tubule. This barrier is referred to as the blood-testis (or lymph-testis) barrier. The barrier divides the tubules into basal and adluminal compartments and excludes large molecules, such as proteins and certain other components of the lymph, from entering the tubule (fig. 1.2).

The presence of various generations of spermatocytes and spermatids derived from individual spermatogonia (see review Clermont, 1972) initiates waves of spermatogenesis that are not random but instead occur in a specific cyclic manner which are referred to as stages. The time taken for the same stage to reappear in a given segment of the seminiferous tubule is constant for each species and ranges from 8 to 16 days. For the rat the cycle, which takes 12.8 days to complete, is divided into 14 stages. A cross-section of the rat testis at any point will reveal adjacent tubules at different stages of the spermatogenic cycle. In the spaces between adjacent tubules is the interstitial tissue of the testis, which consists of a framework of loose connective tissue that supports blood vessels and the interstitial cells (Leydig cells, macrophages

and lymphocytes) (see review Sharpe, 1990). The Leydig cells are the endocrine cells of the testis, and are responsible for the production of the male steroid hormone, testosterone. Testosterone is required for specific androgen-dependent stages of spermatogenesis e.g. stage VII in the rat. In the rat testis, Leydig cells constitute 2.7% of testicular volume; such that each cubic centimetre of the testis contains about 22 million Leydig cells (Mori and Christensen, 1980).

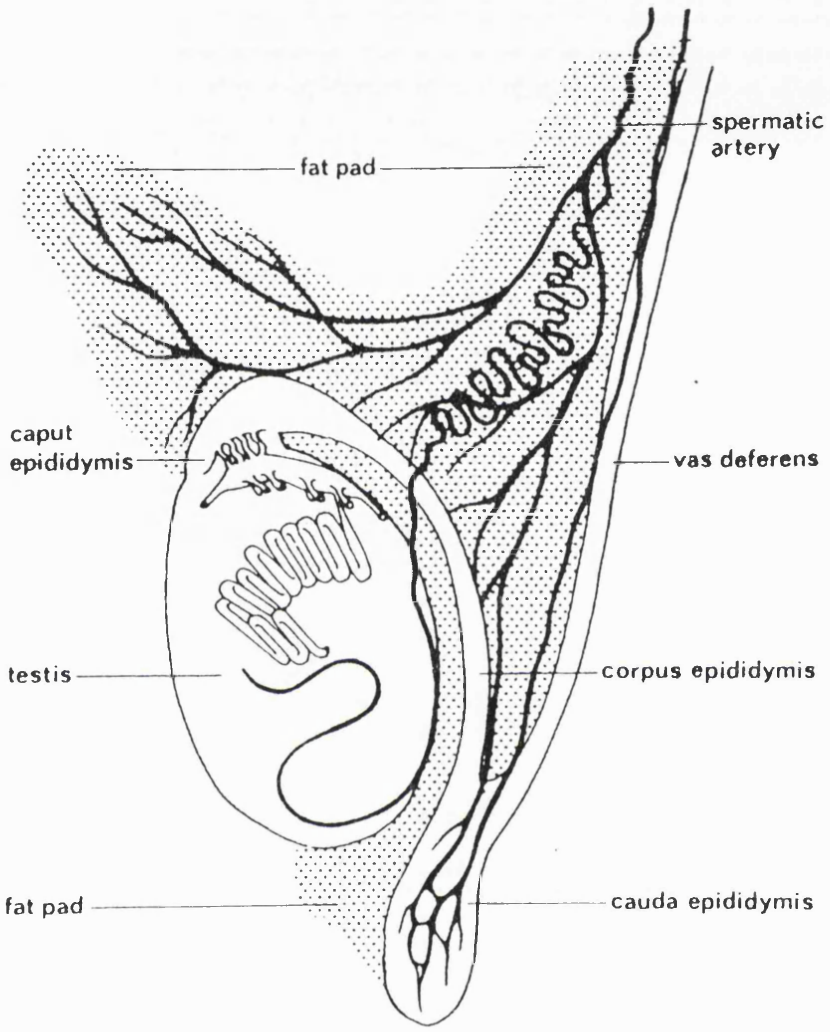

Figure 1.1 Anatomy of the testis. The seminiferous tubules produce spermatozoa which are transported via the rete testis to the caput epididymis. The spermatozoa undergo maturation as they pass through the caput epididymis to the cauda epididymis. The mature sperm are then stored in the cauda epididymis until they are ejaculated (Cooke *et al.*, 1973).

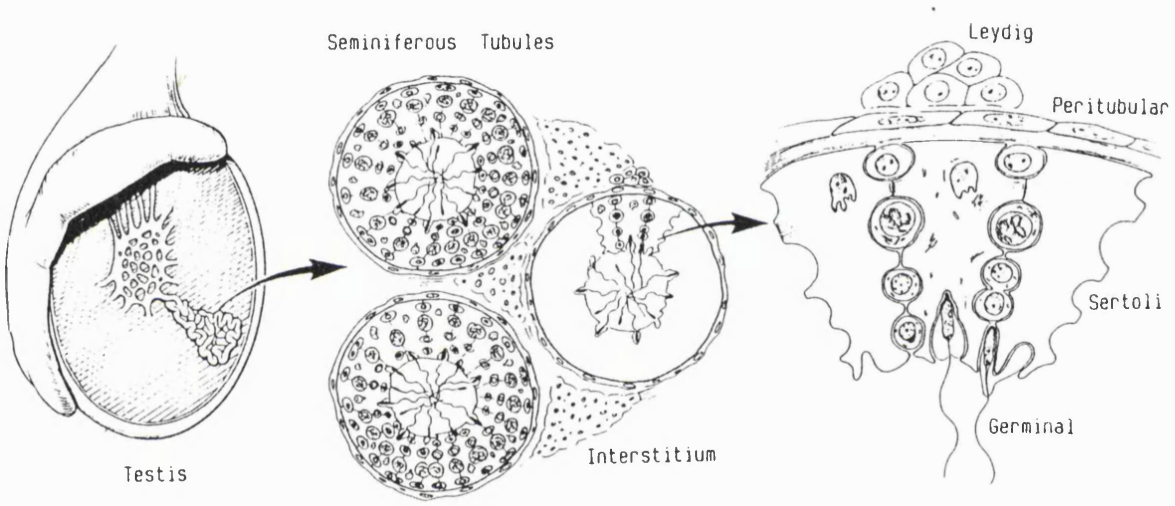

Figure 1.2 Cell biology of the testis. Diagram showing the intact testis on the left, and a cross-section showing the seminiferous tubules which contain the Sertoli cells. Surrounding the tubules is the interstitium which contains Leydig cells, macrophages, together with collagen fibres, a rich blood supply and a sympathetic nerve supply. Note on the right of the diagram the tight junctions at the base of the Sertoli cells; these are responsible for maintaining the blood-testis barrier (see review Skinner 1991).

1.1.2 Ovary: structure and function

Like the testis, the ovary can also be divided into different tissue components: the follicles, corpora lutea and interstitial / stromal tissue. There is a clear distinction between the developmental programme of gametes in the ovary and the testes. In the ovary, oogenesis is complete before birth, whereas in the testis there is a continuous proliferation of germ cells and the production of spermatozoa throughout adult life. The endocrine function of the ovary ensures the regular production of healthy oocytes at a time when they will have a maximum chance of being fertilized. In the mature animal the structure and function of the ovary is continually changing. Gonadotrophins secreted by the anterior pituitary gland stimulate the growth of Graafian follicles, ovulation, and the formation of corpora lutea, the latter being formed from the granulosa and theca cells of the follicle ruptured at ovulation. The time taken for follicles and corpora lutea to develop differs from species to species and is reflected in different patterns of ovarian cycles. Follicles at all stages of development can be found distributed throughout both ovaries at all times, except after the menopause in women.

The mature Graafian follicle is composed of several layers of cells surrounding the oocyte, which is contained within a fluid-filled cavity or antrum. The outermost layers, the theca externa and theca interna, are formed from the adjacent stromal cells. They are supplied by a rich network of capillaries and separated by a basement membrane from the avascular granulosa cells which line the follicular cavity. The granulosa cells are comparable to the Sertoli cells of the testis and are important in maintaining the very specialized conditions within the antral cavity which permit development of the

oocyte. The population of granulosa cells is probably not homogeneous, since those lining the basement membrane (mural granulosa cells) and those surrounding the oocyte (the cumulus oophorus) serve different functions For example, there are microtubular connections between the cumulus and oocyte which are probably important for transferring nutrients and hormonal signals to the oocyte.

The localization of gonadotrophin receptors in the ovaries of various animal species and in women has been extensively studied by injecting radioactive hormone *in vivo* or incubating *in vitro* with tissue slices, cells or membranes (Midgley 1973; Channing and Kammerman., 1974; Amsterdam *et al.*, 1975). Detection of the hormone by immunocytochemistry was also performed (Kobayashi *et al.*, 1990; Kinnunen *et al.*, 1981). The effects of gonadotrophins on follicular growth, ovulation, and luteinization have been shown to be associated with differences in the numbers of luteinizing hormone (LH) and follicle stimulating hormone (FSH) receptors. Iodinated LH binding to the ovarian theca cells increases during follicular development, especially in the preovulatory follicles. The endogenous LH surge and administration of an ovulatory dose of LH or human chorionic gonadotrophin (hCG) causes a down regulation in the LH receptor content of ovulatory follicles. During luteinization and corpus luteum formation, there is resynthesis of LH receptors. In the corpus luteum, the binding of human LH increases from the early luteal phase to the midluteal phase and decreases towards the late luteal phase (Misrahi *et al.*, 1993).

LH has several distinct actions on the ovary. The most important action of LH, however, is to increase the synthesis of steroids by those cell types in the ovary which

have receptors for LH, i.e. the stroma, theca interna and granulosa cells of pre-ovulatory follicles and later the corpus luteum. The final product secreted depends on the relative activity of the steroid-converting enzymes (see section 1.3).

1.2 Glycoprotein hormones (LH, hCG, FSH and TSH)

The glycoprotein hormone family includes the pituitary gonadotrophins (LH and FSH) as well as thyroid stimulating hormone (TSH) and hCG. They consist of two subunits that are joined by non-covalent forces. The 92-amino-acid α-subunit is common to all four hormones. The β-subunit is specific for each hormone although they all show some degree of homology because of their binding to the α-subunit. It has been shown that both subunits of the glycoprotein hormones are required for binding to their respective receptors and it is the β-subunit that confers binding specificity (see review Pierce and Parsons, 1981). LH and hCG share common biological properties and their β-subunits possess a high degree of sequence homology (85% in the first 114 amino-acids (Talmadge *et al.*, 1984)), with their most unique difference being a proline and serine-rich carboxyl terminal extension of the hCG subunit. Hence this β-subunit homology is responsible for LH and hCG binding to a common gonadal receptor.

LH and hCG also have a high degree of disulphide bridging in each subunit (see review Pierce and Parsons, 1981) and contain two types of carbohydrate side chains (Sairam, 1983). There are asparagine (N)-linked chains on all α- and β-subunits and serine (O)-linked chains on the carboxyl terminal extension of hCG β-subunit. The O-linked chains are relatively simple structures and are not required for biological activity since they are absent from the LH molecule. The N-linked carbohydrates are

complex biantennary (hCG) or triantennary (FSH) structures with a central mannose core and branches that terminate with galactose-sialic acid or galactose-sulphate depending on the species and tissue of origin. The carbohydrate moieties on the glycoprotein hormones affect their half-life in the circulation and thus their *in vivo* biological potency. Studies with deglycosylated hormones have been performed with LH, hCG, FSH, and TSH (Sairam, 1983). In general they indicate that removal of N-linked carbohydrates does not interfere with receptor binding but markedly decreases biological responses such as stimulation of the adenylyl cyclase enzyme and steroidogenesis. These results indicate that the carbohydrates are needed on both subunits for full expression of biological activity (Keutmann *et al.*, 1983).

1.2.1 Pituitary release of gonadotrophins

LH and FSH are synthesized in the anterior pituitary gonadotrophs and act on the testis and ovary to modulate steroidogenesis and gametogenesis.

The pituitary gland is located at the base of the brain and it is central to the regulation of the endocrine system. The gland consists of the anterior pituitary (adenohypophysis) and the posterior pituitary (neurohypophysis). The neurohypophysis contains neurones that secrete the peptide hormones, arginine vasopressin (AVP, antidiuretic hormone) and oxytocin. The adenohypophysis releases six principal hormones into the systemic circulation. These include adrenocorticotrophin (ACTH), growth hormone (somatotrophin, GH), prolactin (PRL), TSH and the gonadotrophins LH and FSH. The control of gonadotrophin synthesis and secretion from the gonadotrophs is regulated by gonadotrophin releasing hormone (GnRH) (Redding *et al.*, 1972) (fig. 1.3).

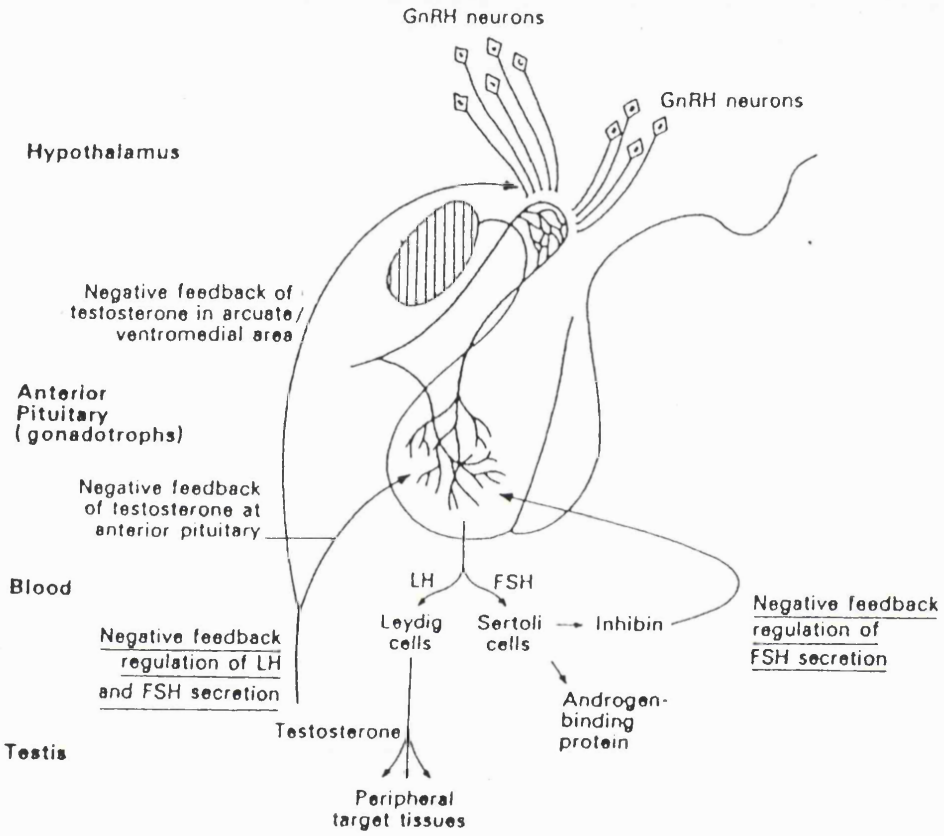

Figure 1.3 Diagrammatic summary of the regulation of FSH and LH secretion in the male (Johnson and Everitt, 1984).

1.2.2 Regulation of LH and FSH release by GnRH

GnRH is a decapeptide which is produced in specific neurons in the brain of all vertebrates. It is secreted into the hypophyseal portal circulation from the median eminence of the hypothalamus with intrinsic frequency. The pulsatile release of GnRH from the hypothalamus is essential in maintaining the responsiveness of the gonadotrophs to GnRH, which in turn are influenced by gonadal sex steroids (testosterone, progesterone and oestradiol), neurotransmitters such as catecholamines (norepinephrine, epinephrine and dopamine), and the endogenous opioid peptides (Giguere *et al.,* 1981; Kamel and Krey., 1983; Adams *et al.,* 1991). Testosterone controls the secretion of GnRH in the male through an extensive series of negative feedback loops.

GnRH actions are initiated by binding to specific receptors on the gonadotroph membrane. After binding to pituitary GnRH receptors, calcium-dependent processes are triggered leading to gonadotrophin release (Borges *et al.*, 1983; see reviews Conn *et al.*, 1987 and Davidson *et al.*, 1991). In addition to the native hormone, many potent agonists (Kolho and Huhtaniemi, 1989) and antagonists (Puente and Catt, 1986) of GnRH have been synthesized which are useful in studying the physiology of the hypothalamo-pituitary-gonadal axis.

Several studies using rat (Haisenleder *et al.*, 1991), primate (see review Marshall and Kelsh, 1986), and sheep (McIntosh and McIntosh, 1985) showed that a pulsatile GnRH signal is required to maintain gonadotrophin secretory activity, because a continuous

GnRH infusion desensitizes the gonadotroph cell to further GnRH stimulation and down regulates GnRH receptors (Clayton, 1982).

1.3 Regulation of steroidogenesis in the gonads by LH

Testosterone is central to male reproduction. Leydig cells are responsible for the production of testosterone in the testis and this is mainly under the control of LH.

In all steroidogenic cells, cholesterol is the precursor for the formation of the steroid hormones (fig 1.4). There are three potential sources of cholesterol. These include uptake of plasma lipoprotein particles, *de novo* synthesis from acetyl CoA and hydrolysis of cholesteryl esters stored in lipid droplets. Lipoprotein-derived cholesterol is of major importance for steroidogenesis in the adrenal and the ovary (see review Gwynne and Strauss III., 1982). In the testis, cholesterol is derived mainly from the *de novo* synthesis (Morris and Chaikoff, 1959; Charreau *et al.*, 1981; Hou *et al.*, 1990).

It has been demonstrated in Chinese hamster ovary (CHO) cells that newly synthesized cholesterol is transported to the plasma membrane through an energy dependent process (Kaplan and Simoni, 1985). This was studied further in mouse tumour Leydig (MA10) cells wherein it was found that the plasma membrane was the major store of cholesterol for steroidogenesis and that under hormone-stimulation, newly synthesized cholesterol was diverted from incorporation into the plasma membrane and directed into the steroidogenic pathway (Freeman, 1987a, 1989).

The first stage of steroid synthesis is the transport of cholesterol to the mitochondria (see review Gower, 1988). The cytoskeleton may also play a role in the intracellular transport of cholesterol (see review Hall, 1985) and there is evidence for the involvement of cholesterol carrier proteins, which may transport cholesterol from the cytosol or plasma membrane to the mitochondria. Three proteins which may be involved in this transport and which are structurally distinct and widely distributed in both steroidogenic and non-steroidogenic tissues have been isolated. When these proteins are added to mitochondria isolated from steroidogenic tissues, they all stimulate the transfer of cholesterol from the outer to inner mitochondrial membrane, and the production of pregnenolone, catalyzed by the cytochrome P450 cholesterol side-chain cleavage (CSCC) complex on the inner mitochondrial membrane. These proteins are: 1) sterol carrier protein-2 (SCP_2) (Noland *et al.*, 1980) which consists of a single polypeptide chain of 122 amino acids and was shown to promote the transfer of cholesterol from lipid droplets to mitochondria; 2) steroidogenesis activator polypeptide (SAP) (Pedersen and Brownie, 1987); and 3) the 8.2 kDa protein (Yanagibashi *et al.*, 1988). Both SAP and the 8.2 kDa protein were shown to stimulate directly cytochrome $P450_{cscc}$ activity in isolated mitochondria in a dose-dependent fashion. The 8.2 kDa protein was found to have homology (except that it lacks two residues at the carboxyl-terminus) with a brain protein called endozepine (diazepam binding inhibitor, DBI) which inhibits the binding of diazepam to benzodiazepine/$GABA_A$ receptors (Besman *et al.*, 1989). The importance of the 8.2 kDa protein in the regulation of steroidogenesis is still uncertain.

The rate-limiting physical step in steroidogenesis in all steroidogenic tissues, is the transport of cholesterol from the outer to the inner membrane of the mitochondria (Crivello and Jefcoate (1980)., Jefcoate *et al.*, (1987)). Acute stimulation of both rat adrenal and luteal cells with trophic hormone has been shown to result in the rapid synthesis of a 28 kDa protein which was speculated to play a role in the regulation of steroidogenesis (Krueger and Roberts Orme-Johnson, 1983; Pon and Roberts Orme-Johnson, 1986; Pon *et al.*, 1986). The synthesis of this protein, as well as the production of steroids in these cells, was shown to be cycloheximide-sensitive. Alberta *et al.* (1989) also reported the mitochondrial localization of a phosphoprotein which rapidly accumulated in adrenal cortex cells after exposure to ACTH or cAMP. Stocco and Kilgore (1988) and Stocco and Chen (1991), while studying this labile protein involved in steroidogenesis, reported the accumulation of several mitochondrial proteins in response to hormone-stimulation. These workers showed that R2C rat Leydig tumour cells which produce steroids constitutively, also show constitutive production of mitochondrial proteins (30 kDa proteins) that are cycloheximide sensitive. This led to the suggestion that steroidogenesis in mitochondria is controlled by a protein with a short half life.

The second stage of androgen synthesis is the intramitochondrial transport of cholesterol. The transport of cholesterol from the outer to the inner mitochondrial membrane is dependent on protein synthesis (Freeman, 1987b). The three proteins discussed earlier (SCP_2, SAP and 8.2 kDa protein) can also be implicated in the transfer of cholesterol from the outer to the inner mitochondrial membrane. Phospholipids and Ca^{2+} ions are also thought to be involved in cholesterol transference.

Recently, Clark *et al* (1994) purified and cloned a candidate protein identified in MA10 cells that is synthesized in response to LH stimulation and that is localized to the mitochondria. This novel protein, called Steroidogenic Acute Regulatory protein (StAR), is required for the short-term increase in steroidogenesis following cell stimulation with steroidogenic agonists. A working model has been proposed for the acute regulation of steroidogenesis in Leydig cells by StAR whereby the precursor protein is rapidly synthesized in response to hormone stimulation and targeted to the mitochondria. The protein is translocated across the outer and inner membrane and undergoes two cleavage events to produce the final mature form(s) of the StAR protein. It is hypothesized that the formation of contact sites between the inner and outer membrane due to the translocation of this StAR protein across the mitochondrial membranes allows for cholesterol to be transferred to the inner membrane and thereby delivered to the cholesterol side-chain cleavage complex. It is also possible this protein may actively bind cholesterol. However, further studies are required to determine the mechanism whereby StAR affects cholesterol transport to the inner mitochondrial membrane and the cholesterol side-chain cleavage complex.

The third stage of androgen synthesis is the side-chain cleavage (SCC) of cholesterol. This reaction, which takes place in the inner mitochondrial membrane, utilizes three molecules of molecular oxygen and three molecules of NADPH; the first two are for the sequential hydroxylation of carbons 22 and 20 to yield 22R hydroxycholesterol and 20α, 22R-dihydroxycholesterol. The third molecule of oxygen is required for cleavage of the carbon-carbon bond between these two carbon atoms to give pregnenolone and isocaproate. The cholesterol side chain cleavage complex therefore comprises a 20-

and 22-hydroxylase and a C-20,22-lyase, all tightly bound to the inner face of the membrane and associated with a specific cytochrome P-450_{SCC} (see review Gower, 1988).

The following stages in androgen synthesis describe the conversion of pregnenolone to testosterone and are catalyzed by non-mitochondrial enzymes. These enzymes include the 5-ene-3β-hydroxysteroid dehydrogenase/3-oxosteroid-4,5-isomerase (3β-HSD), cytochrome P-450 17α-hydroxylase/17,20-lyase (P-450_{C17}) and 17β-hydroxysteroid dehydrogenase (17β-HSD) which are found in the smooth endoplasmic reticulum. Pregnenolone is metabolized to testosterone in Leydig cells of the testis and the theca cells of the ovary. Two pathways for testosterone synthesis exist (fig. 1.4). One involves 5-ene-3β-hydroxysteroids ("delta-5" pathway), and the other involves 4-ene-3-oxosteroids ("delta-4" pathway). The delta-5 steroids can enter the delta-4 pathway at several levels via the action of 3β-HSD in the fourth stage of androgen synthesis.

The fifth stage involves the P-450_{C17} enzyme which catalyzes the 17α-hydroxylation of pregnenolone and progesterone to give 17α-hydroxypregnenolone and 17α-hydroxyprogesterone respectively, followed by the cleavage of the C-17,20 carbon bond via the 17,20-lyase reaction to yield dehydroepiandrosterone (DHEA) and 4-androstenedione, respectively in the sixth stage of androgen synthesis. Hence the production of C_{19} steroids from C_{21} precursors depends on the activity of the P-450_{C17} enzyme. In addition to the cytochrome P-450_{C17}, NADPH and O_2 are needed. It is now well demonstrated that all four of these reactions are mediated by a single enzyme, P-

450_{C17}, bound to smooth endoplasmic reticulum (Shikita and Tamaoki, 1965) which requires a flavoprotein immunologically distinct from mitochondrial ferredoxin to transfer of electrons from NADPH to cytochrome P-450 (Baron *et al.*, 1972).

17β-HSD converts androstenedione into testosterone in the seventh stage of androgen synthesis by catalyzing the reduction of the ketone at C17. Cytochrome P-450 aromatase ($P\text{-}450_{arom}$) may thereafter aromatize androstenedione and testosterone in the final eighth stage, to the corresponding oestrogens, namely estrone and estradiol-17β.

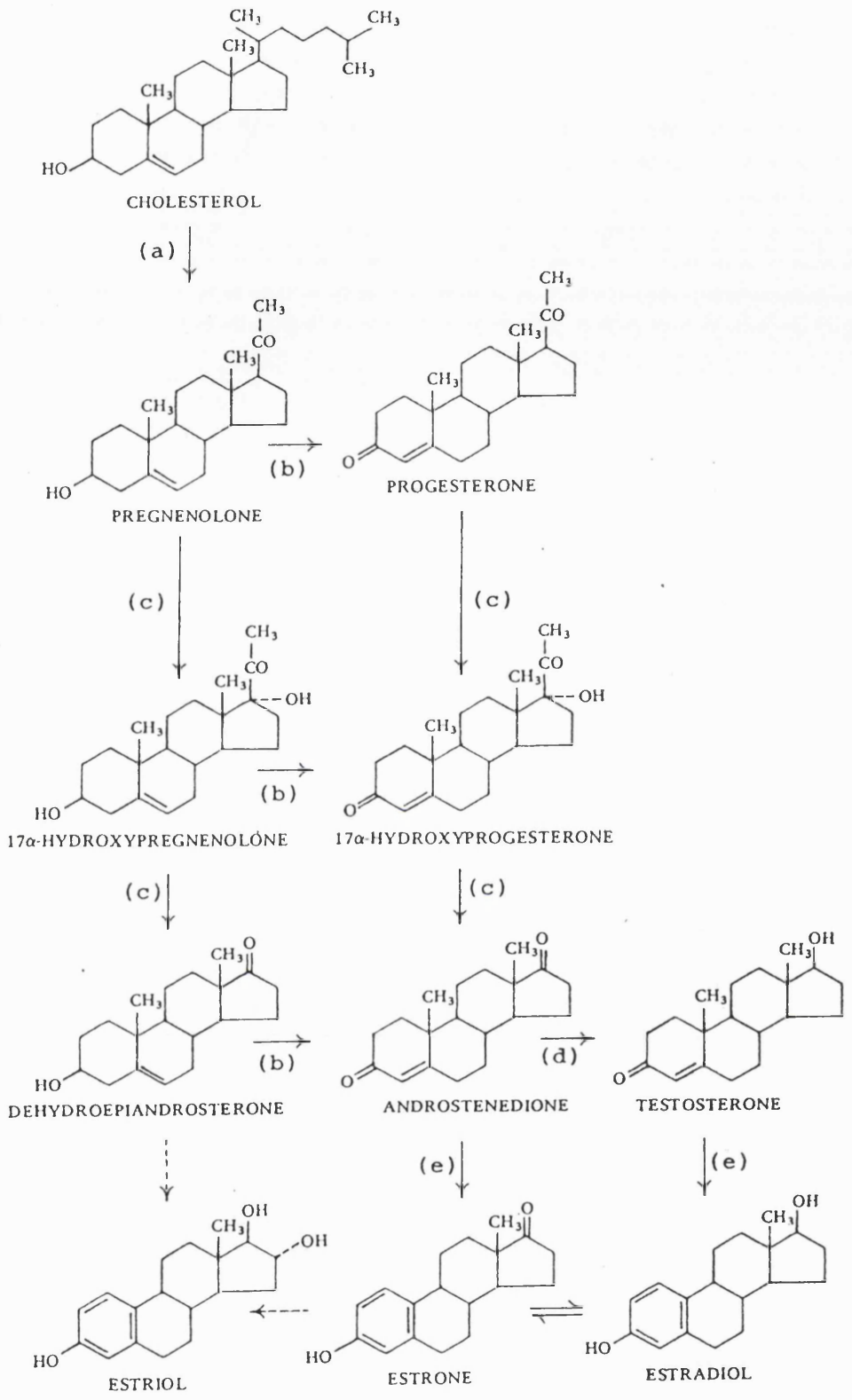

Figure 1.4 Biosynthesis of the male and female steroid hormones from cholesterol. The enzymes involved in each step are the following:- (a) cytochrome P-450$_{SCC}$ (b) 5-ene-3β-hydroxysteroid/3-oxosteroid-4,5-isomerase (3βHSD) (c) cytochrome P-450 17α-hydroxylase/17,20-lyase (P-450$_{C17}$) (d) 17β-hydroxysteroid dehydrogenase 17βHSD) (e) cytochrome P-450 aromatase (P-450$_{arom}$) (adapted from Schulster *et al.*, 1976).

1.4 Mouse tumour Leydig (MA10) cells

MA10 cells are a clonal strain of mouse tumour Leydig cell adapted to culture in the laboratory of Dr. M. Ascoli (Ascoli, 1981). These cells were derived from a transplantable tumour (designated M5480P) that originated spontaneously in C57B1/6 mouse.

MA10 cells contain 10,000 - 20,000 LH/CG receptors per cell with high affinity (K_D= 10^{-9} - 10^{-10} M). Like mouse testis Leydig cells, MA10 cells have the capacity to convert cholesterol into steroid hormones and respond to LH/CG with increased steroid biosynthesis. A comparison of the steroids produced by freshly isolated mouse Leydig cells and MA10 cells revealed some changes in the steroidogenic pathway. The most obvious changes are: a) an increase in the ability of the cultured cells to synthesize 20α-dihydroprogesterone and b) a decrease in the activity of $P450_{C17}$ enzyme in MA10 cells, which is involved in the conversion of progesterone to androstenedione. The major steroid produced by MA10 cells is therefore progesterone rather than testosterone (Ascoli, 1981). Steroidogenic cholesterol in MA10 cells is derived from the plasma membrane (Freeman, 1989) and is under the control of cyclic AMP (Freeman, 1987a).

The MA10 cell system is ideally suited to study gonadotrophin actions and regulation of the expression of differentiated functions of Leydig cells. The advantages of using these cells are: 1) tumour cells are generally easier to grow in culture than normal cells and 2) the retention of differentiated function could be easily detected by measuring hormone binding and / or steroid production.

1.5 LH/CG receptor: structure / activity relationships

1.5.1 Structure of the LH/CG receptor

All the glycoprotein receptors have recently been cloned. The first was the LH/CG receptor cloned from rat ovaries (McFarland *et al.,* 1989) and porcine testis (Loosfelt *et al.,* 1989) (fig. 1.5). This was followed by cloning of the TSH receptor (Libert *et al.,* 1989; Nagayama *et al.,* 1989; Parmentier *et al.,* 1989; Misrahi *et al.,* 1990) and then the FSH receptor (Sprengel *et al.,* 1990). The human (Minegishi *et al.,* 1990) and murine (Gudermann *et al.,* 1992) LH/CG receptors have also been cloned. The deduced amino acid sequence from full-length cDNA reveals that the structure of the LH/CG receptor is similar to that of other G-protein-coupled receptors. A comparison was made between the hydropathy plot for the rat luteal LH/CG receptor and those of the rhodopsin-like G-protein-coupled receptors. Figure 1.6 shows that the carboxyl-terminal half of the LH/CG receptor displays a similar hydropathy plot to that of substance K receptor (SKR), suggesting that this half of the LH/CG receptor possesses seven transmembrane regions (McFarland *et al.*, 1989). Alignment of the transmembrane regions of the LH/CG receptor with the other rhodopsin-like G-protein-coupled receptors gave an amino acid homology of 18 - 26% (McFarland *et al.,* 1989). From the amino acid sequence of the rat luteal LH/CG receptor and by analogy with the topography of other G-protein-coupled receptors, it was postulated that the LH/CG receptor existed in the plasma membrane with the amino-terminal hydrophilic half being extracellular and the carboxyl-terminal half spanning the plasma membrane seven times, ending with a relatively short cytoplasmic tail (fig.1.5) (see review Segaloff *et al.*, 1990). This hypothesis was confirmed by using antibodies directed to

sequences in the amino-terminal (amino acid (aa) residues 194-207) and the carboxyl-terminal sequence (aa residues 660-674). Using these site-specific antibodies in immunofluorescence studies in intact cells, the postulated orientation of the receptor was confirmed. Immunofluorescence was detected with the amino-terminal antibody on the surface of intact luteal cells, whereas immunofluorescence was only detected with the carboxyl-terminal antibody when the cells were permeabilized (Rodriguez and Segaloff, 1990).

In spite of the structural and functional similarities with other G-protein coupled receptors, the receptors for glycoprotein hormones have several distinctive features. They are approximately twice the size of other cloned G-protein coupled receptors and possess a large N-terminal extracellular domain (approximately 340 amino acids) that is N-glycosylated (six N-glycosylation sites on the LH/CG receptor). For the LH receptor this extracellular domain accounts for approximately half of the polypeptide chain. The most striking feature of the extracellular domain of the LH/CG receptor is a 14-fold imperfect repeat sequence of approximately 25 residues. These motifs are termed "leucine-rich repeats" and have been recognized in various proteins comprising the family of leucine-rich glycoproteins (e.g. α_2 serum glycoprotein (Takahashi *et al.*, 1985) and yeast adenylyl cyclase (Hashimoto *et al.*, 1988)). Members of the leucine-rich glycoprotein family have been postulated to interact with both hydrophobic and hydrophilic surfaces, possibly mediated by amphipathic helices formed by the repeat structures. Since the large extracellular domain of the LH/CG receptor is apparently responsible for binding LH and hCG (Keinanen and Rajaniemi, 1986), this domain

may be responsible both for binding of hormone and for interacting with the transmembrane domains to mediate signal transduction (see section 1.5.4).

The C-terminal half of the molecule which includes the seven transmembrane domains is essentially equivalent in size to the entire molecule of other G-protein coupled receptors (e.g. rhodopsin and adrenergic receptors (Nathans and Hogness, 1983; see review Lefkowitz and Caron, 1987)). In adrenergic receptors, an extended third cytoplasmic loop seems to be required for specific interactions with the G-protein (Kobilka *et al.,* 1988; see review O'Dowd *et al.*, 1989; Strader *et al.*, 1989; Cheung *et al.,* 1991). Also Grasso *et al.* (1995) demonstrated that for the rat testicular FSH receptor, the third cytoplasmic intracellular loop (aa residues 533-555) acts as an antagonist of FSH receptor-mediated G-protein activation. These workers used a synthetic peptide corresponding to the receptor's third cytoplasmic loop (aa residues 533-555) and also found that it inhibited FSH receptor mediated signal transduction in intact Sertoli cells. Malek *et al.* (1993) demonstrated the existence of two specific sites in the third inner loop of the dopamine D_2 receptor which are involved in functional G-protein-mediated coupling to adenylyl cyclase. Peptides synthesized against these two sites were found not only to attenuate dopaminergic adenylyl cyclase inhibition in membranes, but also had the ability to activate directly GTPase activity in membranes. However, the LH/CG receptor does not appear to have an extended third cytoplasmic loop, or significant sequence homology with the β-adrenergic receptor's G-protein-coupling domain (McFarland *et al.*, 1989; Loosfelt *et al.*, 1989). The C-terminal 68 residues of the LH/CG receptor, which are located intracellularly, contain potential phosphorylation sites (serine, threonine and tyrosine residues) where

cellular control of receptor activity may occur, as suggested for the β-adrenergic receptor (see review Sibley *et al.*, 1988).

Most members of the family of G protein-coupled receptors have one or more conserved cysteine residues in their carboxy-terminal cytoplasmic tails which are believed to be consensus sites for palmitoylation (Probst *et al.*, 1992). Palmitoylation of this cysteine(s) has been shown to occur in rhodopsin (Ovchinnikov *et al.*, 1988; Papac *et al.*, 1992; Karnik *et al.*, 1993), the β_2-adrenergic receptor (O'Dowd *et al.*, 1989) and the α_2-adrenergic receptor (Kennedy and Limbird, 1993). The palmitate present at this location is expected to insert into the plasma membrane, thus forming a fourth cytoplasmic loop, which may be important for receptor function. A number of recent studies have used site-directed mutagenesis to ascertain the importance of palmitoylation to the function of G protein-coupled receptors. While it was found that mutations that prevented palmitoylation of the β_2-adrenergic receptor result in a loss of G protein activation (O'Dowd *et al.*, 1989), similar mutations in the α_2-adrenergic (Kennedy and Limbird, 1993) and the M_2 muscarinic receptors (van Koppen and Nathanson, 1991) have failed to identify such effects. Kawate and Menon (1994) showed that the LH/CG receptor is similarly palmitoylated at cysteine residues 621 and 622, creating a membrane-anchoring site at the putative cytoplasmic domain. These workers also showed that this palmitic acid-mediated anchoring decreased the ligand-induced receptor internalization and prolonged the retention of the ligand bound receptor on the cell surface. Zhu *et al.* (1995) subsequently showed that residues 621 and 622 on the LH/CG receptor are palmitoylated and that this palmitoylation did not affect coupling of the receptor to G_s. In addition, these workers mutated the two

conserved cysteines to alanines (to prevent palmitoylation) and found that the mutated receptor, although capable of binding hCG and transducing the cAMP signal, failed to insert into the plasma membrane.

Prior to its cloning, extensive studies on the molecular nature and size of the LH receptor were carried out (see reviews Ascoli and Segaloff, 1989 and Segaloff and Ascoli, 1993). One particular approach to elucidate the size of the LH/CG receptor involved the chemical cross-linking of ^{125}I-hCG to MA10 cells which express LH/CG receptors and from this study, it was demonstrated that the LH/CG receptor has a molecular mass of about 83 kDa on denaturing polyacrylamide gels (see review Segaloff *et al.,* 1990). In another study by Kim *et al.* (1987) using indirect immunoprecipitation of biosynthetically labelled LH/CG receptors, the LH/CG receptor in MA10 cells was identified as a 92 kDa protein which was able to bind hCG. This apparent 92 kDa protein of the biosynthetically labelled LH/CG receptor (as determined on SDS gels) was the same whether analyzed in the presence or absence of disulphide-reducing agents. Furthermore, in the presence of protease inhibitors, additional bands were not detected and the yield of the 92 kDa band was unaltered. A similar result was obtained by Metsikko (1984) by radiolabelling the sialic acid residues of rat luteal membranes; this study reported a 90 kDa band that was able to bind hCG and the size of the protein did not differ in the presence or absence of disulphide-reducing agents. Thus, data from the indirect immunoprecipitation of the biosynthetically labelled LH/CG receptor suggested that this receptor is a single polypeptide. The difference in size of the LH/CG receptor observed in immunoprecipitation protocols (92 kDa) versus that observed by chemical cross-linking

of the hormone to the receptor (83 kDa) can be explained as follows. The indirect immunoprecipitation method allows visualization of the free receptor on SDS gels, whereas the cross-linking approach involves the visualization of the hormone-receptor complex on SDS gels whereby the size of the receptor is estimated by subtracting the contribution of the hormone to the molecular mass of the complex. Of the two estimates, the size determined by the indirect immunoprecipitation method (93 kDa) seems to be more accurate. However, larger forms of the receptor have also been reported. Dattatreyamurty *et al.* (1983) reported the purification of LH/CG receptors from corpora lutea which had a molecular mass of 240-280 kDa after SDS-PAGE under reducing conditions. This high molecular weight band was found to be composed of two identical disulphide-linked units of 120 kDa each. Each of the units was further made up of two subunits of 85 and 38 kDa. Wimalasena *et al.* (1983) and Wimalasena and Dufau (1982) described the isolation by lectin affinity chromatography of rat ovarian LH/CG receptor in five molecular species ranging from 12-165 kDa that retained hormone binding activity. Bruch *et al.* (1986) reported a preparation of rat ovarian LH/CG receptor by using reverse immunoaffinity and affinity chromatography having a molecular mass of 240 kDa made of four species, two major 79 and 64 kDa and two minor 55 and 47 kDa proteins, as indicated by SDS-PAGE under reducing conditions.

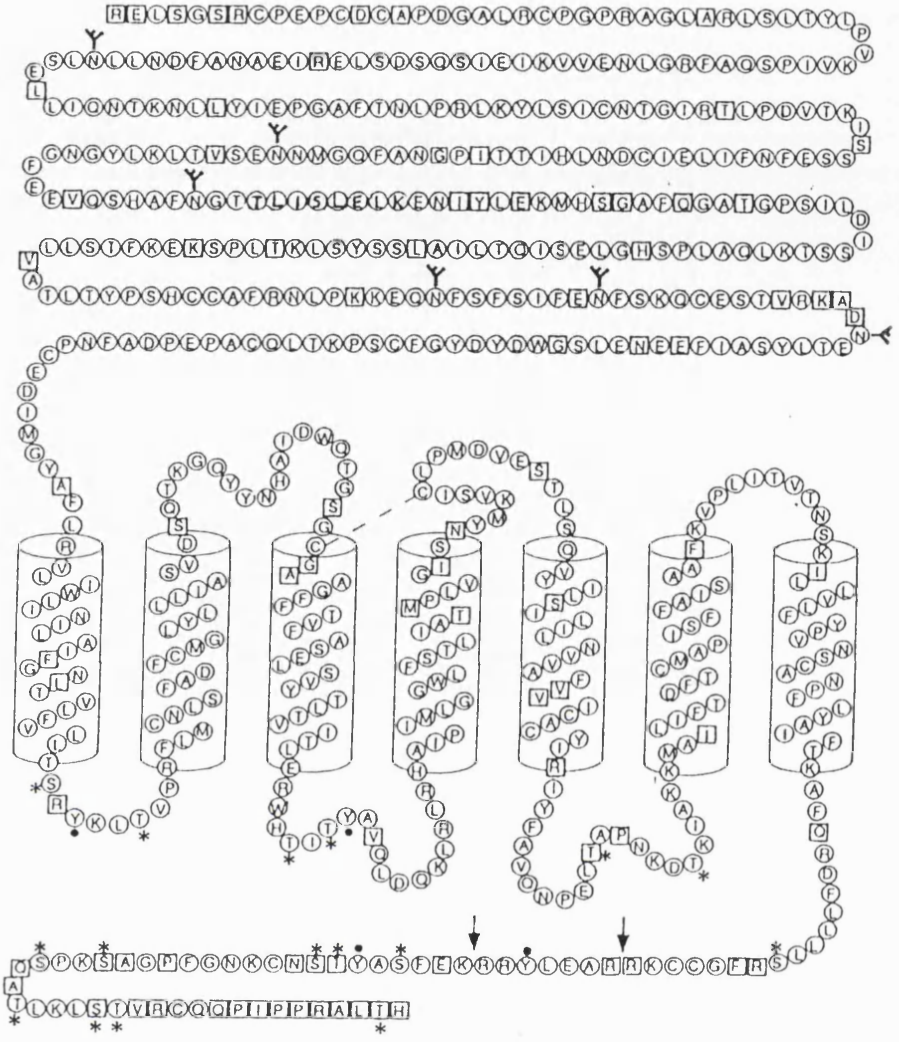

Figure 1.5 Structure of the LH/CG receptor. Amino acids that are identical between the rat luteal (McFarland *et al.,* 1989) and the porcine testicular (Loosfelt *et al.,* 1989) LH/CG receptors are enclosed in circles. Those enclosed in squares are unique to the rat luteal receptor. Amino acids in barrels correspond to the putative transmembrane regions. Those amino acids above the barrels being extracellular. Those below the barrels being intracellular. Potential sites for N-linked glycosylation in the extracellular region are denoted by (Y). A potential disulphide bond between the first and the second extracellular loop regions is noted by the dashed line. Potential intracellular sites for phosphorylation are denoted by asterisks (serines and threonines) or dots (tyrosines). The two arrows in the cytoplasmic tail point to two clusters of basic amino acids which might represent potential tryptic cleavage sites (from Segaloff *et al.*, 1990).

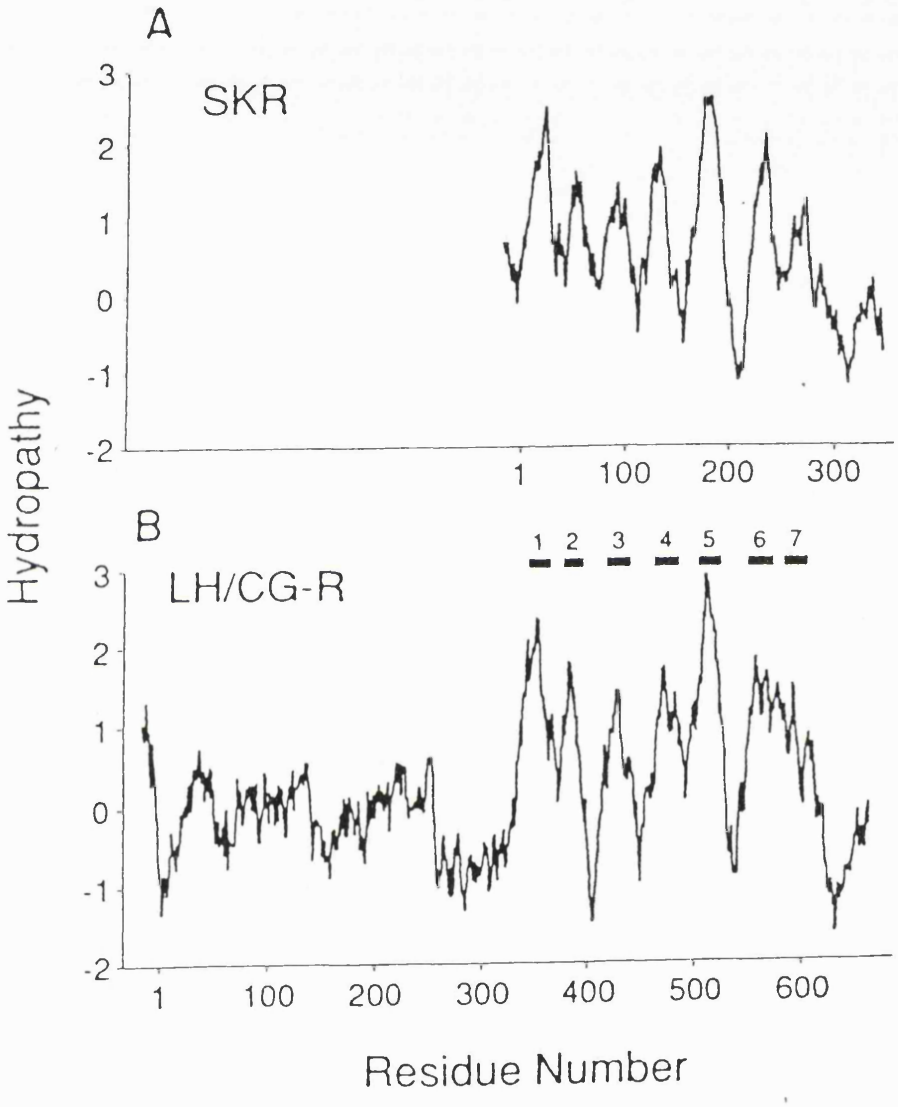

Figure 1.6 Hydropathy plot of the LH/CG receptor. Kyte and Doolittle hydropathy plots (window of 20 residues) for (A) the substance K receptor (SKR) and (B) the LH/CG receptor (LH/CG-R). The seven putative transmembrane regions of the LH/CG receptor are denoted by the numbered solid bars (McFarland *et al.*, 1989).

1.5.2 LH/CG receptor gene and mRNA

The LH/CG receptor gene was isolated from rat genomic libraries (Koo *et al.*, 1991; Tsai-Morris *et al.*, 1991). The coding region has been shown to span 60kb and consists of 11 exons and 10 introns. The first 10 exons encode the N-terminal extracellular domain of the receptor. Exon 11 encodes the C-terminal half of the molecule which includes the seven transmembrane domains and the entire C-terminal cytoplasmic domain. The last exon is similar in size to the intronless gene for the β-adrenergic receptor (Koo *et al.*, 1991).

The mRNA for the LH/CG receptor is expressed not only in testis and ovary, but also in brain (Lei *et al.*, 1993) and in thyroid (Frazier *et al.*, 1990). In both the ovaries and testes there are several transcripts varying from 1.2-7.7 kb. The size and the abundance of these transcripts vary from one species to another and also from ovary to testis. For example in the rat, four transcripts were detected (6.7, 4.3, 2.6 and 1.2 kb (see review Segaloff *et al.*, 1990)) and in MA10 cells, six transcripts were identified (7.7, 4.3, 2.6, 1.9, 1.6 and 1.2 kb (Wang *et al.*, 1991)). Since the cDNA that encodes the full-length LH/CG receptor contains at least 2.1 kb, it is evident that transcripts shorter than 2.1 kb cannot encode the whole receptor. Since one of the LH/CG receptor mRNA species is only 1.2 kb, it might represent a mRNA that encodes for a truncated form of the receptor, although there are no data yet to show that a truncated form of this particular receptor protein exists under physiological circumstances. It is important to note that it is not known which of the larger mRNAs is used for the translation of the LH/CG receptor protein.

1.5.3 LH/CG receptor gene/protein in non-gonadal tissues

LH/CG receptors which were once thought to be present only in the gonads, have now been found in several non-gonadal tissues and cells. Using reverse transcription-polymerase chain reaction (RT-PCR) Tao *et al.* (1995) amplified a 255 bp LH/CG receptor sequence from rat prostates. Northern blotting demonstrated that this tissue expressed 4.3, 3.3, 2.6, 1.8, 0.8 and 0.2 kb LH/CG receptor transcripts. However, the relative abundance in prostatic tissues was lower than in gonadal tissues. Western and ligand blotting demonstrated that prostatic tissues also contain an 80 kDa receptor protein which can bind ^{125}I-hCG and this binding was inhibited by excess unlabelled hCG. These same workers also found that normal human prostatic tissues, biopsies of benign prostatic hypertrophic tissue and prostate cancer tissues also express LH/CG receptor mRNA transcripts and receptor protein (Tao *et al.,* 1995), suggesting that LH may directly regulate prostate functions. Growth and regression of the prostate gland had been shown to be associated with castration (see review Cunha *et al.,* 1987). Previously, these growth changes in the prostate were assumed to be due to changes in androgen levels. However, since LH/CG receptors are present in the prostate, it is possible that these growth changes could at least partly be due to changes in LH levels. Hence LH/CG receptors may have relevance to both physiologic and pathologic regulation of prostate functions.

There have been numerous examples suggesting that the brain may also contain LH/CG receptors, but only recently has evidence emerged for their expression. Cerebrospinal fluid (CSF) and central nervous system (CNS) tissues of humans (Croxatto *et al.,* 1964; Bagshawe *et al.,* 1968) and other animals (Antunes *et al.,* 1979;

Hostetter *et al.,* 1981; Emanuele *et al.,* 1981; Emanuele *et al.,* 1983) contain low levels of LH and/or hCG. The CSF concentrations of these hormones in humans have been shown to increase when the brain contains LH- or hCG- producing tumours (Bagshawe and Harland, 1976; Braunstein *et al.,* 1981; Harris *et al.,* 1988). The presence of hormones in CSF and the CNS raises the possibility that they may act locally if the receptors are present in brain. Evidence for this came from Lei *et al.* (1993) who demonstrated, using RT-PCR and Northern blotting, that the rat brain expresses LH/CG receptor mRNA. This study demonstrated the existence of a major 2.6 kb and minor 1.8 and 4.4 kb transcripts. Western immunoblotting, ligand blotting, and covalent receptor cross-linking studies showed that rat brain also translates an 80 kDa receptor protein that can bind hCG and LH but not FSH. *In situ* hybridization, dot blotting, immunocytochemistry and topical autoradiography localized LH/CG receptor expression to the rat hippocampus, dentate gyrus, hypothalamus, cerebellum, choroid plexus, ependymal cells of third, fourth and lateral ventricles, cortex, brainstem, bovine hypothalamus and human area postrema. Since LH/CG receptors were found in areas of the brain that are involved in the regulation of the biosynthesis and secretion of LH, it has raised the possibility that LH might also act in an autocrine/paracrine manner to regulate its own synthesis.

Shi *et al.* (1993) also found that in human placental trophoblasts, hCG regulates its own synthesis by promoting the differentiation of cytotrophoblasts (which make little hCG) into syncytiotrophoblasts (which make considerable amounts of hCG) (Hoshina *et al.*, 1982 and 1985; Kliman *et al.*, 1986; Daniels-McQueen *et al.*, 1987). It has also

been shown previously that cytotrophoblasts and syncytiotrophoblasts in human placental tissue express LH/CG receptors (Reshef *et al.*, 1990; Lei and Rao, 1992).

LH/CG receptors have also been recently found in human endometrial and myometrial cells and uterine vasculature. Lei *et al.* (1992) used Northern blotting and *in situ* hybridization analyses to reveal that human uterus contains a major 4.3 kb and a minor 2.6 kb LH/CG receptor mRNA transcript and that these transcripts are expressed in endometrial and myometrial vascular smooth muscle cells and vascular endothelial cells. Immunoblot and immunocytochemical analyses revealed that the human uterus also contains a single immunoreactive receptor protein and that this receptor protein is present in endometrial and myometrial vascular smooth muscle and vascular endothelium. They found that the expression of receptor mRNA and/or immunoreactive receptor protein was higher in myometrial than in endometrial blood vessels and higher in vessels of both uterine compartments from the secretory compared to proliferative phase of the menstrual cycle, postmenopause, or pregnancy. A blood vessel seen traversing through perametrium immunostained for the receptor protein only after it entered the myometrium. The blood vessels in tissues that are not targets for LH/CG did not immunostain, whereas those of some (but not all) LH/CG target tissues, immunostained for the receptor protein. These findings suggest that LH/CG may directly regulate blood flow in the human uterus and other target tissues, perhaps by decreasing vascular resistance and/or increasing vascular endothelial cell proliferation. Toth *et al.* (1994) investigated the expression of LH/CG receptor mRNA and protein in human uterine arteries. These workers found that uterine arteries express multiple LH/CG receptor mRNA transcripts and an 80 kDa immunoreactive receptor

protein both in endothelial and smooth muscle cells. The extra- and intramyometrial arteries and the isolated 80 kDa receptor protein bound ^{125}I-hCG, which was inhibited by excess unlabelled hCG. The receptor mRNA, receptor protein and ligand binding were higher in smaller intramyometrial arteries than in larger extramyometrial arteries. Incubation of uterine arteries with highly purified hCG resulted in a concentration-dependent increase in the formation of vasodilatory eicosanoids and decrease in the formation of vasoconstrictive eicosanoids. Uterine blood flow was selectively increased by 16h after hCG administration for ovulation induction in women and was found to be a direct effect of hCG. This study therefore suggests that LH/CG receptors in human uterine arteries have important implications for physiological regulation of uterine blood flow.

Several previous studies have indicated that hCG has immunoreactive properties and alters the functions of human lymphocytes (Younger *et al.,* 1969; Kaye and Jones, 1971; Jenkins *et al.*, 1972; Adcock *et al.*, 1973; Beck *et al.,* 1977; Yagel *et al.,* 1989). However, it has not been determined whether the gene encoding the receptor for LH/CG is expressed in human lymphocytes. Lin *et al.* (1995) isolated total peripheral mononuclear lymphocytes from blood samples of pregnant women and found that these cells contained mRNA transcripts encoding the LH/CG receptors and a 50 kDa receptor protein which bound ^{125}I-hCG. T-lymphocytes isolated from total mononuclear cell fractions also expressed these receptor mRNA transcripts as well as the receptor protein. The levels of receptor transcripts and receptor protein were lower in lymphocytes than in ovarian tissue. Lymphocytes have previously been shown to produce hCG which is structurally and functionally similar to trophoblast hCG

(Harbour-McMenamin *et al.,* 1986). It is therefore possible that lymphocytes may have an autocrine system involving their own hCG and receptors.

In summary, LH/CG receptors, as well as being present in gonadal tissues, have now been identified in prostatic tissue, certain areas of the brain, endometrial and myometrial cells, uterus, uterine arteries, placental tissue and lymphocytes.

1.5.4 Hormone binding region of the LH/CG receptor

Work has been ongoing for a long time in order to identify the hormone binding regions of the LH/CG receptor. In one study by Roche *et al.* (1992), a comprehensive series of overlapping synthetic peptides were used to study the relationship between the primary structure of the ovarian receptor for LH/CG and hormone binding. Twenty-four consecutive, overlapping peptides that replicate the entire extracellular domain of the rat luteal receptor were synthesized, as well as three additional peptides replicating the putative extracellular loop regions. Each peptide was evaluated in radioreceptor assays for interaction with hCG by measuring its ability to inhibit competitively the binding of ^{125}I-hCG to the ovarian membrane LH/CG receptor. They found four independent receptor regions that can interact with the hormone. One site near the NH_2-terminus was localized to receptor amino-acid residues Arg^{21}-Pro^{38}. Two more sites of hormone interaction were identified by peptides replicating residues Arg^{102}-Thr^{115} and Tyr^{253}-Phe^{272}. A fourth binding region was identified in the third extracellular loop, replicated by rat luteal receptor peptide Lys^{573}-Lys^{583}. When the amino acid sequences of the four active rat LH/CG receptor regions were aligned and compared with published sequences for other glycoprotein hormone receptors, three

of the four regions (Arg^{102}-Thr^{115}, Tyr^{253}-Phe^{272} and Lys^{573}-Lys^{583}) showed high sequence homology with the human LH/CG receptor, human TSH receptor and rat FSH receptor and hence may represent contact sites for the α-subunit of hormone. The other binding region, Arg^{21}-Pro^{38}, had low sequence homology with the other glycoprotein hormone receptors and was postulated to be a specific binding determinant for the LH/hCG β-subunits.

In another study, two mutant LH/CG receptors were constructed, one containing exons 1-10 (LH/CG $receptor_{exon\ 1\text{-}10}$) and the other containing exon 1 and 11 (LH/CG $receptor_{exon\ 1\&11}$). These mutants were then functionally expressed in COS 7A cells (Ji and Ji, 1991). The LH/CG $receptor_{exon\ 1\text{-}10}$, which lacks the transmembrane domains, showed high affinity binding for hCG when COS 7A cells (transfected with LH/CG $receptor_{exon\ 1\text{-}10}$) were solubilized in non-ionic detergent. The LH/CG $receptor_{exon\ 1\&11}$ also bound hCG, but with low affinity and this type of receptor was capable of stimulating cAMP production. These results demonstrated that exons 2-10 encode a high affinity LH/CG binding site and exon 11 encodes the site for receptor-modulation to activate G-proteins.

Nagayama *et al.*, (1990) constructed a series of LH/CG-TSH receptor chimeras by homologous substitution of corresponding regions of the extracellular domain in order to locate the site of high affinity hormone binding. Amino-acid residues 1-83 and 316-367 were shown not to be involved, whereas aa residues 83-316 were essential for hormone binding and activation.

These results therefore demonstrate that the N-terminal region of the LH/CG receptor contains the high affinity hormone binding site.

1.5.5 LH/CG interaction with G-proteins and the adenylyl cyclase system

LH/CG interacts with their receptor at the plasma membrane level to activate the adenylyl cyclase system to form adenosine 3':5'-cyclic monophosphate (cAMP) (see review Cooke, 1983), as well as directly activating other transducing systems (see review Rommerts and Cooke, 1988). These alternative signal transduction pathways include the formation of inositol 1,4,5-trisphosphate (IP_3) which has been demonstrated in ovarian cells, but not Leydig cells (see review Rommerts and Cooke, 1988). The activated breakdown of phospholipid by phospholipase C (PLC) results in the liberation of diacylglycerol (DAG). This compound can directly stimulate protein kinase C (PKC) and can be further metabolized by the action of DAG lipase to release arachidonic acid (AA). AA is metabolized to leukotrienes via the lipoxygenase pathway which has been implicated in the regulation of steroidogenesis in Leydig cells (Dix *et al.*, 1984; Sullivan *et al.*, 1988). Calcium is also required to attain maximal LH/CG-stimulated steroidogenesis in all steroidogenic cells including Leydig cells. There are two basic mechanisms by which cytosolic calcium levels can be increased; firstly by releasing calcium ions from intracellular store sites and secondly by moving calcium ions from the extracellular space into the cell through the plasma membrane. The first mechanism usually involves the activation of PLC followed by the formation of the two second messengers DAG and IP_3, the latter being responsible for the release of calcium ions from intracellular stores. Although it has been reported that LH can stimulate the turnover of phospholipids and formation of inositol phosphates in rat

granulosa and bovine luteal cells (Davis *et al.*, 1986, 1987), Ascoli *et al.* (1989) showed that LH/CG does not stimulate inositol phosphate accumulation in MA10 cells. Furthermore, Nikula and Huhtaniemi, (1988) demonstrated that LH-stimulated activation of PKC via DAG in Leydig cells does not occur.

Under physiological conditions, G_s is regulated by hormone receptors, which, when activated by agonists, promote the release of bound GDP and binding of GTP. G_s with bound GTP dissociates to yield $G_{s\alpha}$-GTP and $G_{\beta\gamma}$. $G_{s\alpha}$-GTP is the species that is directly responsible for activation of the cyclase catalytic unit. Hydrolysis of bound GTP to GDP by a GTPase activity intrinsic to $G_{s\alpha}$ terminates the signal. Activation of G_s thus involves binding of GTP and dissociation of the α from the $\beta\gamma$ subunits. Inactivation is associated with the hydrolysis of GTP to GDP and reformation of the $\alpha\beta\gamma$ heterotrimer.

The stimulatory effect of LH/CG on adenylyl cyclase is mediated by the GTP binding protein, G_s. Cholera toxin (CTX) ADP-ribosylates the $G_{s\alpha}$ subunit, blocking its capacity to hydrolyse bound GTP to GDP, such that the G protein α subunit remains locked in the active state (see review Gilman, 1987). Hence adenylyl cyclase is persistently activated in the absence of hormone. CTX has been shown to increase cAMP production and steroidogenesis in intact Leydig cells (Cooke *et al.*, 1977; Dufau *et al.*, 1978).

The adenylyl cyclase system of gonadal cells like those of other cell types (see review Birnbaumer *et al.*, 1990), can be negatively regulated by the inhibitory GTP binding

protein, G_i. This was demonstrated in Leydig cells by the use of pertussis toxin (PTX), which inactivates G_i through ADP-ribosylation of the α_i subunit (Platts *et al.,* 1988). Hence G_i, which is present in Leydig cells, is involved in the negative modulation of cAMP and hence steroidogenesis.

Forskolin was originally discovered as a stimulator of adenylyl cyclase and has been shown to interact directly with the catalytic subunit of adenylyl cyclase (Seamon *et al.*, 1981). It has the ability to activate adenylyl cyclase directly in intact cells and tissues, membranes and detergent solubilised and purified preparations of adenylyl cyclase. Tritiated forskolin was used in a study by Seamon and Daly (1986) to detect high-affinity binding sites for forskolin in membranes from a number of tissues and they concluded that high-affinity forskolin binding sites are associated with complexes of the adenylyl cyclase catalytic subunit and the G_s protein. Forskolin was therefore used in the present study to directly activate the adenylyl cyclase system in Leydig cells.

1.5.6 Regulation of LH/CG receptors

In addition to the stimulatory actions of LH and hCG on steroidogenesis in Leydig and ovarian cells, these hormones also cause a refractoriness or desensitization of that same steroidogenic response. This may involve a loss of LH receptors (down-regulation), an uncoupling of the LH receptor from the adenylyl cyclase, an increase in the metabolism of cAMP due to an increased phosphodiesterase activity and a decrease in the activities in some of the enzymes in the pathways of steroidogenesis (see review Rommerts and Cooke, 1988).

The ability of LH/CG to modulate the number of LH/CG receptors was first demonstrated by Sharpe (1976; 1977) and Hsueh *et al.* (1976; 1977). These investigators found that the binding of radiolabelled hCG to rat testis decreases as a function of time after injecting the rats with either LH or hCG (homologous down-regulation). Subsequent experiments by Hsueh *et al.* (1976); Tsuruhara *et al.* (1977); Huhtaniemi *et al.* (1978; 1981) have shown that the homologous down-regulation of testicular LH/CG receptors is sometimes preceded by a phase of homologous up-regulation. Homologous up-regulation of testicular LH/CG receptors was detected within 4 hours of injection of hCG and was maximal by 6 hours. Within 24 hours, the up-regulated receptors returned to control levels and, at longer times, homologous down-regulation occurred. The magnitude of homologous up- and down-regulation was dependent on the dose of hormone injected. In addition to the ability of exogenous LH or hCG to regulate testicular LH/CG receptors, elevations in the endogenous levels of LH induced by single injections of GnRH also induces down-regulation of rat testicular receptors (Catt *et al.*, 1979a; Dufau *et al.*, 1979).

The homologous up- and down-regulation of LH/CG receptors are due to changes in the number of receptors without changes in affinity. Under optimal conditions, the LH/CG receptors can be up-regulated 1.5- to 2.5-fold and down-regulated almost completely (Tsuruhara *et al.*, 1977; Huhtaniemi *et al.*, 1981).

There are also studies on the regulation and processing of LH/CG receptors based on the fate of radiolabelled LH/CG. Labelled LH/CG injected *in vivo* or incubated with cultured cells *in vitro* has been localized to the plasma membranes, submembranous

smooth and coated vesicles, lysosomes and Golgi membranes (see review Roche and Ryan, 1985). It was therefore postulated that the receptor underwent receptor mediated endocytosis (RME). Catt *et al.* (1979b) and Cooke *et al.* (1986) found the receptor to be in a highly dynamic state, being continually internalized into endoplasmic vesicles and recycled back to the cell surface during short term exposure (up to 24 hours) of Leydig cells to LH/CG *in vitro*.

The whole process of RME for the LH/CG receptor is temperature dependent. At 4°C movement of the hormone / receptor complex inside the cell does not occur and at 21°C there is hormone accumulation within the cytoplasm without degradation or release from the cell. At 34°C (the normal temperature which Leydig cells exist), internalization, degradation and loss of the degraded hormone fragment from the cell occurs (Habberfield *et al.,* 1986).

Recycling of the LH/CG receptor has been demonstrated in rat (Habberfield *et al.*, 1986) and porcine (Genty *et al.,* 1987) Leydig cells. In MA10 cells LH/CG receptors were found to be continually synthesized and not recycled (Lloyd and Ascoli, 1983). Salesse *et al.* (1989) showed that LH and hCG are both internalized by the same pathway.

1.5.7 Desensitization of the LH/CG receptor

When the ability of the hormone / receptor complex to activate adenylyl cyclase is diminished without a loss of membrane receptors, then this phenomenon is termed "desensitization". This can involve homologous (exposure of the cells to LH or hCG

(Ekstrom and Hunzicker-Dunn, 1989)) as well as heterologous (exposure of the cells to other hormones, growth factors, or second messenger analogues (Rebois and Patel, 1985; Inoue and Rebois, 1989)) desensitization (see review Segaloff *et al.*, 1990). Phosphorylation of the β-adrenergic receptor has been shown to be involved in the mechanism of desensitization (Hausdorff *et al.*, 1990; Lefkowitz *et al.*, 1990). Due to the similarity in structure of the β-adrenergic and LH receptors it has been suggested that the desensitization of the LH receptor also occurs as a result of receptor phosphorylation. Minegishi *et al.* (1989) reported that the purified receptor from rat testes can be phosphorylated *in vitro* on serine and threonine residues by the catalytic subunit of the cAMP-dependent protein kinase (PKA) in a hCG-concentration-dependent manner. Agonist-stimulated phosphorylation of transfected rat LH/CG receptors in response to a cAMP analogue and a phorbol ester has also been demonstrated (Hipkin *et al.*, 1993). In these studies, however, stoichiometry of LH/CG receptor phosphorylation was not reported. Lamm and Hunzicker-Dunn (1994) demonstrated that under cell-free conditions, LH/CG receptors in porcine preovulatory follicles are also rapidly phosphorylated by exogenous PKA. However, PKA-dependent phosphorylation of the LH/CG receptor, even in the presence of hCG, occurred at low stoichiometry (0.01 mol phosphate/mol receptor) since the receptor does not possess a strict consensus sequence for PKA phosphorylation (Arg-Arg-X-Ser/Thr). Although PKA catalyzed the phosphorylation of the receptor, PKA did not promote desensitization of hCG-responsive adenylyl cyclase activity. The same workers also demonstrated that hCG-induced desensitization of cAMP synthesis in porcine follicular membranes was unaffected by activation or inhibition of endogenous PKA or by the addition of exogenous active PKA (Lamm *et al.*, 1994). Prior studies had also shown

that elevated cAMP (added to membranes or via agents that increase intracellular cAMP levels in intact cells) does not simulate LH/CG-induced desensitization (Bockaert *et al.,* 1976; Dix *et al.,* 1982; Rebois and Fishman., 1986). Additionally, desensitization of transfected rat LH/CG receptors has been shown to occur under conditions in which cAMP-mediated phosphorylation was greatly reduced or abolished (Hipkin *et al.*, 1993). In contrast, physiologically stoichiometric phosphorylation of β-adrenergic receptor by PKA is increased 2- to 4- fold in the presence of agonist and receptor phosphorylation is highly correlated with desensitization (Lefkowitz *et al.,* 1990; Hausdorff *et al.,* 1990; Lefkowitz, 1993).

Taken together, these results contribute to the accumulating evidence that the mechanism through which LH/CG receptor becomes inactivated or desensitized is distinct from that which is known for other G-protein-linked receptors.

1.5.8 Proteolysis of receptors, proteases, and binding proteins

Many proteins lead a dual existence as both membrane-bound and soluble isoforms. In general terms, soluble and membrane-bound isoforms of the same protein can be generated by one of two possible mechanisms. Firstly, by separate biosynthetic pathways, either by alternative pre-mRNA splicing of a common transcript or by transcription of closely related but distinct genes; and secondly, by post-translational release of the extracellular domain of membrane proteins at a site adjacent to the membrane-spanning sequence by proteolytic cleavage. In recent years numerous instances have been reported in which membrane proteins are apparently specifically released by proteolysis in a direct and sometimes regulated manner to produce active,

soluble forms. These soluble forms, which can act as soluble binding proteins, have been shown to consist of the extracellular domains of their respective receptors. It is likely that these soluble isoforms regulate the effects of cognate ligands by acting as soluble inhibitors of ligand-receptor interactions. Moreover, in cases where the binding proteins are produced by proteolytic release, the cells are desensitized through loss of the extracellular binding site from the cell-surface receptors.

A growth hormone (GH) binding protein has recently been identified in rabbit (Ymer and Herington., 1985), human (Herington *et al.,* 1986; Baumann *et al.,* 1986) and rat (Sadhegi *et al.,* 1990) serum. However, this soluble protein was hard to isolate (Ymer and Herington., 1985) due to its inability to precipitate in the binding assay used to measure the membrane-bound receptor (Herington and Veith, 1977). Using antibodies, it was possible to show that this GH binding protein shared several epitopes with the rabbit liver GH receptor (Barnard and Waters, 1986), indicating the structural similarity of the two proteins. Spencer *et al.*, (1988) isolated, characterized and performed partial amino-acid sequence analysis on purified proteins of the GH receptor from rabbit liver and the GH binding protein from rabbit serum. The existence of the GH binding protein was explained by the sensitivity of the rabbit liver GH receptor to proteolysis, together with their finding that the amino-terminal 37 residues of the binding protein were identical to the amino-terminus of the rabbit liver GH receptor. These workers then reached a plausible mechanism for generation of the GH binding protein by proteolytic cleavage of the rabbit liver GH receptor near the transmembrane region, thereby releasing the soluble hormone binding domain. These observations, showed that these proteins are intimately involved in the growth process. The

mechanism for production of the GH binding protein seems to be species-specific. Immunoprecipitation studies by Sadhegi *et al.* (1990) revealed that the circulating GH binding protein in the rat is generated by translation of an alternatively spliced mRNA. No equivalent alternative mRNA splicing mechanism has been positively identified to date in species other than rodents. Evidence from the rabbit and human, two species with very abundant GH binding protein serum concentrations, suggested that proteolytic cleavage of the extracellular domain of the GH receptor was the primary mechanism for GH binding protein generation in these species. This evidence is based on the presence of potential serine protease cleavage sites close to the transmembrane domain (Leung *et al.*, 1987). The most compelling evidence for this, however, comes from recent studies by Sotiropoulos *et al.* (1993) who have shown that cell models, devoid of endogenous GH receptors, when transfected with full-length rabbit GH receptor cDNA, but not rat GH receptor cDNA, release soluble GH binding proteins *in vitro.* Release of soluble GH binding protein activity must occur via post-translational mechanisms, almost certainly involving proteolysis of the rabbit GH receptor. No specific protease has yet been identified. It is of interest, however, that ubiquitin, a protein with intrinsic proteolytic activity, was reported to be covalently linked to purified rabbit liver GH receptor (Leung *et al.*, 1987).

Several studies have investigated whether a LH/CG binding protein might exist analogous to the GH binding protein. In a study by Tsai-Morris *et al.* (1990), the synthesis and secretion of a LH/CG binding protein was investigated in a eukaryotic expression system (COS-1 cells) expressing a LH/CG receptor cDNA with a 266bp deletion that results in truncation of the open reading frame and omission of the 1st

transmembrane domain. This study demonstrated secretion of LH/CG binding sites into the medium of the cell system used and that the extracellular domain of the LH/CG receptor possesses the required structure for high affinity binding of gonadotrophin. This soluble form of the LH/CG receptor may be of importance in modulating extracellular hormone concentrations by reducing the concentration of free LH available for interaction with membrane receptors, in a manner similar to that of the GH binding protein. Another study by Xie *et al.* (1990), in which a mutated construct of the rat luteal LH/CG receptor encoding for only the extracellular domain was used to transiently transfect human kidney 293 cells, demonstrated that high affinity binding sites for LH could only be detected in the solubilized cells of a eukaryotic cell expression system and were not secreted into the culture medium. This study raised the possibility that a LH/CG binding protein, consisting of the extracellular domain of the receptor might be expressed by gonadal cells but may remain trapped intracellularly.

It has been reported previously that proteolytic cleavage of hormone receptors at the plasma membrane is involved in down-regulation of hormone responses; e.g. for the epidermal growth factor (EGF) receptor, it has been shown that EGF receptor inactivation may be a direct or indirect result of proteolysis, either by preventing recycling of the receptor or by loss of cell surface receptors (Gross *et al.*, 1983). Similarly Hatzfeld *et al.* (1982) suggested that proteolysis of acetylcholine receptors, generated by increased production of plasminogen activator production, may influence the metabolism and concentration of surface receptors. Strulovici and Lefkowitz (1984) showed that the β-adrenergic receptor contains a trypsin-sensitive site which is exposed

on the outer surface of the plasma membrane. Proteolysis at this site releases a fragment of the receptor peptide which, while not directly involved in receptor binding or adenylyl cyclase activation, appears to be important for anchoring the receptor in the plasma membrane. Hence, proteolysis of the β-adrenergic receptor at this or other sites might also play a physiological role in regulating the processes of receptor recycling and receptor degradation. Several reports also indicate that the binding of certain hormones to their receptors triggers a conformational change which exposes on the outer surface of the plasma membrane a trypsin-sensitive site in the receptor sequence; e.g. insulin (Donner and Yonkers, 1983) and glucagon receptors (Iyengar and Herberg, 1984).

The possible involvement of membrane proteases as regulators of LH/CG function has also been suggested. Roche and Ryan (1986) have described the presence of numerous proteolytic enzymes within plasma membranes from luteinized rat ovaries, but their functional significance in the membranes remains uncertain. Protease inhibitors have been shown to inhibit the activation of adenylyl cyclase in rat ovarian and hepatic membrane preparations (McIlroy *et al.,* 1980) and a serine protease inhibitor (aprotinin) decreased the rate of association of hCG to its receptor in ovarian membrane preparations (but not in solubilized preparations of the LH/CG receptor) (Wilks and Hui, 1987). Kellokumpu and Rajaniemi (1985a) reported that a specific proteolytic processing of the LH/CG receptor occurs in rat ovarian membranes and that this processing is triggered by the binding of the gonadotrophin to its receptor. This proteolytic cleavage resulted in the release of two distinct ^{125}I-hCG-receptor fragment complexes with molecular masses of 96 and 74 kDa. Susceptibility to this endogenous

plasma membrane protease activity was increased with hCG binding (Kellokumpu and Rajaniemi, 1985b). Kellokumpu (1987) also investigated the existence of this process in a cultured mouse tumour Leydig cell line (MLTC-1) and concluded that the faster rate of hormone internalization in the MLTC-1 cells meant that this process of proteolytic cleavage was not evident in the MLTC-1 cells.

The regulation of the truncation of LH receptors was investigated recently by West and Cooke (1991) in two types of mouse tumour Leydig cells (MA10 and MLTC-1) and in rat testis Leydig cells and rat tumour Leydig cells (R2C). The MA10 and MLTC-1 cells down-regulate but do not appear to recycle their LH receptors (Lloyed and Ascoli, 1983). In order to distinguish between intracellular and extracellular proteolysis, these workers used NaN_3 (a metabolic inhibitor) to inhibit internalization as described previously by Habberfield *et al.* (1986). Desensitizing the cells with respect to steroid production (i.e. uncoupling of the LH receptor from its transducing system) was achieved by incubating the cells for 2h with LH (Freeman and Ascoli, 1981; Dix *et al.*, 1982). Loss of surface binding sites was also mimicked in the mouse tumour Leydig cells by inhibiting receptor internalization with NaN_3 and was prevented by the addition of protease inhibitors. Incubating the rat testis Leydig cells and R2C cells with protease inhibitors caused a 2- to 3-fold increase in binding sites and a 2- to 3-fold increase in LH-stimulated cAMP production. They concluded that proteolysis of LH/CG receptors in mouse tumour Leydig cells is involved in down regulation of the receptor and that proteolytic cleavage also occurred under conditions where internalization of LH/CG receptor complexes was inhibited. In contrast, in the rat Leydig cells they reported that proteolysis of the receptor is a continuous process

over the first four hours after cell isolation and that this participates in the normal regulation of the hormonal response of the cell *in vitro*. When rat testis Leydig cells and MA10 cells were incubated in the presence of ^{125}I-hCG, a radioactive protein complex with an approximate molecular mass of 80-90 kDa was detected in the incubation medium. They postulated that the peptide constituent of this complex (40-50 kDa) could be the extracellular domain of the LH/CG receptor released as a result of proteolysis and concluded that LH receptors are regulated by proteolysis at the plasma membrane in both mouse and rat Leydig cells.

1.6 Antibodies and glycoprotein receptors

The first report on the production of antibodies against the LH/CG receptor was published over 15 years ago (Luborsky and Behrman, 1979). These original antibodies bound the hCG-receptor complex solubilized from rat luteal tissue, as well as rat testes, but did not recognize the hCG-receptor complex solubilized from ovine or human ovaries. In addition these antibodies did not inhibit the binding of hCG to rat luteal membranes or intact luteal cells, although they inhibited hCG-stimulated progesterone production in these cells. The titre of these antibodies was also reported to be very low. Metsikko and Rajaniemi (1981) reported raising antibodies against a partially purified LH receptor preparation. These antibodies did not bind the hCG-receptor complex, but were able to inhibit the binding of hCG to homogenates of ovarian tissue. The same authors have also reported the production of antibodies raised against an affinity-purified preparation of the rat luteal LH/CG receptor (Metsikko and Rajaniemi, 1984). These were shown to bind the hCG-receptor complex and to inhibit the binding of hCG to a homogenate of rat luteal tissue. These antibodies also

recognized a 90 kDa band in Western blots of the affinity purified rat ovarian LH/CG receptor. Rosemblit *et al.* (1988) also produced polyclonal antibodies against the rat luteal LH/CG receptor. These antibodies recognized a 93 kDa single band on Western blots of partially purified rat luteal receptor, but failed to inhibit the binding of hCG to intact rat luteal cells or to detergent extracts thereof.

Site-directed anti-receptor antibodies also facilitated the study of other glycoprotein receptors. Endo *et al.* (1992) produced a rabbit antibody against a synthetic peptide corresponding to the N-terminal region of the human TSH receptor (aa residues 29-57). Using this antibody, they identified a 104 kDa recombinant protein expressed in CHO-K1 cells. This band existed regardless of the presence or absence of disulphide-reducing agents but was not detected in untransfected CHO-K1 cells. Under reducing conditions, the antibody also bound the rat receptor from FRTL5 cells identifying the same molecular size protein (104 kDa). Therefore the use of this anti-peptide antibody has facilitated the study of the structure of the TSH receptor.

Another use of anti-receptor antibodies has been in the identification of binding regions on receptors. Sakata *et al.* (1992) showed that the TSH binding regions of human TSH receptor reside in two areas within amino-acid residues 12-44 and 308-344. They raised antisera against four overlapping synthetic peptides representing these two regions of the TSH receptor (peptides 12-30, 24-44, 308-328, and 324-344) and investigated their ability to act either as thyroid stimulating antibodies (TSAb) or thyroid stimulation blocking antibodies (TSBAb) using cultured porcine thyroid cells. In addition to this, serum concentrations of triiodothyronine (T3) and thyroxine (T4)

were examined from each rabbit. They concluded from this study that residues 12-30 and 324-344 of the TSH receptor, respectively, are the site (at least a part of the site) where stimulating and blocking type immunoglobulins are directed. In another study trying to identify functional regions of the TSH receptor, Dallas *et al.* (1994) immunized rabbits with recombinant extracellular TSH receptor (ETSHr) protein and evaluated their antibody response by testing serial serum samples for IgG against the ETSHr protein and 26 synthetic peptides spanning the entire ETSHr. They identified three regions of the TSHr (aa residues 292-311, 367-386, and 397-415) through which antibodies blocked TSH binding. Furthermore, antibodies purified on either peptide 292-311 or peptide 367-386 affinity columns blocked both TSH binding and TSH-mediated activation of thyroid cells in culture.

Dattatreyamurty and Reichert *et al.* (1993) raised polyclonal antibodies in rabbits against a synthetic peptide corresponding to a unique region of the FSH receptor (aa residues 9-30), with no sequence homology to the LH and TSH receptor. They examined the characteristics of these antibodies relevant to receptor function and found that binding of [^{125}I]human FSH to membrane-bound receptors was inhibited in a concentration-dependent manner by the anti-FSH receptor anti-peptide antibody. Immunofluorescence staining of cultured rat Sertoli cells showed binding of this antibody to plasma membranes receptors. When detergent-solubilized membrane preparations from rat Sertoli cells were fractionated by SDS-PAGE under non-reducing conditions and then subjected to Western blot analysis, the anti-receptor peptide antibody, but not pre-immune rabbit serum, specifically recognized intact FSH receptor as a 240 kDa protein under non-reducing conditions and a 60 kDa protein was

identified under reducing conditions. Quintana *et al.* (1993) also prepared a polyclonal antibody against a synthetic peptide comprising residues 19-29 of the rat FSH receptor. The specificity of this antibody was documented using human embryonic kidney (293) cells and stable transfectants of 293 cells expressing the recombinant LH/CG and FSH receptors. From this study, they found that this antibody inhibited the binding of FSH but not that of LH/CG to their cognate receptors. This antibody also identified the recombinant receptor as a heterogeneous glycoprotein with a molecular mass of 58-83 kDa under non-reducing conditions and 69-81 kDa under reducing conditions.

1.7 Rationale for the use of anti-peptide antibodies to study the LH receptor

It has been known for a number of years that antibodies capable of recognizing an intact protein can be produced by short synthetic peptide immunogens which correspond in sequence to particular regions of that protein. Production of such antibodies is known to be most readily achieved by immunization with synthetic peptides bound to carrier proteins. Use of this anti-peptide antibody approach offers a number of advantages in the study of membrane receptors. This is due to the site-directed nature and pre-determined specificity of anti-peptide antibodies; features that distinguish them from antibodies produced by other procedures (reviewed by Lerner, 1982). By careful selection of the peptide sequences used as immunogens, it should be possible to raise antibodies that are specific for a protein in more than one species, but also that do not recognize other homologous proteins. Also antibodies can be raised to different parts (e.g. N- and C-terminus) of the same protein. Such antibodies are potentially more useful as probes than those raised using the whole protein as

antigen. For these reasons, the anti-peptide antibody approach was adopted for the present study, in order to study LH/CG receptor structure and function.

1.8 Aims of this project

The aim of the studies reported in this thesis was to elucidate the structure/activity relationships of the LH/CG receptor using specific site-directed anti-peptide antibodies as tools.

Antibodies to the LH/CG receptor have been available previously, but due to their poor titre and/or species and sex specificity, it has not been feasible to use such antibodies to study receptor function in different gonadal and non-gonadal cells. Therefore, the initial requirement was to produce peptide-specific antibodies that would recognize the receptor protein specifically and in its native form in both sexes in more than one species. Antibodies to both the extracellular and cytoplasmic domains of the receptor were to be raised in order to study possible cleavage of the receptor. The antibodies were also to be exploited as potential agonists/antagonists of ligand-induced signal transduction and steroidogenesis. The ability of the antibodies to interfere with ^{125}I-hCG binding and LH action in MA10 cells was also to be investigated. In order to examine the species and sex specificity of the anti-peptide antibodies, MA10 cells, rat ovarian tissue and rat testicular tissues were to be used. The antibodies were to be used as probes to study the distribution of LH/CG receptors in immunocytochemical analyses and in addition to this, it was proposed to investigate possible proteolytic fragments generated after receptor cleavage.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Pregnant mare's serum gonadotrophin (PMSG) was obtained from Intervet laboratories (Cambridge, U.K.) and crude hCG from Serono laboratories, (Welwyn Garden City, Herts, U.K.). Collagenase (197U/mg, Batch 49C948) was from Worthing Biochemical Corporation, (New Jersey, U.S.A.). MA10 cells were a gift from Dr. M. Ascoli of the University of Iowa, U.S.A. Cyclic AMP antisera was obtained from Dr. M. Schumacher, Hamburg. Progesterone antisera was provided by Dr. M. J. Sauer, MAFF, Central Veterinary Laboratory (New Haw, Weybridge, Surrey, U.K.). Dulbecco's Modified Eagles Medium, penicillin/streptomycin, Waymouth's MB752/1 medium (with L-Glutamine), Ca^{2+}-Mg^{2+} free PBS and donor horse serum were purchased from Life Technologies Ltd, (Middlesex, U.K.). Dextran T500, sephadex G-25, and PD-10 columns were from Pharmacia biotech Ltd, (St. Albans, Herts, U.K.). [^{3}H]-progesterone (specific activity 103Ci/mmol), nitrocellulose Hybond-C membranes, Kodak Xray film, carrier free Na^{125}I (specific activity 100Ci/g) were purchased from the Radiochemical Centre, Amersham, Bucks, U.K. Endoglycosidase F-N-glycosidase F-free was obtained from Boehringer Mannheim Biochemica, (East Sussex, U.K.). Bio-Rad dye reagent, Tween-20, goat anti-rabbit IgG alkaline phosphatase conjugate and low range molecular weight prestained markers were obtained from Bio-Rad Laboratories Ltd, (Hemel Hempstead, Herts, U.K.). Centriprep-30 concentrators were purchased from Amicon, Inc. (Bevenly, MA0195, U.S.A.). Chloramine T, polyethylene glycol 6000 (PEG), glycerol, charcoal, diethylether, and nonidet P-40 were obtained from BDH Laboratories supplies, (Merck Ltd, Lutterworth, Leicestershire, U.K.). Scintillation fluid (Ultima Gold) was obtained from Canberra Packard Ltd, (Pangbourne, Berks, U.K.). N^{α}-Fluorenylmethoxycarbonyl-amino acid

pentafluorophenyl esters (Fmoc amino-acid pfp esters), polyamide-Kieselguhr resin, 1-hydroxybenzotriazole (HOBT) and ovalbumin were purchased from Milligen, (Watford, Herts, U.K.). Maleimidobenzoyl-*N*-hydroxysulphosuccinimide ester (sulphoMBS), and Sulpholink™ coupling gel were purchased from Pierce, (Chester, Chesire U.K.). ELISA plates and sterile plasticware were obtained from Nunc, Nunclon, Denmark. Ovine luteinizing hormone (batch oLH-26, potency: 2.3U/mg) and hCG (batch CR-127, potency: 14,900IU/mg) were donated by NIADDK, (NIH, Bethesda, MD, U.S.A.). All other reagents were purchased from Sigma - Aldrich Chemical Company Ltd, (Poole, Dorset, U.K.) or BDH, U.K. All aqueous solutions and buffers were made up in double distilled water.

2.2 Methods

2.2.1 Animals

Highly luteinized ovaries were obtained from 21 day old rats after injection with 50IU pregnant mare's serum gonadotropin (PMSG) followed 56h later with 25IU hCG. 30 day old animals were sacrificed by cervical dislocation (i.e. 7 days after hCG administration.

2.2.2 Preparation of crude rat Leydig cells

Crude rat testis Leydig cells were isolated from adult male Sprague-Dawley rats (200-250g) as described essentially by Platts *et al.* (1988) and modified by Choi and Cooke (1992). The rats were first anaesthetized with 300:1 (v/v) oxygen:halothane and killed by cervical dislocation. The rat's abdomen was washed with ethanol and the testes dissected out into a small volume of dissection medium (Dulbecco's Modified Eagles Medium DMEM 1.34% (w/v), 10mM HEPES, 0.1% (w/v) BSA, 500µg/ml streptomycin, 250IU/ml penicillin, pH 7.4). The testes were decapsulated and each placed in 3.5ml of medium (containing 7ml/rat dissection medium, 0.5mg/ml collagenase, 750µl/35ml trypsin inhibitor (1% solution), pH 7.4), and incubated at 37^{o}C in a shaking water bath (60-65 strokes/min) until the Leydig cell clumps were dispersed (approx. 45min; the time of digestion depended on the age of the rats). Dissection medium was added to make the volume up to 25ml and the universals were inverted 40 times. The suspension was left to settle and the medium removed with a 50ml syringe and filtered through a fine mesh gauze (60µm) into a 50ml universal container. The settled suspension was washed again with dissection medium by

inverting 20 times and then left to settle again. The supernatant was removed and filtered as above. The filtered supernatant was then centrifuged for 10min at 300*g* (bench top centrifuge), and the resulting cell pellet was resuspended in a small volume (1-2ml) of dissection medium.

2.2.3 MA10 cell culture

Stock cultures of MA10 cells were maintained in T75 culture flasks in Waymouth's MB752/1 medium (with L-Glutamine) + 15% (v/v) horse serum at 37^{O}C in an atmosphere of 5% (v/v) CO_2 in air. The medium was changed every two days. The stocks were split every 3-4 days depending on the cell density.

The cells were subcultured by washing the cells twice with 5ml Ca^{2+}-Mg^{2+} free phosphate buffered saline (PBS) before the addition of 3-4ml trypsin solution (0.5% (w/v) porcine trypsin and 0.2% (w/v) EDTA). The flasks were then incubated at 37^{O}C for 2-3min. Waymouths medium containing serum was then added to the flasks in order to inhibit the trypsin. The cell suspension was then centrifuged at 1000*g* for 10min and the cell pellet was resuspended in fresh medium. Cell numbers were determined using a haemocytometer and viability of the cells was scored by the exclusion of 0.4% (v/v) trypan blue dye. The experimental cultures were plated at a density of 1x10^{5} cells/ml in Waymouth's medium in 6, 12, 24, or 96 well plates and used 2-3 days after subculture.

2.2.4 Diaphorase Cytochemistry

This method is essentially as described by Aldred and Cooke (1983). Cells plated in 24 well plates were washed with 0.01M PBS and then incubated with 400µl 0.01M phosphate buffer pH 7.4 containing 0.001% (w/v) nitroblue tetrazolium and 0.003% (w/v) NADH for 30min at 37°C. After this incubation the reagent mixture was removed and the cells were fixed with 50% (v/v) ethanol/10% (v/v) formaldehyde solution. The cells were then incubated at room temperature for a further 30 min.

The percentage of positively stained cells (non viable) was estimated by counting a minimum of 5 fields of about 500 cells each under the light microscope.

2.2.5 3β-HSD Cytochemistry

This method was derived from Cooke *et al.* (1983). Cells ($2x10^5$) were placed in a 1.5ml microtube and an equal volume of 6% (w/v) dextran was added, before incubating the cells at -20°C for 1h in order to make the cells permeable. The thawed cells were then centrifuged and 400µl of the freshly prepared reagent mixture (5α-androstan-3β-ol-17-one (2mg/ml in DMF), nitroblue tetrazolium (1mg/ml in PBS), nicotinamide (1.6mg/ml in PBS), NAD^+ free salt (3mg/ml in PBS), phosphate buffer pH 7.4 0.01M, ratio 0.1:1.0:0.7:0.8:4.0 (v/v)) was added and incubated at 37°C for 1h. Aliquots (100µl) were centrifuged using a cytospin centrifuge at 1000*g* for 5min. The cells were allowed to dry and then fixed in ethanol/formaldehyde (50%:10%, (v/v)) solution for 10-30min. One drop of gelatine jelly was added and a cover-slip placed on top. A minimum of 5 fields were counted. The colourless cells were negative and

dark blue/purple cells were positive. Results were expressed as a percentage stained of total cell number in each field counted.

2.2.6 Synthesis of LH receptor peptides (continuous flow synthesis)

Peptides corresponding to residues Arg^{48}-Glu^{65} and Tyr^{187}-Asp^{206} (N-terminal peptides) and to residues Cys^{622}-Ala^{636} (C-terminal peptide) of the rat ovarian LH receptor were synthesized according to the method of Atherton and Sheppard, (1989) using the solid phase N^{α}-Fluorenylmethoxycarbonyl-polyamide (Fmoc-polyamide) method with N^{α}-Fmoc-amino acid pentafluorophenyl (pfp) esters. Continuous-flow synthesis was carried out on a Polyamide-Kieselguhr (Pepsyn KA) resin (1g) using a Cambridge Research Biochemicals Pepsynthesizer, with DMF as solvent. The resin, consisting of a polydimethylacrylamide gel held within the pores of an inert macroporous rigid Kieselguhr matrix containing an acid-labile 4-hydroxymethylphenoxyacetyl-norleucyl derivative, was purchased with the first amino acid attached (cysteine for the two N-terminal peptides). A cysteine residue was also attached to the N-terminus of the C-terminal peptide to facilitate coupling to the carrier protein, ovalbumin.

Prior to each coupling step, the N^{α}-Fmoc protecting group was removed by a 10min incubation with DMF containing 20% (v/v) piperidine, a weak base. After this deprotection step the resin was washed for 10min with DMF. The next amino acid was then coupled by recirculation of the corresponding N^{α}-Fmoc-amino acid pfp ester (3-fold molar excess over free amino groups) through the resin column for 60min in the presence of the catalyst 1-hydroxybenzotriazole (HOBT). Following the coupling step the resin was again washed for 10min in DMF.

Each peptide was cleaved from the resin support and side-chain protecting groups removed using 100ml of a 95%(v/v) trifluoroacetic acid (TFA) solution in distilled water, containing 2.5%(v/v) 1,2-ethanedithiol (EDT) and 2.5%(w/v) phenol as free radical scavengers. The time allowed for cleavage was normally at least 90min (for peptides containing 4-Methoxy-2,3,6-trimethylbenzene sulphonyl (Mtr) side chain protecting group for arginine residues the cleavage time was increased). Following cleavage, the peptide solution was filtered to remove the resin particles and then TFA and most of the scavenger molecules were removed by rotary evaporation at 30°C under vacuum. Residual scavengers and the products of the side-chain protecting groups were then removed by precipitation of the peptide from diethylether (80ml), followed by two washes of the precipitate with diethylether (80ml). The resultant peptides were then dried under vacuum and stored dry at -20°C (see *Chapter 3*).

2.2.7 Peptide characterization and purification

The purity of each peptide was assessed by reverse phase high performance liquid chromatography (HPLC) using a 90min gradient of 5-40% (v/v) acetonitrile in 0.1%(v/v) aqueous TFA at a flow rate of 1ml/min on a 300A pore-size, C_{18} column (Aquapore RP-300) attached to a Varian 5000 liquid chromatograph. Peptide samples (50μg of each) were loaded and detected by their absorption of light at 220nm.

The amino acid composition of each peptide was determined using a 4151 Alpha Plus amino acid analyzer (LKB) following their hydrolysis in 5M HCL containing 0.04% (v/v) 2-mercaptoethanol and 0.1% (w/v) phenol for 24h at 110°C (see *Chapter 3*).

2.2.8 Peptide conjugation

The following method, adapted from the procedure described by LaRochelle *et al.* (1985), is designed for the attachment of cysteine-containing peptides to carrier proteins. Ovalbumin 10mg was dissolved in 625µl 10mM sodium phosphate pH 7.4 and dialysed overnight at 4°C against 2l of the same buffer. The following day the solution was briefly centrifuged to remove insoluble particles and 10µl 50mM *N*-ethylmaleimide was added to 500µl of the supernatant (8mg protein) and incubated for 10min at 25°C to block free thiol groups on the ovalbumin. The cross-linking agent maleimidobenzoyl-*N*-hydroxysulphosuccinimide ester (sulpho-MBS:200µl) was then added slowly and incubated at 25°C for 30min. Excess *N*-ethylmaleimide and sulpho-MBS was removed by gel filtration using a sephadex (20 x 1cm) G-25 column equilibrated with 50mM sodium phosphate, pH 6.0. Fractions (1ml) were collected and their optical density was measured at 280nm. The peak fractions containing the activated carrier protein (3-4ml) were pooled and stored at 4°C. After dissolving 8mg peptide in 1ml 50mM Tris-HCl, 1mM EDTA pH 8.0 and 40µl 0.5M dithiolthreitol (DTT) was added and incubated for 1h at 25°C under nitrogen to ensure that the peptide thiol (-SH) groups were fully reduced. The reduced peptide was separated from the excess DTT by chromatography at 4°C on a sephadex G-10 (20 x 1cm) column equilibrated in 50mM sodium phosphate pH 6.0. Fractions (1ml) were collected, and the optical density of each fraction was measured at 230nm. The fractions containing the peptide were pooled together (3-4ml), mixed with the activated carrier and the pH was adjusted to 7.4 using 0.1M NaOH. This solution was then incubated for 4h at 25°C and dialysed against phosphate buffered saline pH 7.4. The

protein concentration was determined by the Lowry procedure (Lowry *et al.*, 1951) and the dialysed conjugate was stored at -70°C (see *Chapter 3*).

2.2.9 Production of anti-peptide antibodies

Antisera against the conjugates were raised in Dutch half lop female rabbits (starting weights 2kg) as follows. The conjugates (160μg protein) in 0.5ml 10mM sodium phosphate: 150mM NaCl (PBS) pH 7.2 were first emulsified with 1.5ml complete Freund's Adjuvant and then injected intradermally at multiple sites along the back. Additional ("booster") injections of antigen (100μg) in incomplete Freund's adjuvant were made after 10 and 12 weeks and then the animals were bled from the ear vein (test bleed 40ml) after another 2 weeks. The blood was allowed to clot in glass tubes for 16h at 4°C. Both red and white blood cells were removed by sedimentation at 1000*g* for 10min and the serum aspirated. Antisera were then treated at 56°C for 30min to inactivate complement and subsequently stored at -70°C. Control (pre-immune) sera were obtained from the rabbits prior to the first injection of peptide-conjugates (see *Chapter 4*).

2.2.10 Affinity purification of antibodies

2.2.10.1 Preparation of the column

Peptide-specific antibodies were purified from crude serum by chromatography on columns containing immobilized peptides. The latter were prepared by reaction of peptide with Sulpholink™ gel, which consists of a matrix containing iodoacetyl groups which react with peptide sulphydryl groups. Peptides (3mg) were dissolved in 500μl

50mM Tris-HCl: 5mM EDTA, (pH 8.5), and 50µl 0.5M DTT was then added and incubated for 1h at 25°C under nitrogen in order to ensure that the peptide SH groups were fully reduced. The reduced peptide was separated from excess DTT by chromatography at 4°C on a column (20 x 1cm) of Sephadex G-10 equilibrated in 50mM Tris-HCl: 5mM EDTA, (pH 8.5). 1ml fractions were collected and their absorbance was measured at 230nm. The fractions containing the peptide (3-4ml) were pooled (avoiding contamination with the DTT peak) and stored at 4°C.

Sulpholink™coupling gel (3ml) was washed with 20ml 50mM Tris-HCl: 5mM EDTA, (pH 8.5) in a sintered glass filter funnel at 25°C to remove the storage buffer (10mM EDTA, 0.05% sodium azide, 50% glycerol), then transferred to a 10ml screw-capped glass tube and centrifuged briefly and the supernatant was removed and discarded.

The peptide from the G-10 column was then added to the Sulpholink™gel and incubated at room temperature with gentle rotation for 25min in the dark (to prevent light-catalyzed oxidation of liberated iodide to iodine, which will react with peptide tyrosine residues). The incubation was continued in the dark for a further 30min without rotation, and the supernatant was again removed after centrifugation. Excess peptide was washed out on a sinter funnel with 30ml 50mM Tris-HCl: 5mM EDTA, (pH 8.5). In order to block excess iodoacetyl groups on the gel, 5ml 50mM cysteine in 50mM Tris-HCl: 5mM EDTA, (pH 8.5) was added and incubated for 1h at 25°C with gentle rotation. Non-covalently bound peptide was removed by transferring the gel back to the sinter funnel and washing with 50ml 1M NaCl. The gel was then

washed with 50ml PBS and transferred onto a 5ml chromatography column which was stored in PBS containing 0.02% sodium azide at 4°C.

2.2.10.2 Purification and elution

Each antibody serum sample (3-10ml) was repeatedly passed through the relevant peptide column for 2-3h. The column was then washed with 10mM sodium phosphate, 800mM NaCl, (pH 7.2) to remove non-specifically-bound protein. Bound IgG was then eluted with 5M $MgCl_2$, followed immediately by 10-fold dilution in distilled water as described by Baldwin (1994). Following elution, purified antibodies were dialysed overnight in 4l of PBS. All the above steps were carried out at 4°C. Purified antibodies were concentrated to a volume of 1-2ml, depending on the initial serum volume, using the centriprep-30 concentrators and were stored at -70°C. Protein content was later determined by the Bio-Rad or Lowry method. Using this procedure 0.5-1.0 mg of each affinity-purified antibody was obtained from each ml of serum (see *Chapter 4*).

2.2.11 ELISA for screening anti-peptide antisera

The abilities of antisera and affinity-purified antibodies to recognise synthetic peptides were assessed by ELISA.

Maxisorp 96 well microtiter plates were coated by addition to each well of 20ng synthetic peptides in coating buffer (50mM sodium carbonate buffer, pH 9.6) followed by drying down overnight in an evacuated desiccator at room temperature. The wells were then washed five times with 200µl wash buffer (phosphate buffered saline

containing 0.05% (v/v) Tween-20 and 0.02% (w/v) sodium azide (PBSA-T)) and further incubated with 200µl blocking buffer (PBSA-T containing 5% (w/v) non-fat milk powder) for 2h at 37°C. The wells were washed five times with wash buffer as before, and incubated with 100µl diluted primary antibody or pre-immune serum overnight at 37°C. Dilutions of antibody or pre-immune serum were carried out in antibody buffer (PBSA-T containing 1% (w/v) milk powder). The wells were then washed five times with wash buffer as described above and incubated for 2h with 100µl goat anti-rabbit IgG-alkaline phosphatase conjugate, diluted to 1/3000 in antibody buffer. The wells were again washed five times with wash buffer and then incubated with 100µl substrate (1mg/ml *p*-nitrophenyl phosphate (disodium salt) in 10mM diethanolamine, 1mM $MgCl_2$, pH 9.8) at room temperature. Bound antibody was detected by the addition of the substrate which yielded yellow *p*-nitrophenol upon hydrolysis. The latter was quantified by its absorbance at 405nm after 30min using an Anthos microplate reader (Denley, U.K.).

2.2.12 Membrane preparation and solubilization

Ovarian tissue from pseudopregnant rats (about 100mg) was homogenized in 1ml PBS (10mM sodium phosphate, 140mM NaCl, pH 7.4) containing 5mM EDTA and 5mM N-ethylmaleimide. After centrifugation at 120*g* for 5min, the supernatant was aspirated and then centrifuged at 27,000*g* for 30min. The crude membrane pellet was solubilized in 1 ml Triton X-100 PBS containing 5mM EDTA, 5mM N-ethylmaleimide, and 20% (v/v) glycerol, by stirring on ice for 30min. The suspension was centrifuged at 100,000*g* for 1h at 4°C and the resulting supernatant was concentrated using the Amicon centriprep-30 concentrators. The membranes from rat testes and liver were

prepared the same way. The solubilized membranes were then used for electrophoresis or stored at -70°C.

The membranes of MA10 cells and the endothelial cell line were prepared as described previously by Hipkin *et al.* (1992). Briefly, culture flasks containing the cells were washed twice with ice-cold buffer A (0.15M NaCl and 20mM HEPES, pH 7.4) and subsequently scraped into buffer A containing protease inhibitors (1mM phenylmethylsulfonylfluoride (PMSF), 1μM pepstatin-A, 1μM leupeptin, and 1mM EDTA). The cells were pelleted by centrifugation, lysed by vortexing in buffer A containing 0.5% (v/v) Nonidet P-40, 10% (v/v) glycerol and the protease inhibitors (buffer B), and centrifuged at 100,000*g* (Beckman ultracentrifuge) for 30min. The supernatant was concentrated using the centriprep-30 concentrators and the amount of protein was assayed by the Bio-Rad protein assay method.

2.2.13 Two phase separation membrane preparation

Membranes were prepared as described previously by Levi *et al.* (1982) with slight modifications as follows: Culture flasks containing MA10 cells were washed twice in ice-cold buffer A (0.15M NaCl and 20mM HEPES, pH 7.4), scraped into buffer A containing protease inhibitors (1mM phenylmethylsulfonylfluoride, 1μM pepstatin-A, 1μM leupeptin, and 1mM EDTA), and transferred to a 25ml centrifuge tube. The cell suspension was centrifuged at 220*g* for 10min at 4°C. The pellet was resuspended in 10ml buffer A (containing the protease inhibitors plus 1mM DTT and 0.2mM $MgCl_2$) and left for 3min at 4°C. The suspension was then homogenized (50 strokes) using a Wheaton-Downce homogenizer and a tight fitting pestle. Sucrose 1M (3.6 ml) was

then added immediately to bring the homogenate to iso-osmolarity, and centrifuged at 1000*g* for 5min at 4°C to remove unbroken cells and nuclei. The membrane fraction was then pelleted by centrifugation at 2,500*g* for 30min at 4°C.

2.2.13.1 Preparation of Dextran/PEG system

The dextran/polyethylene glycol aqueous two-phase system used to separate cell surface membranes from other cellular organelles was as originally described by Brunnette and Till (1971). The two phase system was prepared by mixing 200g of 20% (w/v) dextran T500 (calibrated by optical rotation using a polarimeter), 103g of 30% (w/v) PEG 6000, 100ml distilled water, 333ml 0.2M phosphate buffer (pH 6.5) and 80ml 10mM $ZnCl_2$. This solution was mixed, then allowed to separate into two phases at 4°C overnight and then stored separately.

The pellet from the 2,500*g* centrifugation was resuspended in 10ml of the lower phase and then 10ml of the upper phase was added and gently mixed. The mixture was then centrifuged at 1000*g* for 15min after which time the plasma membranes could be seen at the re-established interface. The plasma membranes were collected, resuspended in Tris buffer (10mM Tris HCl, 1.0mM DTT, pH 7.5), centrifuged for 30min at 2,500*g* and then suspended in the Tris buffer. Protein concentration was determined by the Bio-Rad method and the plasma membrane preparation was either used immediately, or stored at -70°C for not more than 4 days.

2.2.13.2 Adenylyl cyclase activity

Adenylyl cyclase was assayed by measuring the rate of formation of cAMP from ATP using the cAMP RIA. The assay was carried out as described essentially by Levi *et al.* (1982). Plasma membranes (10μg) were pre-incubated with/without antibodies for 1h, and then 1mM ATP, 5mM $MgCl_2$, 0.1% (w/v) BSA, 1mM EDTA, 0.5mM 3-isobutyl-1-methylxanthine (IBMX), 1.0mM DTT, 40mM Tris-HCl pH 7.5, 10mM creatine phosphate, 13.2U/ml creatine phosphokinase was added. The membranes were then stimulated for 1h with either 1μg/ml LH, 0.1mM guanosine 5'-[β,γ-imido]triphosphate (p(NH)ppG), 1μg/ml LH + 0.1mM p(NH)ppG or 10mM sodium fluoride (NaF). Each reaction was terminated by the addition of perchloric acid (final concentration 0.115M) and neutralised with tripotassium orthophosphate (final concentration 0.154M).

2.2.14 Protein assays

Two types of protein assays were used throughout this study, because certain reagents interfered with the two different protein assays.

2.2.14.1 Bio-Rad method

The stock solution of concentrated Bio-Rad dye was diluted 1/5 in distilled water and filtered. Aliquots of solubilized membrane were diluted 1/10 with distilled water. A standard BSA curve was constructed in the range 0-1mg/ml in distilled water. Standard or sample (20μl) were added to 1ml dye, mixed and incubated at room temperature for 5-10min, after which the extinction was measured at 595nm. The Bio-rad assay was used when Triton X-100 was present.

2.2.14.2 Lowry method

Reagent solution [2% (w/v) Na_2CO_3 in 0.1M NaOH, containing 0.5% (w/v) SDS, 0.01% (w/v) $CuSO_4.5H_2O$ and 0.02% (w/v) Na,K,tartrate] was made up immediately prior to use. Globulin-free BSA standards (0-100μg/ml) and samples (200μl) were diluted in distilled water. These were incubated with 1ml aliquots of reagent solution for 20min at 20°C. This was followed by incubation with a 1/2 dilution of Folin-Ciocalteau reagent in distilled water (100μl) for 30min at 20°C. Absorbance at 750nm was determined for each sample and the resultant standard curve obtained for protein content (0-100μg) was used to determine sample protein concentrations.

2.2.15 Enzymatic deglycosylation

The deglycosylation of the receptors with endoglycosidase-F (N-glycosidase F-free) was carried out according to the method described previously by Sojar and Bahl (1989). Deglycosylation with endo-F was carried out using the denatured receptor. A 1mg sample (solubilized receptor) in 250μl 100mM sodium acetate buffer pH 6.5, containing 0.5% (w/v) SDS and 0.5% (w/v) DTT was denatured by heating at 100°C for 5min. The denatured receptor was incubated overnight at 25°C with 2U of endo-F in 250μl 100mM sodium acetate pH 6.5, containing 1.25% (v/v) Nonidet P-40 and 10mM 1,10-phenanthroline hydrate. Enzyme reaction was terminated by heating in a boiling water bath with 300μl Laemmli's buffer (Laemmli, 1970). The SDS-PAGE was carried out in a 10% (w/v) gel under reducing conditions.

2.2.16 SDS-PAGE

A 40% stock acrylamide/bisacrylamide solution (ratio 37:1 (w/w)) was used to prepare the required percentage gels used, using the following equation:

$$V_r = C \times V_t/40\%$$

where V_r = required volume of 40% solution, C = final concentration of gel and V_t = total volume of mixture required

The separating gel (10%) was prepared by mixing 19.35ml distilled water, 10ml 40% acrylamide/bisacrylamide solution, 10ml separating gel buffer (1.5M Tris-HCl, pH 8.8), 0.2ml 20% (w/v) SDS, 7.5µl N,N,N',N'-tetramethylethylenediamine (TEMED), and 450µl 0.1% (w/v) ammonium persulphate. This solution was then poured into a vertical slab gel (size 16cm x 20cm) and overlayed with water and left to set for 2h at room temperature.

The stacking gel (5%) was prepared by mixing 6.95ml distilled water, 2.5ml 40% acrylamide/bisacrylamide solution, 2.5ml stacking gel buffer (0.5M Tris-HCl, pH 6.8), 50µl 20% (w/v) SDS, 5µl TEMED, and 100µl 0.1% (w/v) ammonium persulphate.

The water was removed from the top of the separating gel and the stacking gel solution was poured onto the separating gel, with a spacer comb placed between the plates. This solution was allowed to set for 2-4h at room temperature, after which the comb was removed and the gel placed in a tank (Protean II xi Vertical Electrophoresis Cells, Bio-Rad) containing 1.5l running buffer (0.025M Tris, 0.19M glycine, 0.1% (w/v) SDS, pH 8.3).

Aliquots of 50-100μg protein were mixed with an equal volume of Laemmli's sample buffer (0.0625M Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) ß-mercaptoethanol, 0.05% (w/v) bromophenol blue) and incubated for 15-30min at room temperature before being applied to the gel. Prestained low range markers of known molecular weight between 27.5-106 kDa were used as standards. The standards and samples were then pipetted into the chambers left by the comb. Proteins were stacked within the gel at 30mA constant current and then increased to 90mA to run the proteins through the separating gel until the dye front was 1cm from the base of the gel. The gel was then removed and used for protein staining, Western or ligand blotting.

For protein staining, the gels were placed in a container containing staining solution (10% (v/v) acetic acid, 25% (v/v) isopropanol, 0.025% (w/v) Coomassie blue R-250) and incubated overnight on a shaking platform. The following day, the gels were incubated with destaining solution (10% (v/v) acetic acid, 10% (v/v) isopropanol) for 5-6h again on a shaking platform.

2.2.17 Western blot analysis

After SDS-PAGE, the resolved proteins from acrylamide gels were transblotted onto nitrocellulose Hybond-C transfer membranes using an LKB 2117 Multiphor II electrophoresis unit at a constant current of 150mA for 90min in transfer buffer (39mM glycine, 48mM Tris, 0.0375% (w/v) SDS and 5% (v/v) methanol). After transfer, each piece of nitrocellulose was placed in a 150ml sterilin plastic bottle and washed for 10min in wash buffer (Tris buffered saline (TBS): 20mM Tris, 500mM

NaCl, pH 7.5) on a roller mixer. To prevent non-specific binding of antibodies, 50ml blocking solution (TBS-containing 0.2% (v/v) Tween 20 (TTBS) and 5% (w/v) milk powder) was added and incubated for 2h with gentle rolling at room temperature. The membranes were then washed with wash buffer (TTBS) twice for 5min. Primary antibody (crude antisera were routinely used at a dilution of 1/500, whereas affinity purified antibodies were used at 2μg/ml) or pre-immune IgG (at the same concentration, or dilution) was added in antibody buffer (1% (w/v) milk powder in TTBS) and incubated overnight at room temperature. Membranes were then washed three times for 15min with 100ml TTBS with gentle agitation and then 30ml antibody buffer containing 10μl goat anti-rabbit IgG-alkaline phosphatase conjugate (1/3000) was incubated for 1-2h at room temperature. The membranes were again washed 3 times with 100ml TTBS and then further washed twice for 10min with TBS to remove the Tween-20. The binding of antibody to the receptor was detected using the chromagens 5-bromo-4-chloro-3-indolylphosphate (15mg) and nitroblue tetrazolium (30mg) mixed in 100ml sodium carbonate buffer (0.1 M sodium bicarbonate, 1mM $MgCl_2$, pH 9.8).

2.2.18 Dot and slot blots

Increasing amounts of proteins (100ng-100μg) were immobilized on nitrocellulose membranes using a milliblot system. Proteins were reacted with antibodies and antibody binding was detected as described for the Western blots.

2.2.19 Iodination of hCG

Highly purified hCG (CR-127) was iodinated with ^{125}I according to the method of Thorell and Johanson (1971) using lactoperoxidase, to a spec ific activity of 100Ci/g and was purified by Sephadex G-25M chromatography.

A G-25M Sephadex chromatography column (PD-10) was washed with 30ml 0.01M PBS (0.9% (w/v) NaCl, 0.1% (w/v) BSA, pH 7.4) containing 5% (w/v) BSA, to block non-specific binding.

hCG (10μg) in 50μl 0.2M PBS (pH 7.4), was added to a tube containing a magnetic microflea situated on top of a magnetic stirrer, together with 15μl 0.2M phosphate buffer (pH7.5), 10μl $Na^{125}I$ (1mCi), and 10μl lactoperoxidase (0.5mg/ml solution in 0.05M PBS). This was then reacted together for 1min by the addition of 10μl 0.007% (v/v) H_2O_2. The reaction was stopped by the addition of 1ml 0.01M PBS containing 0.1% (w/v) NaN_3 and 10mM KI.

The reaction mixture was added to the column and allowed to enter the PD-10 column before continuing. ^{125}I-hCG was eluted with 0.01M PBS (0.9% (w/v) NaCl, 0.1% (w/v) BSA, pH 7.4). 8 drops/tube were collected in the first 4 tubes and 4 drops / tube in the remaining 26 tubes. The fractions were then measured for radioactivity in a γ-counter. The fractions that form the radioactive peak (see *Appendix*; fig.A1.2) were pooled. The ^{125}I-hCG was diluted with 0.01M PBS (0.9% (w/v) NaCl, 0.1% (w/v) BSA, pH 7.4) to give a concentration of 1ng^{125}I-hCG/μl, stored at -20^{o}C and used within 4 weeks of preparation. Estimation of the specific activity of ^{125}I-hCG was

carried out by competitive binding on crude rat testis Leydig or MA10 cells with known concentrations of purified unlabelled hCG. The results were expressed as a percentage of control binding and the concentration of pure hCG that displaced 50% of the activity was assumed to equal the concentration of the radioactive hCG (see *Appendix*; fig.A1.3).

2.2.20 Iodination of ScAMP-TME

2'-monosuccinyladenosine-3',5'-cyclic monophosphate tyrosyl methyl ester (ScAMP-TME) was radioiodinated as follows and as described by Brooker *et al.* (1979).

The C_{18} Sep-pak (Waters) column was washed with 20ml methanol, followed by 20ml of distilled water.

ScAMP-TME (800ng) (20µl of 40µg/ml succinyl-cAMP tyrosyl methyl ester in 0.005M sodium acetate; pH 4.75) was mixed with 20µl 0.5M potassium dihydrogen phosphate pH 7.0, and 10µl of $Na^{125}I$ (1mCi). 25µg of chloramine T (5µl of a 5mg/ml solution, in 0.5M potassium dihydrogen phosphate pH 7.0) was then added and the tube was capped and vortexed immediately for 1min at room temperature. The reaction was stopped by the addition of 50µl of sodium metabisulphite (5mg/ml sodium metabisulphite solution in distilled water). Sodium acetate buffer 0.1M (800µl) pH 4.75 was then added to the mixture.

The reaction mixture was loaded onto the C_{18} column. Propan-1-ol:0.1M sodium acetate buffer pH 4.75 (17.5:82.5) was used for elution and 1ml fractions were

collected in small glass tubes. The radioactivity was then measured using a γ-counter. The peak activity fractions containing the eluted ^{125}I-ScAMP-TME were pooled (see *Appendix*; fig.A1.4) (usually fractions 2-10)(Xml) in a glass scintillation vial and propan-1-ol was added as appropriate (Yml) to give a final propan-1-ol concentration of 30% (v/v) using the equation:

$$(\text{Xml} \times 17.5\%) + (\text{Yml} \times 100\%) = (\text{X}+\text{Yml}) \times 30\%$$

$$17.5\text{X} + 100\text{Y} = 30\text{X} + 30\text{Y}$$

$$70\text{Y} = 12.5\text{X}$$

$$\text{Therefore Yml} = 12.5/70 \times \text{Xml}$$

2.2.21 Binding of ^{125}I-hCG to crude rat Leydig cells

500μl cell suspension was incubated in LP4 tubes with 1ml dissection medium (containing 0.1% (w/v) methyl cellulose) for 2h at 37^{o}C in a shaking water bath (70 strokes/min) in order to allow the cells to recover from the collagenase treatment (methyl cellulose was added to help keep the cells in suspension). Iodinated hCG (40μl) was added to each tube, in the presence of increasing concentrations of unlabelled hCG (CR-127) (0-250ng/ml), and incubated overnight at 4^{o}C. Next day, the reaction was stopped by placing the tubes on ice for 15min and then washing the cell pellet twice with ice cold dissection medium (centrifuging each time at 1000*g*). The cell pellet was dissolved in 500μl 0.5M NaOH and counted in a γ-counter. Non-specific binding was determined by the addition of 300IU/ml crude hCG (this value was chosen as a saturating dose of hCG from a study in which 1-1000IU/ml of crude hCG was used to displace iodinated hCG (see *Appendix*; fig. A1.1)).

2.2.22 Ligand blot

Gels were prepared as described previously in the presence or absence of SDS, and proteins were mixed with Laemmli's sample buffer (without β-mercaptoethanol) and treated as described in section 2.2.17.

Proteins were transferred onto nitrocellulose membranes as described for the Western blot. The membrane protein samples were rinsed in PBS and incubated for 4h at room temperature in PBS containing 0.1% (w/v) BSA, 0.25% (w/v) gelatin, 1% (w/v) haemoglobin and 10% (v/v) glycerol. The nitrocellulose strips were then incubated overnight in 5ml PBS containing 0.1% (w/v) BSA, 10% (v/v) glycerol and $1x10^6$ cpm ^{125}I-hCG in the presence or absence of excess unlabelled crude hCG (10IU/ml) or in the presence or absence of antibodies. The strips were then washed in 10ml PBS containing 0.1% (v/v) Triton X-100 for 30min and finally rinsed in PBS, dried, and subjected to autoradiography on Kodak X-ray film at -70°C for 2-3 days (Keinanen *et al,* 1987). The molecular size of the receptor protein was determined by running molecular weight standards in an adjacent lane.

2.2.23 ^{125}I-hCG Competitive binding study

Antibody and pre-immune control IgG at concentrations 10 and 1μg/ml were incubated in 6 well plates containing MA10 cells (plated at a density of $1x10^6$ cells/well) for 1h at 37°C in Waymouth's medium + 0.1% (w/v) BSA. Cells were then incubated for 48h at 4°C in the presence of ^{125}I-hCG (500,000 cpm/well). The level of binding was determined by aspirating the medium, washing twice with ice cold PBS + 0.1% (w/v) BSA to remove unbound hormone and then dissolving the cells in 0.5M NaOH, before

counting in a γ-counter. Non-specific binding was determined in the presence of 300IU/ml unlabelled crude hCG.

2.2.24 Effects of antibodies on agonist stimulated cAMP generation and progesterone production

MA10 cells were grown in Waymouth's medium + 15% (v/v) horse serum and were sub-cultured at a density of $1x10^5$ cells/ml, in 96 well plates. Cells were preincubated for 1h with and without antibodies or pre-immune IgG in medium containing 0.1% BSA at antibody concentrations ranging from 0.01-100μg/ml. The cells were then challenged for a further 2h with 10ng/ml LH. In a second series of experiments, cells were incubated with a constant antibody concentration (10μg/ml), before being challenged with a range of LH concentrations (0-1000ng/ml). The reaction was then terminated by the addition of perchloric acid (final concentration 0.115M) and neutralized with tripotassium orthophosphate (final concentration 0.154M). Progesterone and cAMP were both measured in the medium by RIA.

Similar experiments were carried out in which cells were preincubated for 1h with and without antibodies or pre-immune IgG at a concentration of 10μg/ml in medium containing 0.1% BSA. The cells were then challenged for a further 2h with either LH (0-1000ng/ml), dbcAMP (0-100mM), cholera-toxin (0-50μg/ml) or forskolin (0-100μM). The reaction was then terminated and neutralized as described above and both progesterone and cAMP were measured in the medium by RIA.

2.2.25 Radioimmunoassays

When cAMP and progesterone production were being measured, the cell incubations were stopped as described above using perchloric acid and the plates stored at -20°C. This effectively lysed the cells so that any measurement of these products was a combination of extracellular and intracellular levels.

2.2.25.1 Cyclic AMP RIA

cAMP was determined by the method of Steiner *et al.* (1972) with the acetylation modification described by Harper and Brooker (1975). For the cAMP standard curve (range 10-5000fmol cAMP/100µl), cAMP standards in ethanol were aliquoted in triplicate then dried under nitrogen and redissolved in 100µl sample medium (medium as treated in experiment i.e. acidified and neutralized). Treated medium (100µl) was aliquoted for totals, non-specific binding (NSB), and zero concentration of cAMP (Bo). Samples were diluted as necessary in treated medium to a final volume of 100µl. Both the standards and the samples were then acetylated by the addition during vortexing of 5µl of the acetylating mixture, (2.7:1 triethylamine:acetic anhydride). The tubes were left to stand for at least 1h in a fume hood. Bovine gamma globulin (100µl) (0.3% (w/v) in 0.1M PBS (0.2M $NaH_2P0_4.2H_20$, 0.2M $Na_2HP0_4.12H_20$, 0.15M NaCl, 0.3mM NaN_3, pH 7.0)) was added to the totals and NSB. The cAMP antibody (MS1) was diluted in 0.3% (w/v) bovine gamma globulin (1/150 dilution) and 100µl of antibody solution was added to all tubes except totals and NSB. Iodinated cAMP in 0.1M PBS, (20,000cpm/100µl) was then added and the tubes vortexed and incubated overnight at 4°C.

PEG 6000 16% (w/v) (in tap water) (1.6ml) was dispensed into each tube (except the totals) to precipitate the antisera and bound cAMP. The tubes were vortexed and centrifuged at 3,000*g* for 30min at 4°C. The supernatant (free cAMP) was then aspirated to waste, the tubes dried and the pellets measured in a γ-counter.

2.2.25.2 Progesterone RIA

A standard curve for progesterone (range 0.25-31.79pmol/ml) was prepared by aliquoting standards in triplicate. Treated medium (100µl) was aliquoted for totals, non-specific binding (NSB) and zero concentration of progesterone (Bo). Samples were diluted as appropriate in treated medium to a final volume of 100µl. PAS-gelatin buffer (PGB) (100µl) was added to totals and NSB tubes. Progesterone antibody (100µl: diluted 1/4000 in PGB) (characterized previously for use in an EIA (Hodges *et al.*, 1988)) was added to all tubes except totals and NSB. [^{3}H]- progesterone (100µl) (specific activity 103Ci/mmol) in PGB containing 10,000cpm was added to all tubes. The solution was mixed by vortexing and incubated overnight at 4°C. The following day, ice-cold PGB (500µl) was added to the total tubes. Dextran-coated charcoal suspension 500µl (containing 2.5mg charcoal/ml and 0.25mg dextran/ml in 0.01M phosphate buffer (0.2M $NaH_2PO_4.2H_2O$, 0.2M $Na_2HPO_4.12H_2O$, pH 7.0)) was added to the remaining tubes at 4°C in order to precipitate the free [^{3}H] progesterone. The tubes were then mixed by vortexing and centrifuged at 1000*g* for 10min at 4°C. The supernatant was decanted into scintillation vials and 2ml scintillation fluid was added. The vials were then vortexed and counted for 5min in a liquid scintillation counter to determine the proportion of antibody bound [^{3}H] progesterone.

CHAPTER 3

PEPTIDE SYNTHESIS

3.1 Introduction

3.1.1 Peptide synthesis: The solid phase

Solid phase peptide synthesis was first described by Merrifield (1963). In 1964, Merrifield described the technique in more detail with substantial changes, which resulted in greatly improved chemical efficiency.

The principle of solid phase synthesis basically involves the elaboration of the growing chain (peptide) while it is attached to a stable, solid particle (resin). It remains attached to this particle throughout all the synthetic steps and is separated from soluble reagents and solvents by simple filtration and washing. Finally, the desired product is detached from the solid support and purification and characterisation are carried out in free solution.

3.1.1.1 Instrumentation

The Cambridge Research Biochemicals "Pepsynthesizer" was used for continuous flow solid phase synthesis. Essentially, a reaction vessel in the form of a glass column equipped with sintered glass or polytetrafluoroethylene (PTFE) filters at the top and bottom is required, together with a peristaltic-type pump for flowing solvent and reactants through the resin bed to waste and for recirculation. Addition of activated N^{α}-fluorenylmethoxycarbonyl (Fmoc) amino acids takes place directly on the top of the column. Since negligible pressure is generated in the system, thin flexible PTFE tubing is used for connections within the recirculating loop. This minimizes the total loop volume and hence the final dilution of the applied activated Fmoc-amino acid.

The glass reactor column is fitted with upper and lower PTFE filters of coarse porosity (100μm) to avoid unnecessary back pressure.

3.1.1.2 Protecting groups

Unambiguous formation of a peptide bond between two structurally similar amino acids requires that the amino group of one and the carboxyl group of the other be prevented from participating in the coupling reaction (fig. 3.1).

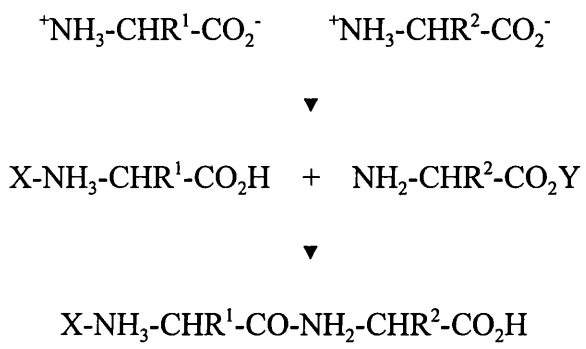

Figure 3.1 Peptide bond formation. X and Y represent amino- and carboxyl-protecting groups, R^1 and R^2 represent different side chain groups.

Since chemically reactive groupings are often present in the side chains of naturally occurring amino acids, additional protecting groups may also be required for these. Protecting groups have to be chosen so as to be easily introduced and to be chemically stable under the conditions of peptide synthesis. In addition they must be easily removable under mild conditions at the end or at intermediate phases in the synthesis. Protecting groups which are retained until assembly of the peptide chain is complete are known as "permanent" and those which are removed at intermediate stages are

known as "temporary". The permanent protecting groups require greater stability to the synthesis reaction conditions than those whose required lifespan is shorter.

3.1.1.2.1 Amino-protecting groups

In the early stages of peptide synthesis, it was realized that urethane derivatives were particularly suitable for protection of amino groups. These were easily prepared and chemically stable. The urethane nitrogen atom is usually inert to the subsequent peptide synthesis reaction conditions. The Fmoc group (ring-substituted benzyl urethane) is labile to bases and thus makes the Fmoc group ideal for temporary protection (fig. 3.2).

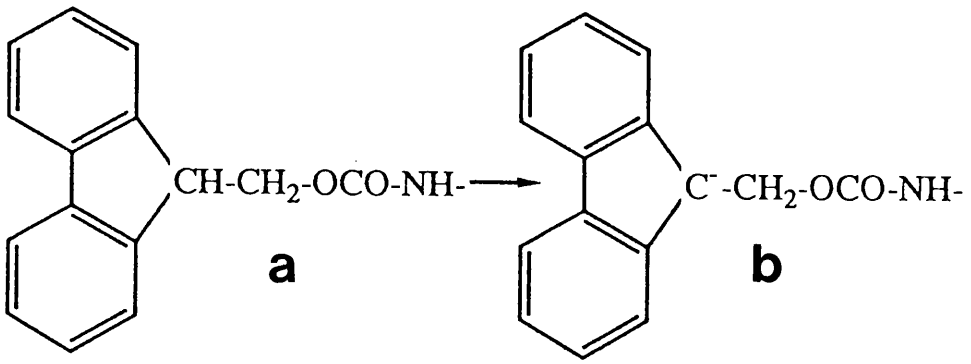

Figure 3.2 The Fmoc amino-protecting group. The Fmoc group (a) is labile to bases because of the special characteristics of the dibenzocyclopentadiene structure. Resonance stabilization of the derived cyclopentadienide anion (b) imparts exceptional acidity to the 9-hydrogen atom, enabling its removal by bases (such as piperidine).

3.1.1.2.2 Carboxy-protecting groups

Initially, simple methyl -$C0_2CH_3$ or ethyl -$C0_2C_2H_5$ ester groups were used for masking carboxy functions, though their cleavage by aqueous alkaline hydrolysis was not straight forward. Amino acid benzyl -$C0_2CH_2C_6H_5$ and *t*-butyl -$C0_2CMe_3$ esters became more popular in peptide synthesis since their cleavage conditions were analogous to the corresponding urethanes used for amino protection.

3.1.1.2.3 Side-chain protecting groups

Most of the amino acids commonly encountered in proteins contain functional side chains. The need for protecting these side chains depends upon the severity of the reaction conditions. Since in solid phase synthesis the reaction conditions tend to be rather severe, side chain protection is almost always required. It is also convenient in solid phase synthesis if the majority or all of the permanent protecting groups are cleaved simultaneously in a single step at the end of the synthesis.

3.1.1.3 Peptide-resin bond

The most important step in solid phase synthesis is the chemical linkage of the growing peptide to the resin support. It has to be easily formed, stable to repeat cycles of acylation and deprotection reactions and yet easily cleaved at the end of the synthesis without damage to newly formed peptide bonds. Resins can now be bought with the first amino acid attached.

3.1.1.4 Formation of the peptide bond

In order to form a peptide bond between two amino acids it is necessary to activate the carboxyl group of one of the amino acids. Simple alkyl esters of protected amino acids undergo aminolysis at too slow a rate to be generally useful for peptide bond synthesis. Phenyl esters are more reactive, and when electronegative substituents are present in the aromatic ring it makes them even more reactive. Pentafluorophenyl esters which were used in this study are an example of this (fig. 3.3).

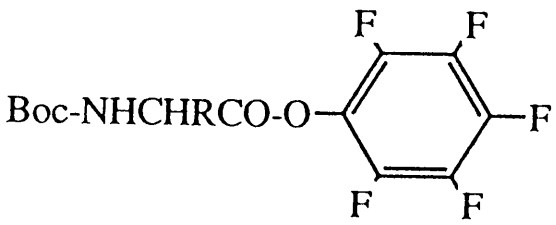

Figure 3.3 Pentafluorophenyl ester amino acid.

1-Hydroxybenzotriazole (acylation catalyst) is commonly used as a catalyst in these reactions. In this study pentafluorophenyl esters of Fmoc amino acids were used. Pentafluorophenyl esters are efficient acylating agents and their chemical structures provide little opportunity for side reactions. They are cleaved cleanly and rapidly by solutions of secondary bases in DMF, conditions which leave even particularly sensitive *t*-butyl derivatives entirely unaffected. Hence selectivity between amino and side-chain deprotection reactions is obtained.

3.1.2 Choice of peptides and theoretical considerations

Antibodies bind to proteins via interaction between functional binding sites on the immunoglobulin molecule known as paratopes, and antigenic determinants on the antigen known as epitopes. A small linear peptide may not represent the complete structure of the corresponding epitope in the protein. The peptide may constitute only a part of a large epitope but nevertheless react with antibodies specific for that epitope. The reactivity of epitopes with antibodies depends on the peptide's ability to assume a correct conformation and it is likely that not all of its amino acid residues will make contact with the antibody. In an attempt to produce anti-peptide antibodies which are cross-reactive with the native protein of interest, it is important to predict immunogenic sequences in a protein and such sequences consist mainly of residues exposed on the protein surface and tend to be hydrophilic. A study by Palfreyman *et al.* (1984) concluded that the success rate (i.e. anti-peptide antibodies produced that recognize the native protein) could be maximized simply by choosing a peptide length of at least 10 residues, of which a significant number are hydrophilic.

Alignment of the available amino acid sequences for rat ovarian and porcine testicular LH/CG receptors facilitated the choice of sequences both within the N- and C-termini of the receptor which had high sequence homologies (fig. 3.4). The following rat ovarian amino acid sequences were therefore chosen for their high homologous regions (relative to porcine testis) and for their high content of hydrophilic amino acids.

PEPTIDE 1 Residues 48----------65

Rat **R G** L **N E** V V **K** I **E** I **S Q S D** S L **E**

Pig **R G** L **N E** V V **K** I **E** I **S Q S D** S L **E**

PEPTIDE 2 Residues 187----------206

Rat **Y** L **E K** M **H S G** A F **Q G** A **T G** P **S** I L **D**

Pig **H** L **K K** M **H N D** A F **R G** A **R G** P **S** I L **D**

PEPTIDE 3 Residues 622----------636

Rat **C K R R** A **E** L **Y R R K E** F **S** A

Pig **C K H Q** A **E** L **Y R R K D** F **S** A

Figure 3.4 Alignment of the amino acid sequences for rat ovarian and porcine testicular LH/CG receptors peptide regions. The rat ovarian sequence was chosen to raise antibodies in this study (Hydrophilic residues are shown in bold).

3.2 Results

3.2.1 Calculation of yield

The yields obtained in the synthesis of each peptide are listed in table 3.1 These are given as a percentage of the theoretical maximum mass of peptide that can be obtained given the amount of solid support used, along with its known amino acid substitution ratio (0.09mmol per 1g resin) and the calculated maximum amount of each amino acid residue that could couple in each step. The percentage yield is, therefore, calculated by summing the integral residue weights for each amino acid. In order to take account of the terminal residues amino and carboxyl groups which together contain an H_2O, 18 is added to the residue weight. The total weight is then multiplied by 9×10^{-5} to give the mass of 0.09 mmol peptide, which corresponds to 100%, and is then divided by the actual mass of the peptide obtained in the synthesis. This figure is then multiplied by 100% to get the final percentage yield.

Table 3.1 Calculation of peptide yield obtained

PEPTIDE 1 19-Residues wt = 2100.05	PEPTIDE 2 21-Residues wt = 2206.02	PEPTIDE 3 15-Residue wt = 1912.01
Add 18 to take account of the terminal residues Amino and Carboxyl groups wt + 18 = 2118.05	Add 18 to take account of the terminal residues Amino and Carboxyl groups wt + 18 = 2224.02	Add 18 to take account of the terminal residues Amino and Carboxyl groups wt + 18 = 1930.01
Multiply by (9×10^{-5}) to give the mass of 0.09mmols peptide which corresponds to 100% = 0.191g	Multiply by (9×10^{-5}) to give the mass of 0.09mmols peptide which corresponds to 100% = 0.200g	Multiply by (9×10^{-5}) to give the mass of 0.09mmols peptide which corresponds to 100% = 0.174g
Actual mass = 165mg	Actual mass = 170mg	Actual mass = 80mg
(0.165/0.191) X 100% = 86.4 % yield	(0.170/0.200) X 100% = 85.0 % yield	(0.080/0.174) X100% = 46.0 % yield

3.2.2 Amino acid analysis

Peptide 1

N-terminus, extracellular domain residues 48-65

^{48}R-G-L-N-E-V-V-K-I-E-I-S-Q-S-D-S-L-E^{65}-C

Table 3.2 Amino acid analysis values for peptide 1

Residue	Expected yield	Obtained yield
Arg(R)	1	0.68
Asp(D)	2	1.91
Glu(E)	4	4.24
Gly(G)	1	0.76
Ile(I)	2	1.89
Leu(L)	2	1.94
Lys(K)	1	0.65
Ser(S)	3	2.40
Val(V)	2	1.07

Peptide 2

N-Terminus, extracellular domain residues 187-206

^{187}Y-L-E-K-M-H-S-G-A-F-Q-G-A-T-G-P-S-I-L-D^{206}-C

Table 3.3 Amino acid analysis values for peptide 2

Residue	Expected yield	Obtained yield
Ala(A)	2	2.55
Asp(D)	1	1.00
Glu(E)	2	2.05
Gly(G)	3	2.81
His(H)	1	1.06
IIe(I) + Leu(L)	(2+1)=3	2.85
Lys(K)	1	1.06
Met(M)	1	0.95
Phe(F)	1	1.07
Pro(P)	1	1.10
Ser(S)	2	1.65
Thr(T)	1	0.96
Tyr(Y)	1	0.85

Peptide 3

C-Terminus, Cytoplasmic domain residues 622-636

^{622}C-K-R-R-A-E-L-Y-R-R-K-E-F-S-A^{636}

Table 3.4 Amino acid analysis values for peptide 3

Residue	Expected yield	Obtained yield
Ala(A)	2	1.60
Arg(R)	4	4.20
Cys(C)	1	0.30
Glu(E)	2	1.65
Leu(L)	1	0.70
Lys(K)	2	2.10
Phe(F)	1	1.02
Ser(S)	1	0.65
Tyr(Y)	1	1.00

3.2.3 HPLC analysis of peptides

Reverse-phase HPLC analysis of each peptide, as described in *Materials and Methods*, section 2.2.7, revealed the presence of a single major peak in the chromatograph. The results for peptides 1,2 and 3 are illustrated in figs 3.5, 3.6 and 3.7 respectively. Estimation of the purity of the peptides was made by comparing the area under the peak with the total area of the absorbance trace yielded by gradient elution of the HPLC column. For peptides 1, 2 and 3 the chemical purity was assessed to be 89, 75 and 83% respectively.

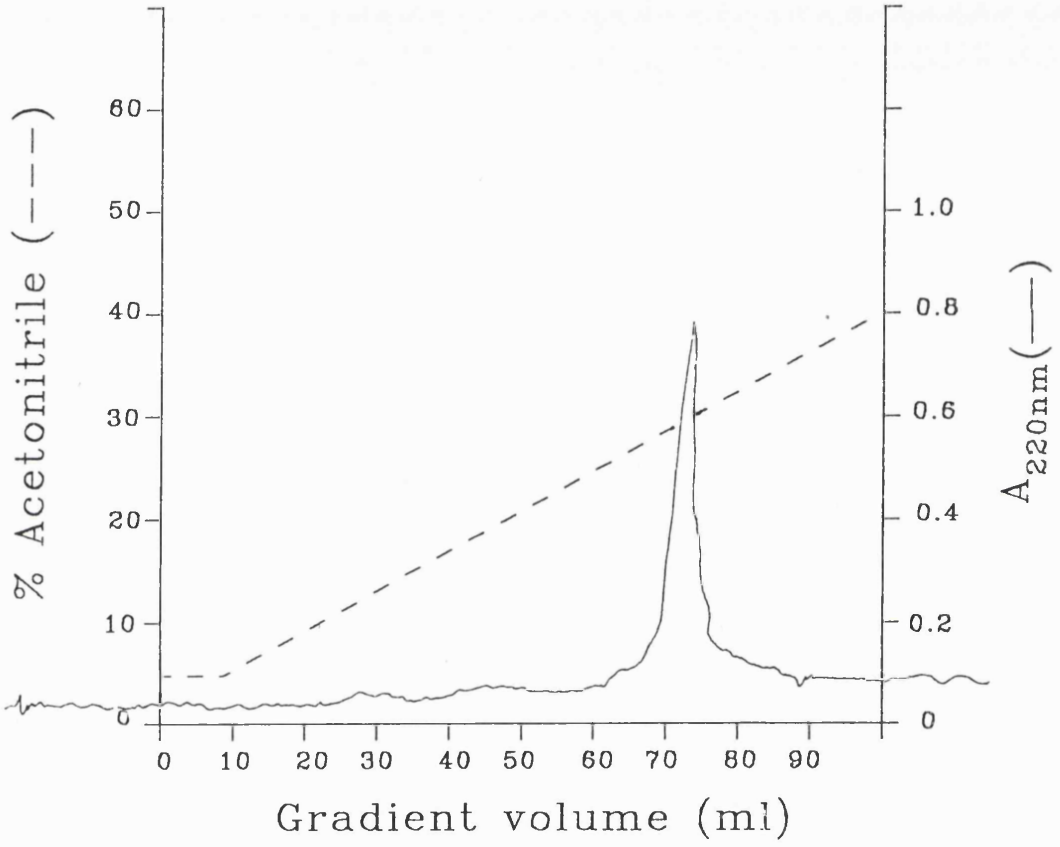

Figure 3.5 HPLC profile of peptide 1. HPLC chromatograph obtained from the extinction at 220nm of the eluate in a 5%-40% (v/v) acetonitrile gradient in 0.1% (v/v) aqueous TFA at a flow rate of 1ml/min from a 300A pore-size, C_{18} column following loading of 50μg of peptide 1.

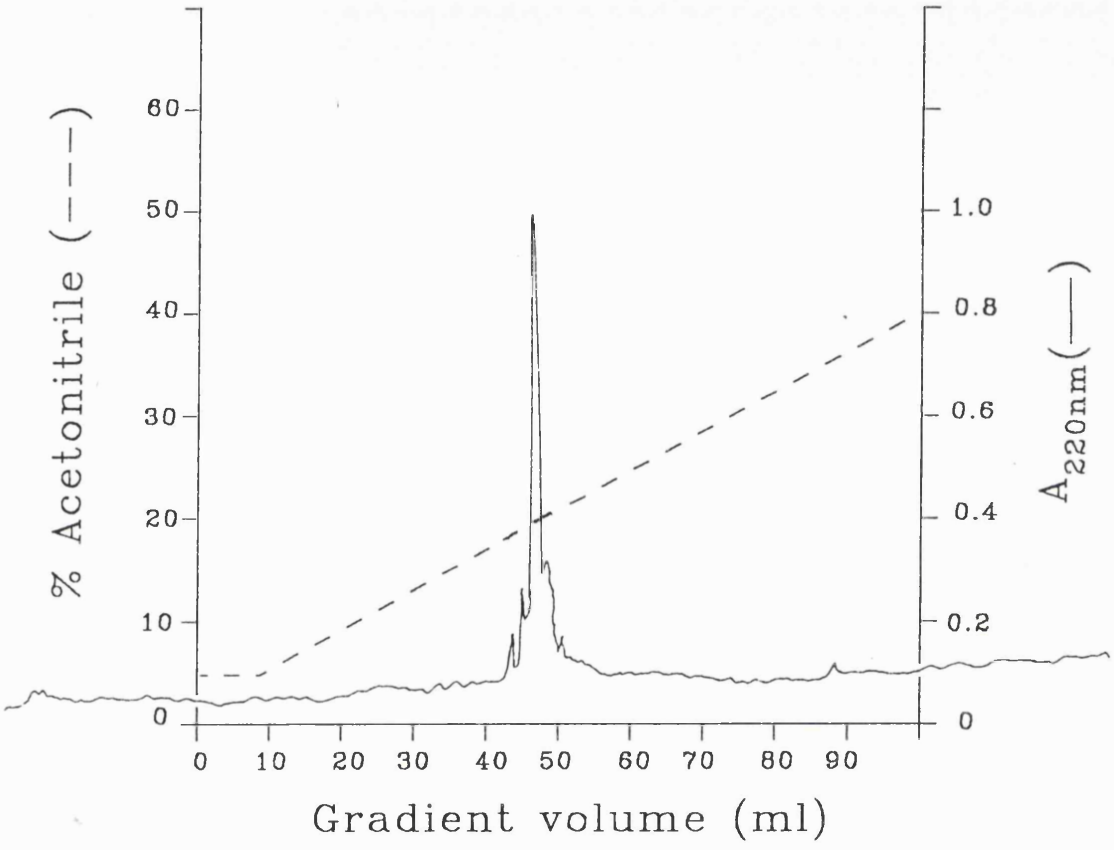

Figure 3.6 HPLC profile of peptide 2. HPLC chromatograph obtained from the extinction at 220nm of the eluate in a 5%-40% (v/v) acetonitrile gradient in 0.1% (v/v) aqueous TFA at a flow rate of 1ml/min from a 300A pore-size, C_{18} column following loading of 50µg of peptide 2.

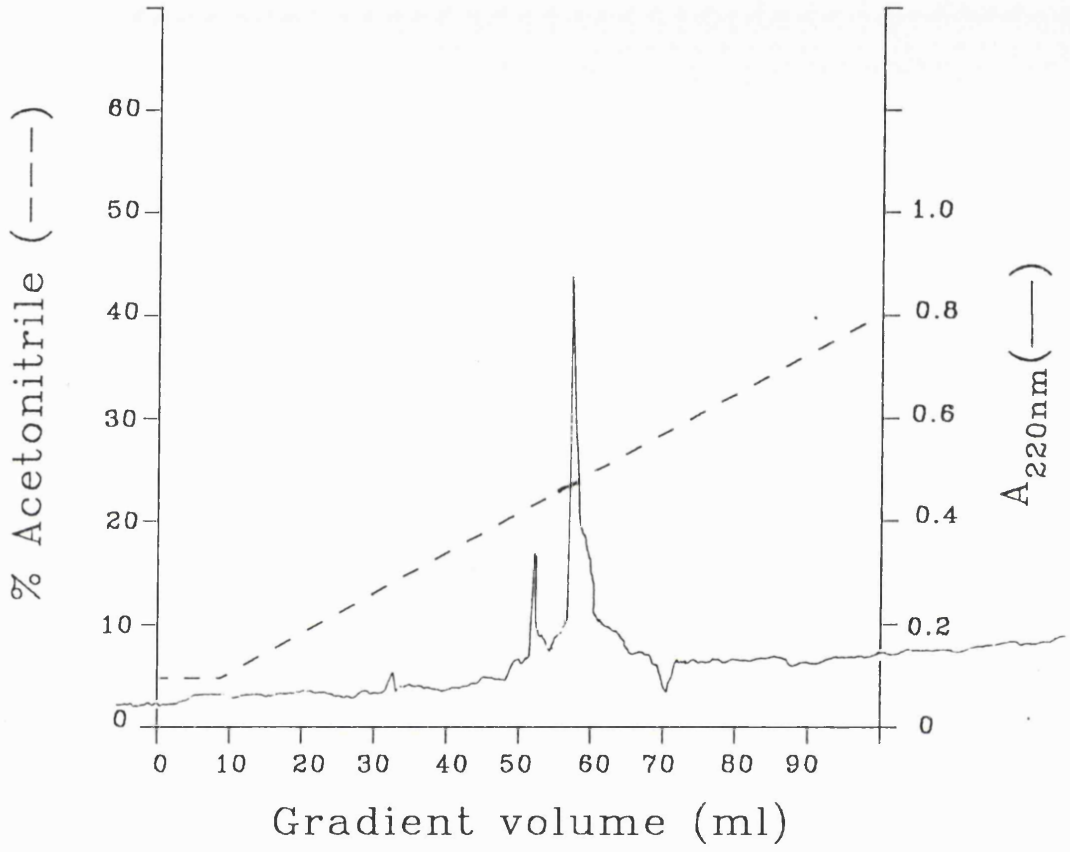

Figure 3.7 HPLC profile of peptide 3. HPLC chromatograph obtained from the extinction at 220nm of the eluate in a 5%-40% (v/v) acetonitrile gradient in 0.1% (v/v) aqueous TFA at a flow rate of 1ml/min from a 300A pore-size, C_{18} column following loading of 50μg of peptide 3.

3.3 Discussion

The yields obtained for the three peptides (86.4%, 85.0% and 46.0%) compares favourably with those reported in the literature for other peptides produced by the Fmoc polyamide procedure (see review Fields and Noble, 1990). The amino acid composition of the peptides as assessed by hydrolysis closely resembled that predicted from their sequences, suggesting that the addition of a three-fold molar excess of amino acid at each coupling step was sufficient to ensure saturation of coupling sites on the growing peptide chain and that each deprotection step had proceeded to completion. In peptide 1, the low value for valine is due to the difficulty in hydrolysing the V^{53}-V^{54} bond. The C-terminal cysteine is not detected in the amino acid analysis because it is destroyed during the hydrolysis. Serine, which is also affected by the hydrolysis step, also gave a lower yield than expected. Glutamine is converted to its glutamic acid and asparagine is converted to aspartic acid during hydrolysis and so in the amino acid analysis, the allocation for glutamine and asparagine is transferred to their corresponding acids. For peptide 2, alanine gave a high value because of the shoulder peak which appeared in the trace. Isoleucine and leucine did not resolve because of their close position (having similar structures), hence they appeared under the same peak. A lower than expected value for serine was obtained again due to its damage by hydrolysis. For the same reasons cysteine and serine gave low values in the analysis of peptide 3.

The estimation of purity of the three peptides by HPLC by the procedure described above were >70% for all three peptides (89, 75 and 83%). In some cases, multiple minor peaks were also observed in the absorbance trace which account for the slightly

lower apparent purity. These peaks arise probably as a result of partial oxidation of sulphur-containing amino acids (C and M), which is a particular problem with cysteine-containing peptides. Similarly, in some cases partial failure to remove protecting groups from some residues, particularly arginine, is known to result in the appearance of multiple peaks on HPLC (it is known that the Mtr side-chain protecting group for arginine is difficult to remove from peptide molecules and is recommended to allow peptides containing Mtr groups longer times for deprotection). Since from HPLC and amino acid analysis the purity and composition of the peptides was sufficiently high, they were used as immunogens without further purification.

CHAPTER 4

PRODUCTION OF ANTIBODIES, PURIFICATION AND CHARACTERIZATION

4.1 Introduction

4.1.1 Conjugation of peptides and antisera production

In order to elicit an immune response to peptides in the size range of those synthesized for this study, it is necessary to conjugate the peptides to a large carrier protein. There are a number of reasons for this. Firstly, in small peptides, closely related amino acid sequences may exist in the animals used for antiserum production and therefore the peptide may not be recognized as foreign. Small peptides may also lack a defined structure which is necessary for their recognition as antigens. Finally, peptide-conjugates are less prone than peptides to rapid degradation in the tissues and circulation of the immunized animal, where they must remain relatively intact in order to elicit an immune response.

It is important that the coupling procedure of the carrier protein to the peptide does not itself interfere with the region of the peptide against which antibodies are sought. This is why if the peptide does not naturally contain a cysteine, a residue of this amino acid can be added to one end of the peptide during synthesis. The resultant -SH group provides a unique point by which the peptide can be attached to the carrier protein as described in *Materials and Methods* section 2.2.8. This strategy avoids the wasteful crosslinking of peptide to peptide or protein to protein and utilizes a specific site on the peptide designed to be remote from the position of the epitope of interest. Many different carrier proteins have been used successfully for the production of anti-peptide antibodies. In this study, ovalbumin, which is a highly soluble protein, was used to

conjugate all three peptides. Immunization in this way elicits an antibody response not only to the peptide but also to the carrier protein itself.

4.1.2 Immunization

All the anti-peptide antibodies were produced in rabbits following the procedure outlined in *Materials and Methods* section 2.2.9. Nine rabbits were used in this study. Rabbits 1-3 were immunized with peptide 1, rabbits 4-6 with peptide 2 and rabbits 7-9 with peptide 3. In all cases the initial immunizations were with a water in oil emulsion of the peptides conjugated to carrier protein in Freund's complete adjuvant. In subsequent booster injections, incomplete Freund's adjuvant was used. This method has been widely employed to produce anti-peptide antibodies for the study of membrane proteins (see review White, 1976).

Rabbits are used in the production of polyclonal antiserum for many reasons including relative cost, ease of maintenance, robustness in the face of extensive immunization, size (large enough to yield 30-40ml serum per month) and ease of bleeding. Dutch half lop rabbits, which appear to have strong immune responses, were employed for production of polyclonal antisera in this study.

Emulsification of the peptide-conjugate with an oily adjuvant helps to sequester the immunogen at the site of injection, from which it is slowly released. It also nonspecifically enhances the immune response. Freund's complete adjuvant contains mineral oil, detergent and heat-killed *Mycobacterium tuberculosis*, which attract macrophages and other cells of the immune system to the injection site. This is used

for the initial injection of conjugate as mentioned above, whereas incomplete adjuvant which lacks Mycobacteria, is used for subsequent injections.

4.1.3. Monitoring of antibody production

4.1.3.1 Reaction with the LH receptor peptide antigens

The titre of peptide-specific antibodies produced in serum was monitored by examining the reactivity of serial dilutions of serum with the peptide antigen using an ELISA, as outlined in *Materials and Methods* section 2.2.11. The estimated relative binding of the serum to the microtiter wells coated with the relevant peptide is then plotted against the dilution of serum in the wells. Comparison of anti-peptide titres of different antisera can be made by comparing the dilutions at which the binding of the serum to the antigen is equivalent to half its maximum value. Thus those antisera which react with the peptide with half maximum efficiency at comparatively high dilutions have a high affinity for their peptide antigen.

Those antibodies produced using a peptide coupled to a carrier protein, which react with the peptide in the ELISA, may be elicited entirely by a region or regions in the peptide, or by domains on both the peptide and the carrier protein. Additionally, the synthetic peptide resembles, but is not structurally identical to, its corresponding domain in the intact, native or denatured protein. Thus, it is clear that strong recognition of peptide in an ELISA by an antibody does not necessarily imply strong recognition of the intact protein.

4.1.3.2 Reaction with intact LH receptors

The reactivity of the anti-peptide antibodies with their specific peptide sequences in intact LH receptor protein was examined using many techniques which are discussed in chapter 5. One of these techniques, dot blotting, described in *Materials and Methods* section 2.2.18, was used to examine the reactivity of the antibodies with a solubilized ovarian membrane preparation and a liver preparation, in addition to the synthetic peptides. Ovarian tissue from superovulated rats contains a high number of LH/CG receptors, whereas the liver tissue is thought to be devoid of such receptors.

4.1.4 Purification of antibodies

Because of the relatively high ratio of serum protein to peptide-specific antibody found in all antisera, most studies have purified anti-peptide antibodies by affinity chromatography before using them as probes for membrane protein structure and function. Affinity purification removes non-specific antibodies, some of which recognize the carrier protein, along with other serum proteins, particularly rabbit serum albumin. Some of these proteins, such as non-specific IgG and serum albumin, are much more concentrated in serum than are the peptide-specific antibodies. Thus purification removes the possibility of a non-specific effect due to the presence of these irrelevant proteins when the antibodies are subsequently used as probes.

In this study, peptide-specific antibodies were purified from serum prior to their use in structural / functional studies. Anti-peptide antibodies were isolated from serum by affinity chromatography using columns containing immobilized peptides, as described

in *Materials and Methods* section 2.2.10. In order to minimize steric hindrance of the binding of antibody during affinity chromatography, peptides were immobilized by the same unique site that was used for attachment to a carrier protein. This is chosen to be as far as possible from the epitope of interest. All peptides were coupled to the Sulpholink coupling gel via their terminal cysteine residues. This matrix (containing iodoacetyl groups linked to agarose) was chosen because it reacts fast with the free sulphydryl residues. Following serum recirculation, non-specifically bound material was eluted from the column first, and then peptide-specific IgG was eluted using 5M $MgCl_2$.

4.2 Results

4.2.1 Monitoring of antibodies (reaction with synthetic peptides)

Figure 4.1 shows a typical plot of A_{405} versus serum dilution obtained in an ELISA versus the immunized peptide from serum pooled from rabbits no.1, 2 and 3, immunized with a conjugate of the synthetic peptide no.1. Similar results are shown for the other rabbits (rabbits no.4, 5 and 6 immunized with conjugate of the synthetic peptide no.2 and rabbits no.7, 8 and 9 immunized with conjugate of the synthetic peptide no.3) in figs 4.2 and 4.3. Serum samples were taken from the animals before (pre-immune serum) and 10 weeks after the initial injection, 2 weeks after the first booster injection and then 2 weeks after the second booster. In each of these plots, the error bars represent the mean ± S.E.M. values for the three rabbits (n=3 for the three rabbits immunized with the same peptide-conjugate) at each serum dilution. Significant reactivity of each antiserum with its relevant peptide is revealed in these plots, while

recognition of the peptide by pre-immune serum from each of the immunized rabbits was low. It is also clearly seen from these graphs that the titre of the serum was higher after each booster injection.

Figure 4.4 again shows a plot of A_{405} versus serum dilution for rabbits no.1, 2 and 3 obtained in an ELISA. Similar plots are shown in figs 4.5 and 4.6 (for rabbits no.4, 5, 6 and 7, 8, 9 respectively). These plots reveal removal of the majority of the reactivity of each antiserum with its peptide antigen following its recirculation through a column containing the peptide. A comparison of the estimated dilution at which the binding of each combined serum sample to the antigen, both prior to and following recirculation on the peptide column, is half the maximum value is shown in table 4.1. These dilution values indicate that for all three sets of pooled antisera, the anti-peptide antibodies were effectively removed by passage of the serum through an immunoaffinity column.

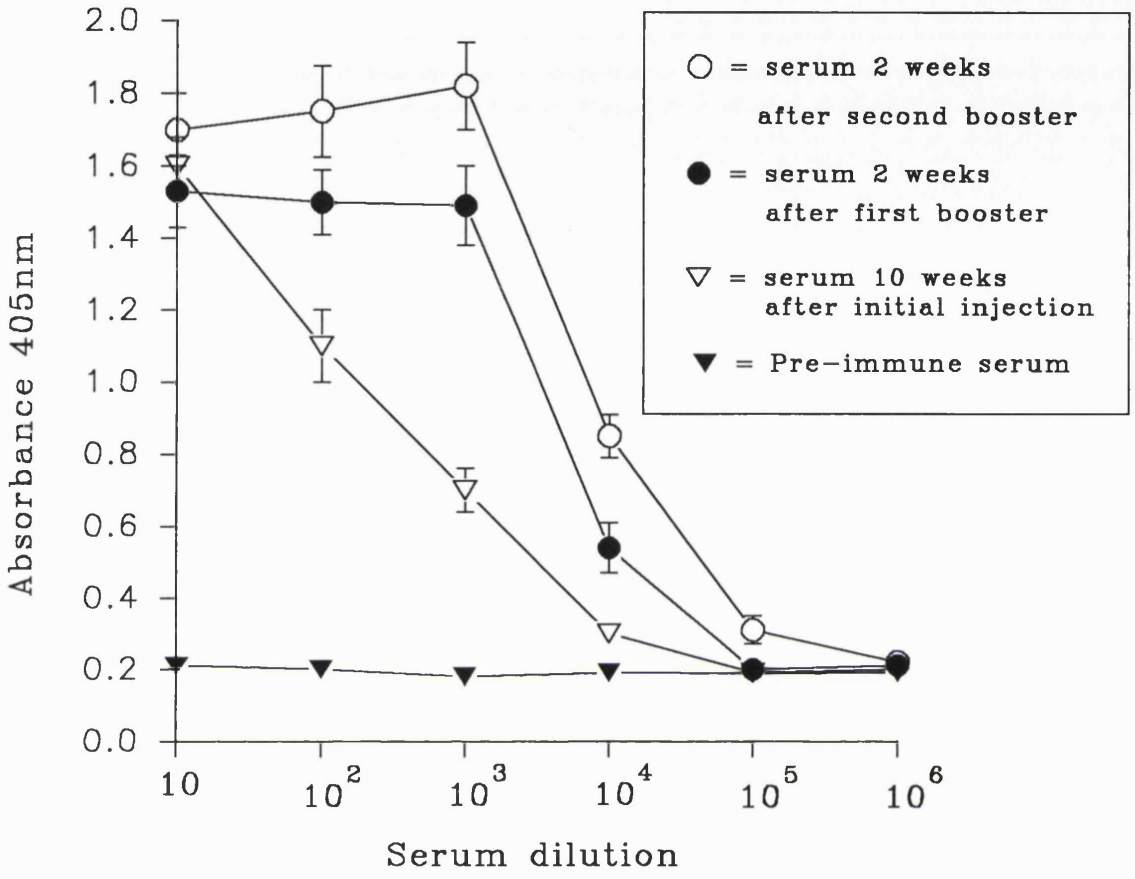

Figure 4.1 Recognition of peptide 1 by crude antisera in an anti-peptide ELISA. Plot of A_{405} versus serum dilution for serum obtained 2 weeks after the first and second booster injections, 10 weeks after the initial injection and pre-immune serum obtained from rabbits no.1, 2 and 3 which were immunized using an ovalbumin-conjugate of peptide no.1. Background absorbency has been subtracted in each case. The plate was coated with peptide 1 and blocked and incubated with both primary and secondary antibody, as outlined in *Materials and Methods* section 2.2.11. Bound antibody was detected by the addition of P-nitrophenyl phosphate, which yielded yellow P-nitrophenol upon hydrolysis. The latter was quantified by its absorbency at 405nm. Serum dilutions were assayed in quadruplicate. The error bars represent mean ± S.E.M. values for the three rabbits (n=3 for rabbits no. 1, 2 and 3).

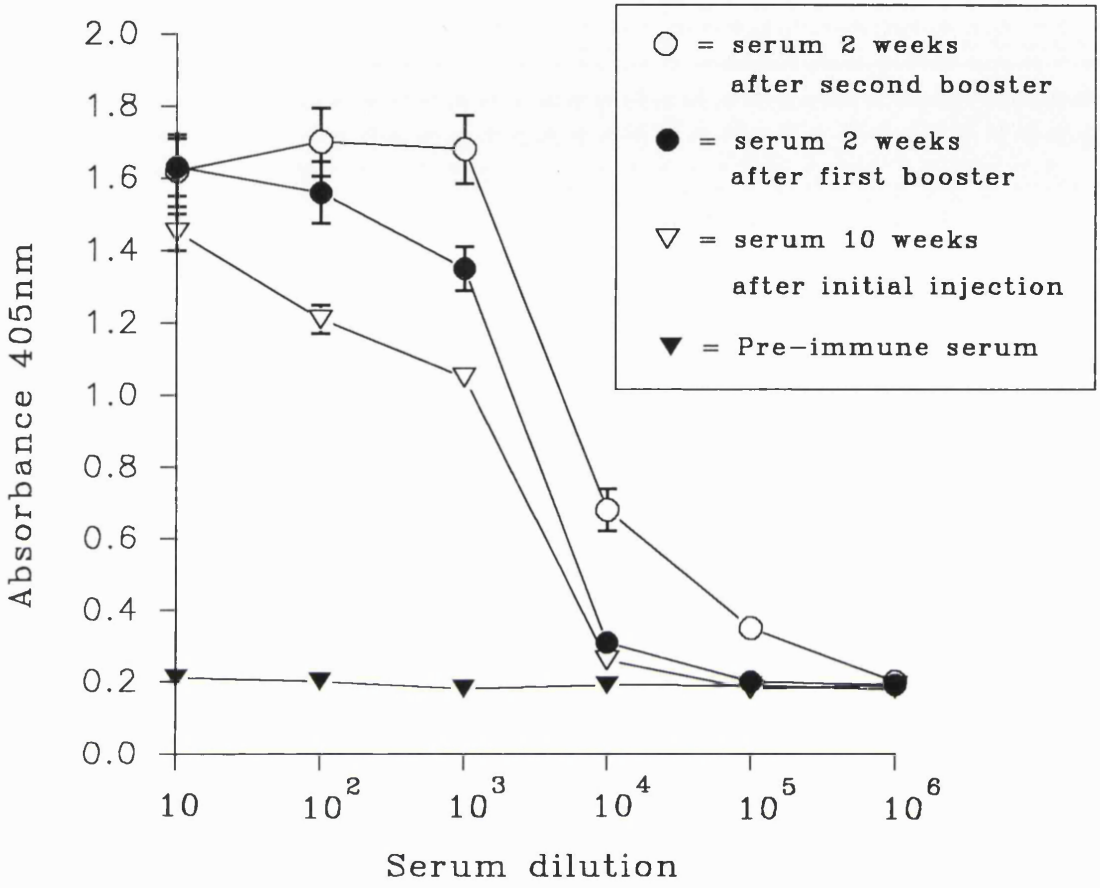

Figure 4.2 Recognition of peptide 2 by crude antisera in an anti-peptide ELISA. Plot of A_{405} versus serum dilution for serum obtained 2 weeks after the first and second booster injections, 10 weeks after the initial injection and pre-immune serum obtained from rabbits no.4, 5 and 6 which were immunized using an ovalbumin-conjugate of peptide no.2. Background absorbency has been subtracted in each case. The plate was coated with peptide 2 and blocked and incubated with both primary and secondary antibody, as outlined in *Materials and Methods* section 2.2.11. Bound antibody was detected by the addition of P-nitrophenyl phosphate, which yielded yellow P-nitrophenol upon hydrolysis. The latter was quantified by its absorbency at 405nm. Serum dilutions were assayed in quadruplicate. The error bars represent mean ± S.E.M. values for the three rabbits (n=3 for rabbits no. 4, 5 and 6).

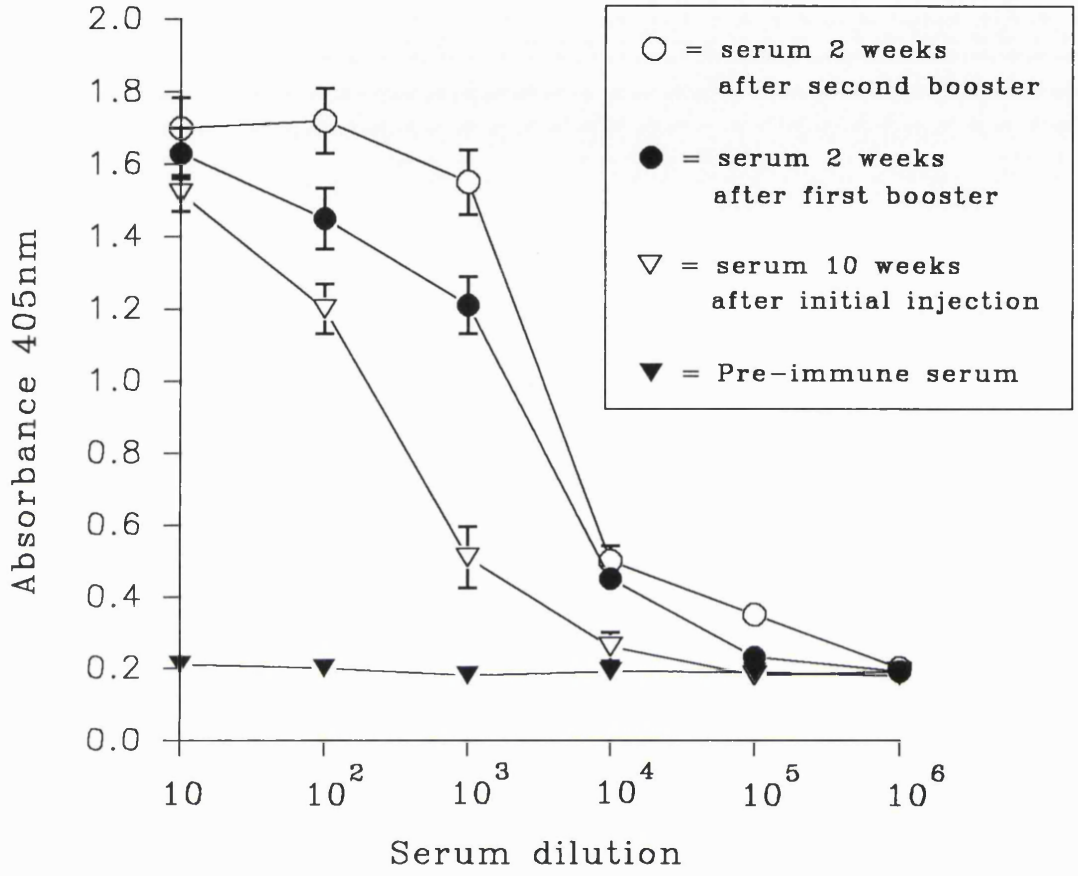

Figure 4.3 Recognition of peptide 3 by crude antisera in an anti-peptide ELISA. Plot of A_{405} versus serum dilution for serum obtained 2 weeks after the first and second booster injections, 10 weeks after the initial injection and pre-immune serum obtained from rabbits no.7, 8 and 9 which were immunized using an ovalbumin-conjugate of peptide no.3. Background absorbency has been subtracted in each case. The plate was coated with peptide 3 and blocked and incubated with both primary and secondary antibody, as outlined in *Materials and Methods* section 2.2.11. Bound antibody was detected by the addition of P-nitrophenyl phosphate, which yielded yellow P-nitrophenol upon hydrolysis. The latter was quantified by its absorbency at 405nm. Serum dilutions were assayed in quadruplicate. The error bars represent mean ± S.E.M. values for the three rabbits (n=3 for rabbits no. 7, 8 and 9).

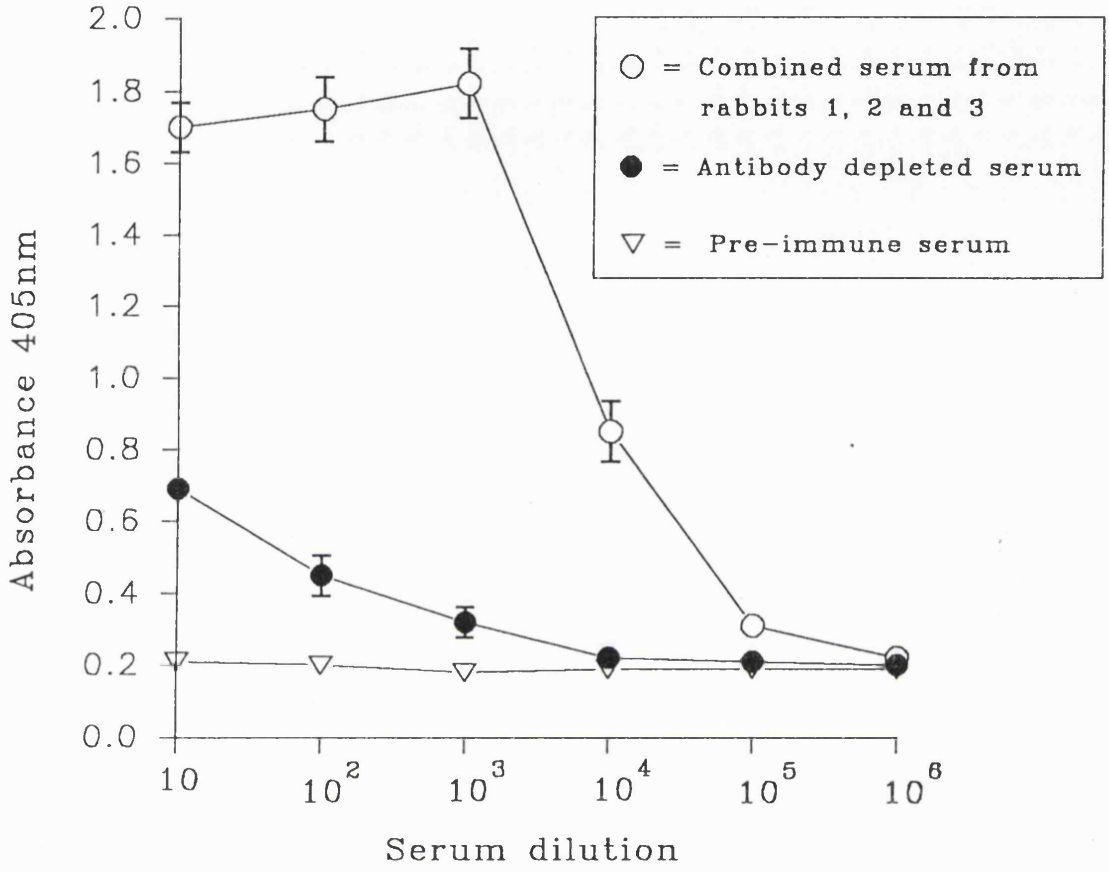

Figure 4.4 Antibody-peptide ELISA of antiserum, recirculated and pre-immune serum for rabbits no.1, 2 and 3. Plot of A_{405} versus serum dilution for antiserum, recirculated serum and pre-immune serum obtained from rabbits no.1, 2 and 3 which were immunized using an ovalbumin-conjugate of peptide 1. All of the A_{405} values shown have been corrected for background absorbency. The combined serum was recirculated through a column to which peptide 1 had been bound. The assay was carried out, using a plate coated with peptide 1, as outlined in the legend for fig.4.1. Serum dilutions were assayed in quadruplicate. The error bars represent the mean ± S.E.M. values for the three rabbits (n=3 for rabbits 1, 2 and 3).

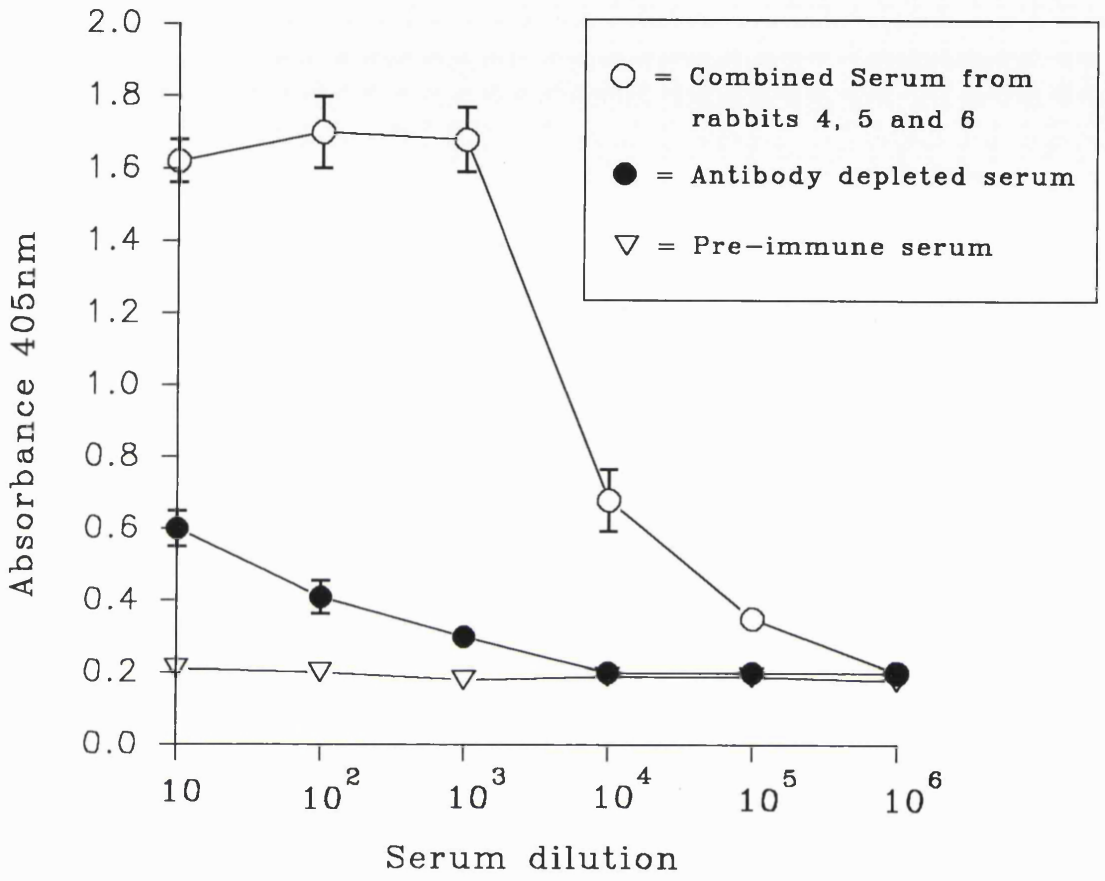

Figure 4.5 Antibody-peptide ELISA of antiserum, recirculated and pre-immune serum for rabbits no.4, 5 and 6. Plot of A_{405} versus serum dilution for antiserum, recirculated serum and pre-immune serum obtained from rabbits no.4, 5 and 6 which were immunized using an ovalbumin-conjugate of peptide 2. All of the A_{405} values shown have been corrected for background absorbency. The combined serum was recirculated through a column to which peptide 2 had been bound. The assay was carried out, using a plate coated with peptide 2, as outlined in the legend for fig.4.1. Serum dilutions were assayed in quadruplicate. The error bars represent the mean ± S.E.M. values for the three rabbits (n=3 for rabbits 4, 5 and 6).

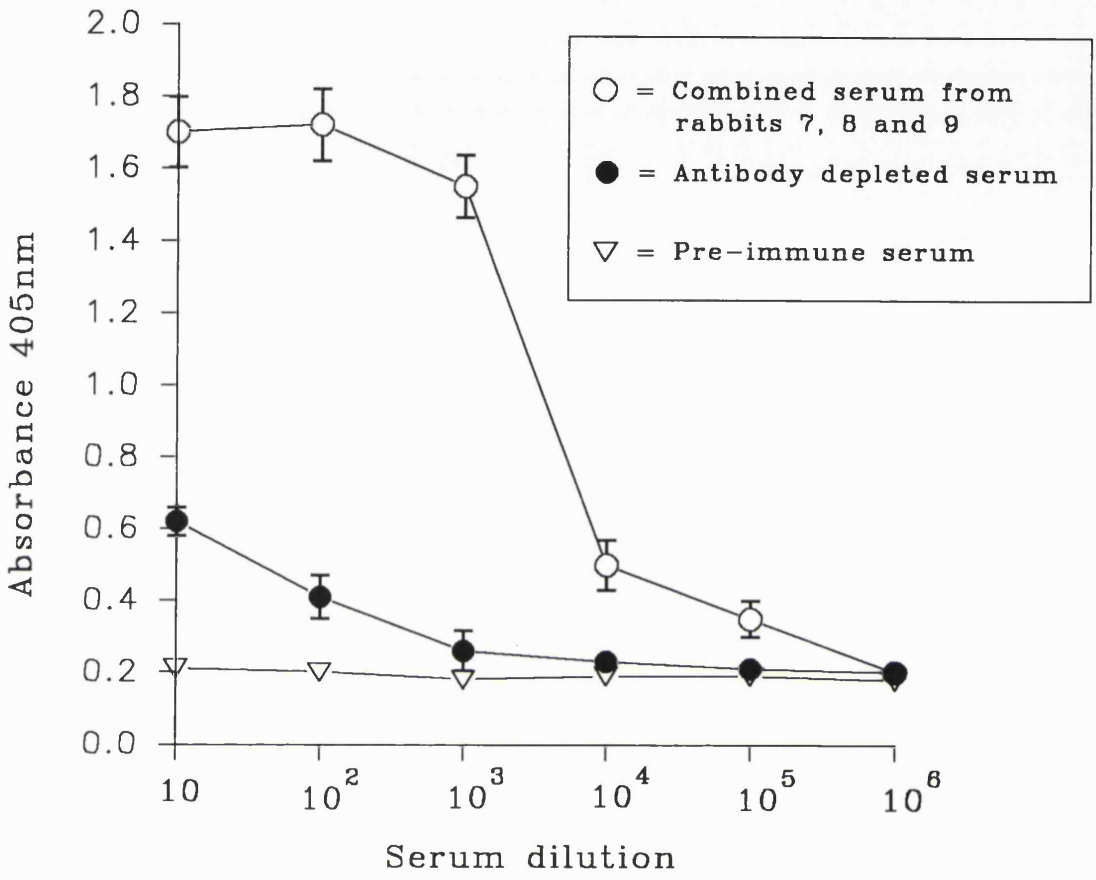

Figure 4.6 Antibody-peptide ELISA of antiserum, recirculated and pre-immune serum for rabbits no.7, 8 and 9. Plot of A_{405} versus serum dilution for antiserum, recirculated serum and pre-immune serum obtained from rabbits no.7, 8 and 9 which were immunized using an ovalbumin-conjugate of peptide 3. All of the A_{405} values shown have been corrected for background absorbency. The combined serum was recirculated through a column to which peptide 3 had been bound. The assay was carried out, using a plate coated with peptide 3, as outlined in the legend for fig.4.1. Serum dilutions were assayed in quadruplicate. The error bars represent the mean ± S.E.M. values for the three rabbits (n=3 for rabbits 7, 8 and 9).

Rabbits no.	Antibody	Crude antiserum dilution	Antibody depleted serum dilution
1, 2 and 3	1	1/12000	1/1100
4, 5 and 6	2	1/10000	1/1000
7, 8 and 9	3	1/8000	1/800

Table 4.1: Table of estimated dilutions of antisera. Estimated dilutions of combined serum samples, both prior to and following recirculation through a column containing the immunizing peptide, which showed binding to the peptide in ELISA which was half of the maximum value.

4.2.2 Affinity-purification and concentration of antibodies

The recovery of peptide-specific antibody after purification was estimated following peptide ELISA for various dilutions of samples of the pooled original crude antiserum and the affinity purified and concentrated antibody (figs 4.7-4.9). Before purification, serum samples from rabbits immunized with peptide 1 were pooled and the combined serum was then affinity purified using a column containing the immobilized peptide 1. The affinity purified antibody was then concentrated using the Amicon concentrators. Similarly serum samples from rabbits immunized with peptide 2 were pooled for antibody 2 purification, and serum samples from rabbits immunized with peptide 3 were used for antibody 3 purification. As seen from figs. 4.7-4.9 the titre

of the affinity purified antibody is much greater than that of the crude serum especially at higher dilutions. This is apparent for all three antibodies. These figures also demonstrate that in the antibody depleted serum, most of the relevant antibody has been removed from the serum after passage through the column containing the relevant immobilized peptide, and that the column eluate contained no antibody.

4.2.3 Cross-reactivity of antibodies

The cross-reactivity of each antibody with peptides other than the specific peptide against which the serum was raised was examined. Figure 4.10 shows that antibody 1 reacted only with peptide 1 and there was no reaction with antibodies 2 and 3 against this peptide. Similarly antibodies 2 and 3 only recognized their corresponding peptides 2 and 3. In each case the absorbances have been corrected by subtraction of the blank absorbances obtained using pre-immune IgG. Specific absorbances shown are the mean + S.E.M of three independent experiments.

4.2.4 Reaction of antibodies with intact protein in dot blots

The specificity of the antibodies was also confirmed in the dot blot analysis. Again there was found to be no cross-reactivity between the antibodies and the different peptides and in addition to this, all three antibodies bound to solubilized proteins from superovulated rat ovaries (positive control) but failed to react with liver proteins (negative control) and blank wells (fig. 4.11).

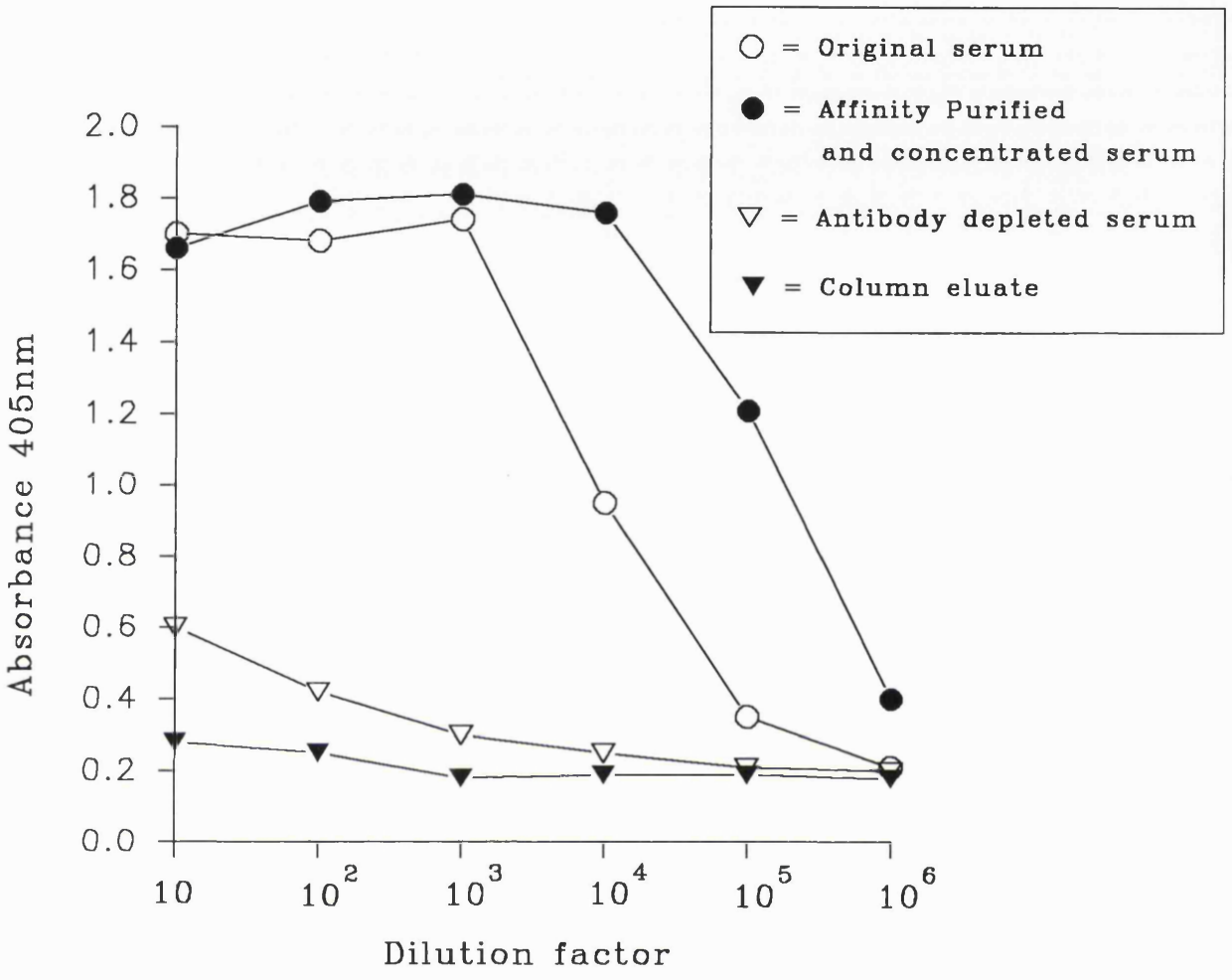

Figure 4.7 Recognition of peptide 1 by affinity-purified antibody 1 in an anti-peptide ELISA. Plot of A_{405} versus dilution factor of various fractions obtained during affinity purification of anti-peptide 1 antibodies from antiserum. All of the A_{405} values shown have been corrected for background absorbency. The assay was carried out using a plate coated with peptide 1, as outlined in the legend for fig.4.1. Serum dilutions were assayed in quadruplicate. The error bars representing the SD value for each data point are smaller than the radius of the data point symbols.

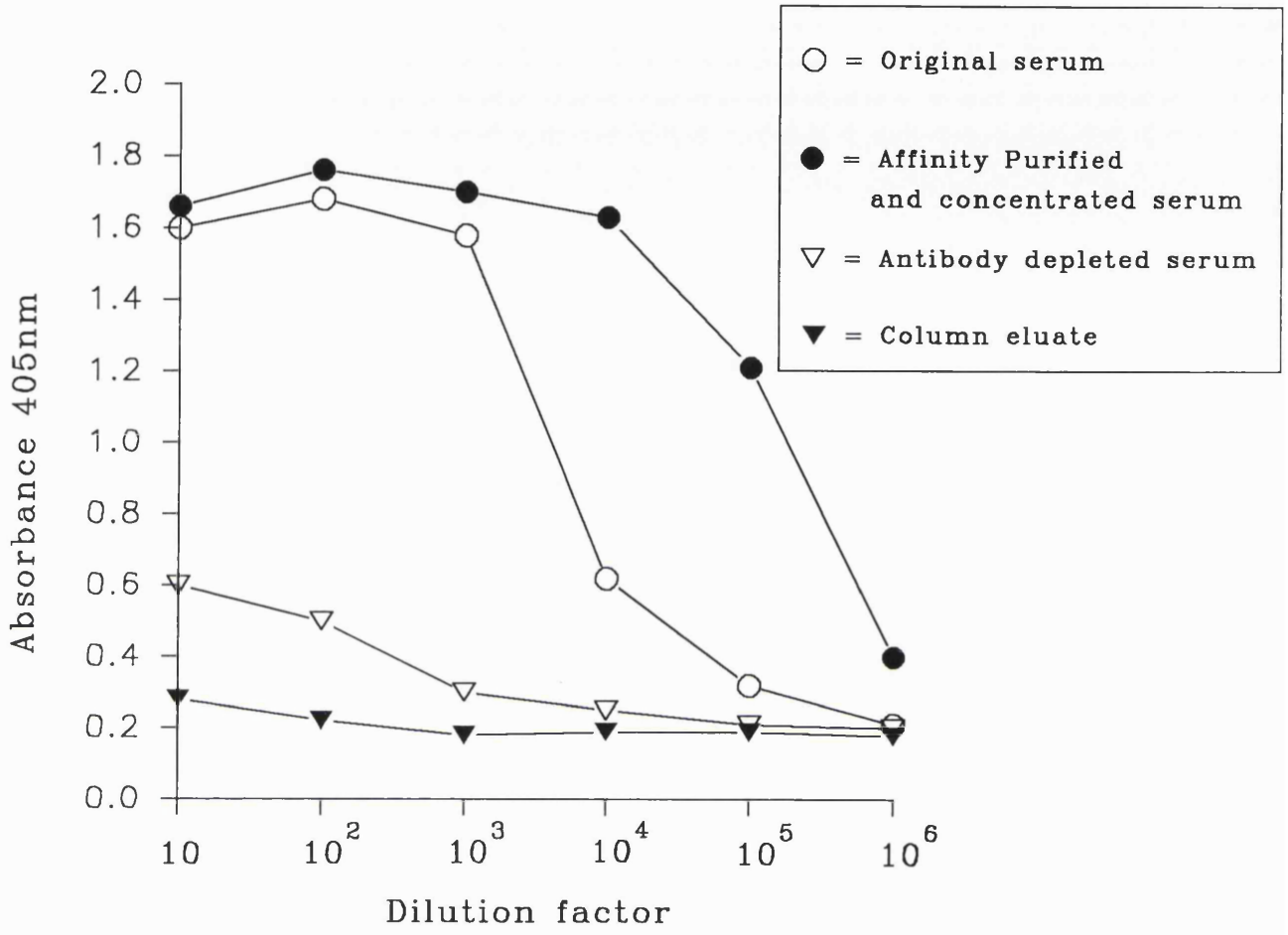

Figure 4.8 Recognition of peptide 2 by affinity-purified antibody 2 in an anti-peptide ELISA. Plot of A_{405} versus dilution factor of various fractions obtained during affinity purification of anti-peptide 2 antibodies from antiserum. All of the A_{405} values shown have been corrected for background absorbency. The assay was carried out using a plate coated with peptide 2, as outlined in the legend for fig.4.1. Serum dilutions were assayed in quadruplicate. The error bars representing the SD value for each data point are smaller than the radius of the data point symbols.

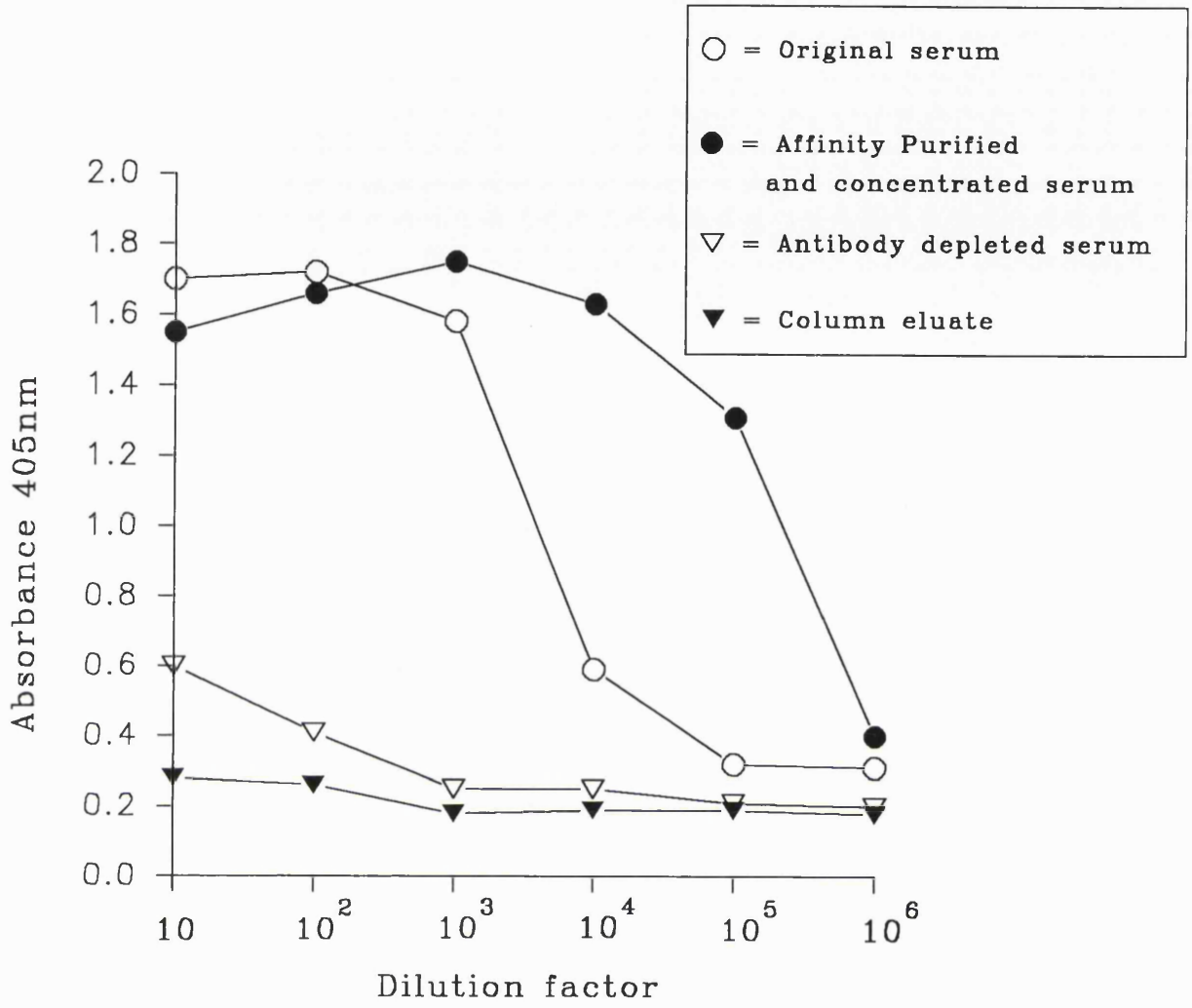

Figure 4.9 Recognition of peptide 3 by affinity-purified antibody 3 in an anti-peptide ELISA. Plot of A_{405} versus dilution factor of various fractions obtained during affinity purification of anti-peptide 3 antibodies from antiserum. All of the A_{405} values shown have been corrected for background absorbency. The assay was carried out using a plate coated with peptide 3, as outlined in the legend for fig.4.1. Serum dilutions were assayed in quadruplicate. The error bars representing the SD value for each data point are smaller than the radius of the data point symbols.

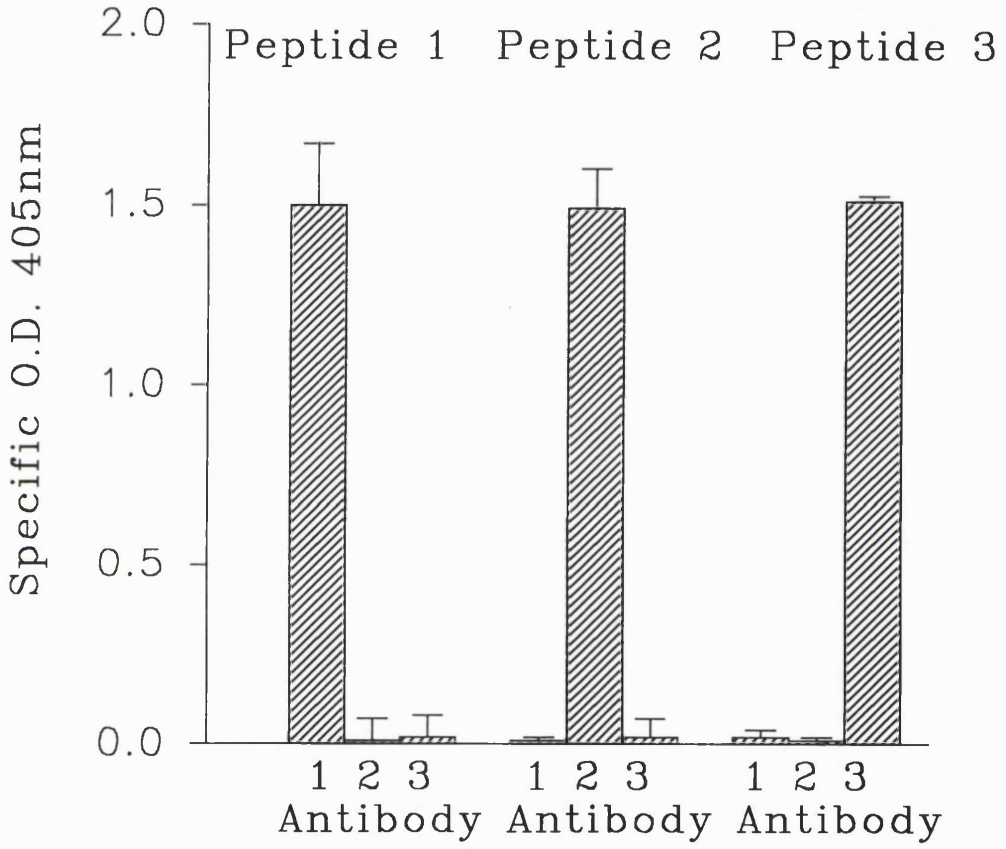

Figure 4.10 Cross-reactivity of affinity purified antibodies in an anti-peptide ELISA. Plot of specific absorbency versus the three different antibodies obtained in an assay against the different peptides. Plates were coated with either peptide 1,2 or 3. Affinity purified antibodies 1,2 and 3 (10μg/ml) were added and the assay carried out as described in the legend for fig. 4.1. The error bars represent + S.E.M of three independent experiments (n=3) with triplicate determinations in each experiment.

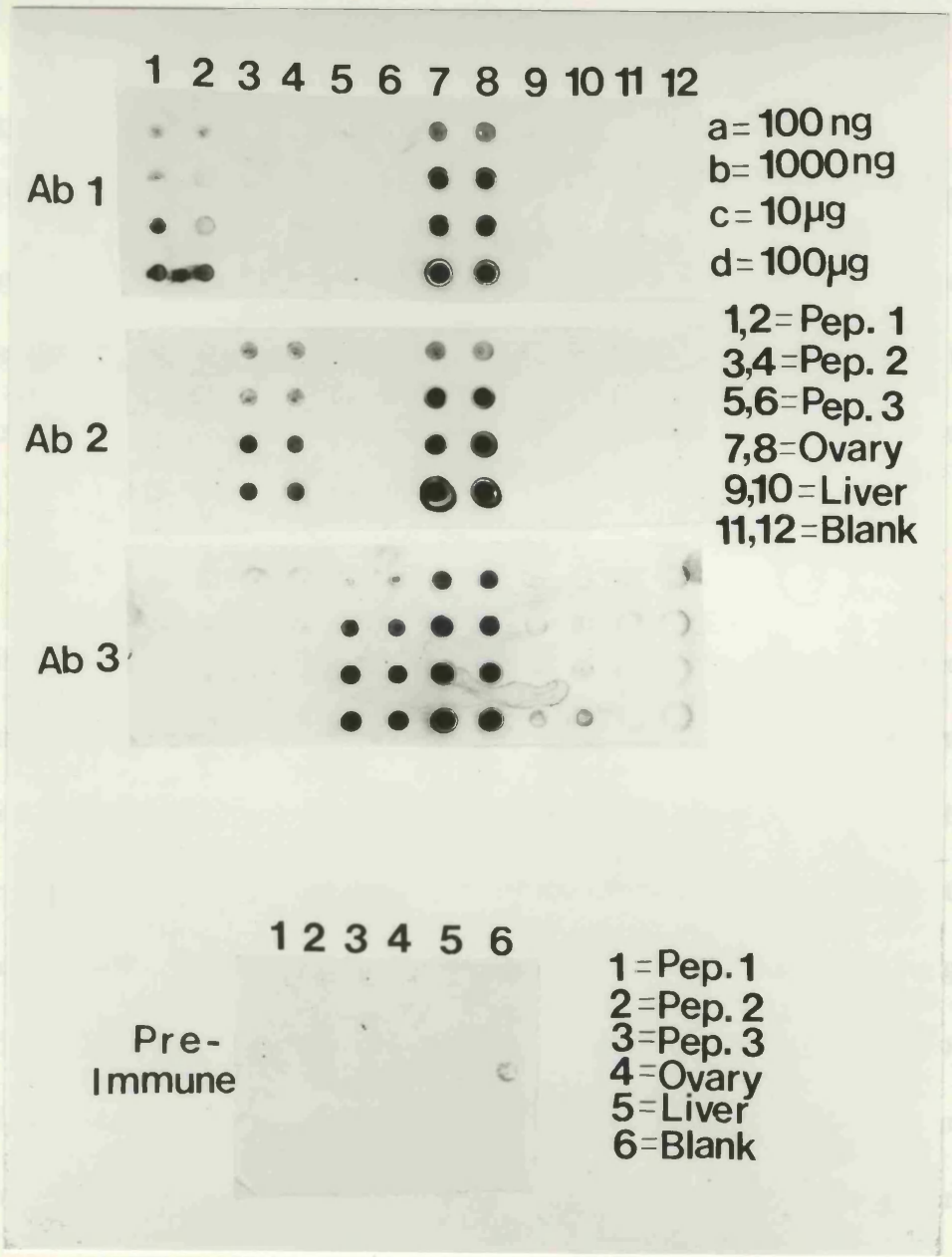

Figure 4.11 Affinity purified antibody specificity and recognition of the intact LH/CG receptor. Dot blots containing (a)=100ng, (b)=1000ng, (c)=10µg and (d)=100µg of peptide 1 (lanes 1 & 2), peptide 2 (lanes 3 & 4) peptide 3 (lanes 5 & 6), solubilised rat ovarian membranes (lanes 7 & 8), solubilised rat liver membranes (lanes 9 & 10) no protein (lanes 11 & 12). These were reacted with antibodies indicated (10µg/ml) or with pre-immune IgG (10µg/ml), and binding of the antibodies to the peptides and membranes was detected as described in *Materials and Methods* section 2.2.17.

For the pre-immune panel, lanes 1, 2 and 3 refer to peptides 1, 2 and 3 respectively. Lanes 4 and 5 refer to ovarian and liver membranes and lane 6 contains no protein. Experiments were repeated three times with similar results for peptides, ovarian and liver membranes.

4.3 Discussion

Peptide-ELISAs revealed that all of the immunization procedures were successful in generating antibodies which recognized the synthetic peptides. Antisera obtained at different stages of immunization were tested and were found to have the highest titres two weeks after the animals were administered with the second booster injections (see figs 4.1-4.3).

The specificity of recognition by each antiserum of its particular peptide is supported by the extremely low reactivity of pre-immune serum from each of the immunized rabbits with the peptides. More convincing evidence for the specificity of the antisera for the peptides is provided by the observed removal of the majority of the reactivity of the serum for the peptide following its circulation through a column containing this peptide (figs 4.4-4.6 and Table 4.1). Also antibodies produced against any one of the peptides failed to show any cross-reactivity with other peptides in the ELISAs and in the dot blot analyses (figs 4.10 and 4.11). In the latter, all three antibodies recognized a specific protein in solubilized membranes from superovulated rat ovaries. No reaction was seen with solubilized liver membranes which are thought to be devoid of LH/CG receptor proteins. Pre-immune IgG failed to react with both the peptides and membranes. Immunodot blotting thus indicated that the anti-peptide specific antibodies recognize a protein specific to the ovarian but not the liver membranes consistent to recognition of the LH/CG receptor (fig 4.11).

A major problem with affinity purification of polyclonal antibodies can be the loss of a significant amount of the specific antibodies. A possible contributor to this loss is

that antibody molecules with the highest avidity for the antigen, i.e. those binding the peptide on the solid support most tightly, may not be removed under the elution conditions used and are thus selected out by the procedure. Some of the protein may also be lost during concentration of the IgG, due to trapping in the centriprep-30 membranes used for this step. However in the present studies, it is apparent that no major losses occurred because it was found that there was at least a 40 fold increase in the titre of the purified concentrated antibody over the crude serum (figs. 4.7-4.9). Hence elution with 5M $MgCl_2$ allowed good recovery of peptide-specific antibodies. All subsequent studies were therefore carried out using concentrated affinity-purified antibodies.

It was concluded that the methodology used resulted in three LH receptor anti-peptide antibodies, which could be purified by affinity chromatography, were specific for the corresponding peptides and recognized specific proteins in ovarian but not liver membranes. Western blotting was therefore carried out in chapter 5 to identify the size and nature of these proteins of interest.

CHAPTER 5

ANTIBODY RECOGNITION OF THE INTACT RECEPTOR PROTEIN

5.1 Introduction

In this chapter further characterization of the antibodies using native and denatured LH/CG receptors from membranes, as well as the receptor in intact cells and tissue sections, is described. This was carried out using SDS-PAGE, Western, slot and ligand blotting on the various membrane samples (see *Materials and Methods* sections 2.2.16-2.2.18 & 2.2.22). Immunocytochemistry was also used to confirm the binding of the antibodies to the LH/CG receptor in rat testicular sections and MA10 cells.

Western blotting demonstrates the recognition of proteins in their denatured state. However, the native and denatured forms of the same protein differ immunogenically. This is due to the fact that regions/domains of a polypeptide which are inaccessible to an antibody when the protein is in its native conformation become exposed upon denaturation. This results in alteration of most epitopes. Consequently recognition of a denatured protein by an antibody does not imply that the antibody will also bind to the protein in its native conformation and similarly antibodies may react with a polypeptide when it is in its native, but not in a denatured state. Therefore in this study, ligand, slot blotting and immunocytochemical studies were performed to confirm the recognition of the antibodies with the LH/CG receptor in its native state (i.e. in the absence of any reducing agents). Another member of the glycoprotein hormone receptor family, the TSH receptor, and an antiserum raised against the extracellular domain of the TSH receptor were also used in Western blotting studies in order to study the cross-reactivity of the different antibodies with these two related receptors.

5.1.1 Western and ligand blotting

The potential high specificity and affinity of antibodies makes them sensitive tools for the identification and characterization of proteins separated by gel electrophoresis. Very small quantities of a protein of interest from a complex mixture of proteins can be characterized by Western blotting. There are many detection techniques available, of which two were used in the current study; the enhanced chemiluminescence (ECL) and the alkaline phosphatase system. Although the ECL system has many advantages over other detection methods, this system gave rise to numerous problems, the main one being the high background levels in all Western blots (which was probably due to the high sensitivity of this method). The cost of the kit also imposed financial limitations on its use. Enzyme linked (alkaline phosphatase) detection of antibody binding was therefore favoured. This detection system is based on the use of alkaline phosphatase-conjugated secondary antibody reagents. Detection is achieved using appropriate chromogenic substrates which form insoluble, stable coloured reaction products at the sites on the blot where the enzyme-conjugated secondary antibody is bound. In this study, the substrates used for visualization were 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium which, when acted upon by alkaline phosphatase, form purple coloured bands (see *Materials and Methods* section 2.2.17).

Direct demonstration of specific ligand-receptor interaction by using radiolabelled ligand avoids the use of specific antibodies, which simplifies the detection system and leads to greater sensitivity. In this study, the ligand blotting technique was used to identify directly the LH/CG receptor after size separation by SDS-PAGE under non-reducing conditions in crude ovarian extracts, MA10 cells and liver membranes. In

these experiments the ligand used was ^{125}I-hCG for which binding was directly assessed by autoradiography.

5.1.2 Cross-reactivity with the TSH receptor protein and antibody

The thyroid stimulating hormone receptor (TSHr) is a cell surface receptor for the glycoprotein hormone TSH which is important in controlling the growth and function of the normal thyroid. The cDNA sequence of this receptor predicts a polypeptide with a molecular mass of 84 kDa and six potential glycosylation sites (Nagayama *et al.*, 1989; Misrahi *et al.,* 1990). From hydrophobicity plots it was suggested that this receptor spans the membrane seven times and is therefore closely related to two other G protein-linked glycoprotein receptors: the LH and the FSH receptors.

In this study, membranes from chinese hamster ovary (CHO) cells transfected with a cDNA encoding the extracellular domain (residues 1-415) of the human TSHr (designated ExG2) (Harfst *et al.*, 1992) and an antibody raised to this recombinant protein (antiserum R14) (gift from Prof. A.P.Johnstone) were used to establish cross-reactivity with the LH receptor antibodies and MA10 membranes in Western blots.

5.1.3 Immunocytochemistry

All the immunocytochemistry data presented in this chapter were obtained in collaboration with Dr. Katja Teerds from the department of Cell Biology & Histology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands. The work was carried out at the University of Utrecht using the three antibodies raised in this study, on formaldehyde fixed rat testicular tissue sections and MA10 cells.

5.2 Results

5.2.1 Protein staining

The efficiency of transfer of proteins from the gels to the membranes was checked each time by staining the gels after transfer. Figure 5.1 shows staining of the MA10 membranes after PAGE. The gel was cut in half and each half was stained. Panel A represents staining after protein transfer and panel B before transfer. The electrophoretic protein transfer was approximately 60% efficient.

Figure 5.2 shows the protein pattern of both MA10 and ovarian membranes after staining.

5.2.2 Reaction with intact protein in slot blots

Slot blots of solubilized proteins from MA10 cells as well as proteins prepared from rat testes were positive with all three antibodies but not with pre-immune IgG. There was no binding of the antibodies with liver proteins nor with proteins prepared by the same method from an endothelial cell line (fig. 5.3).

5.2.3 Western blotting with rat ovarian, testicular, and MA10 membranes

On Western blots of membranes prepared from superovulated rat ovaries all three antibodies recognized a protein that migrated as a 95-100 kDa broad band. A somewhat sharper band of 95 kDa was labelled on blots of MA10 cell membranes, with the pre-immune IgG being negative in both membranes (fig. 5.4). With rat testicular membranes, similar results to those from the MA10 membranes were

obtained, with all three antibodies recognizing the 95 kDa protein and failing to react with liver membranes prepared the same way. Pre-immune IgG was again negative (fig. 5.5).

Treatment of MA10 cell membranes with endoglycosidase F produced two immunoreactive bands of apparent molecular mass 75 and 64 kDa respectively, both of which were recognised by all three antibodies but not pre-immune IgG (fig. 5.6).

5.2.4 Cross-reactivity with the TSH receptor protein and antibody

Membranes prepared from CHO cells transfected with a cDNA encoding for the extracellular domain (aa residues 1-415) of the human TSH receptor (hTSHr), ExG2, and its corresponding antiserum, R14, were used to establish cross-reactivity with the LH receptor antibodies. Fig. 5.7 shows that the ExG2 protein (60 kDa) was recognized by the R14 antibody only and not by the LH receptor antibodies. Antibodies 1, 2 and 3 only recognized the 95 kDa protein found in MA10 membranes whereas the R14 antibody and pre-immune IgG failed to bind to the 95 kDa protein band. The 68 kDa protein band obtained using the R14 antibody in both the ExG2 and MA10 membranes is a non specific band obtained using this antibody (discussed previously by Harfst *et al.* (1994)).

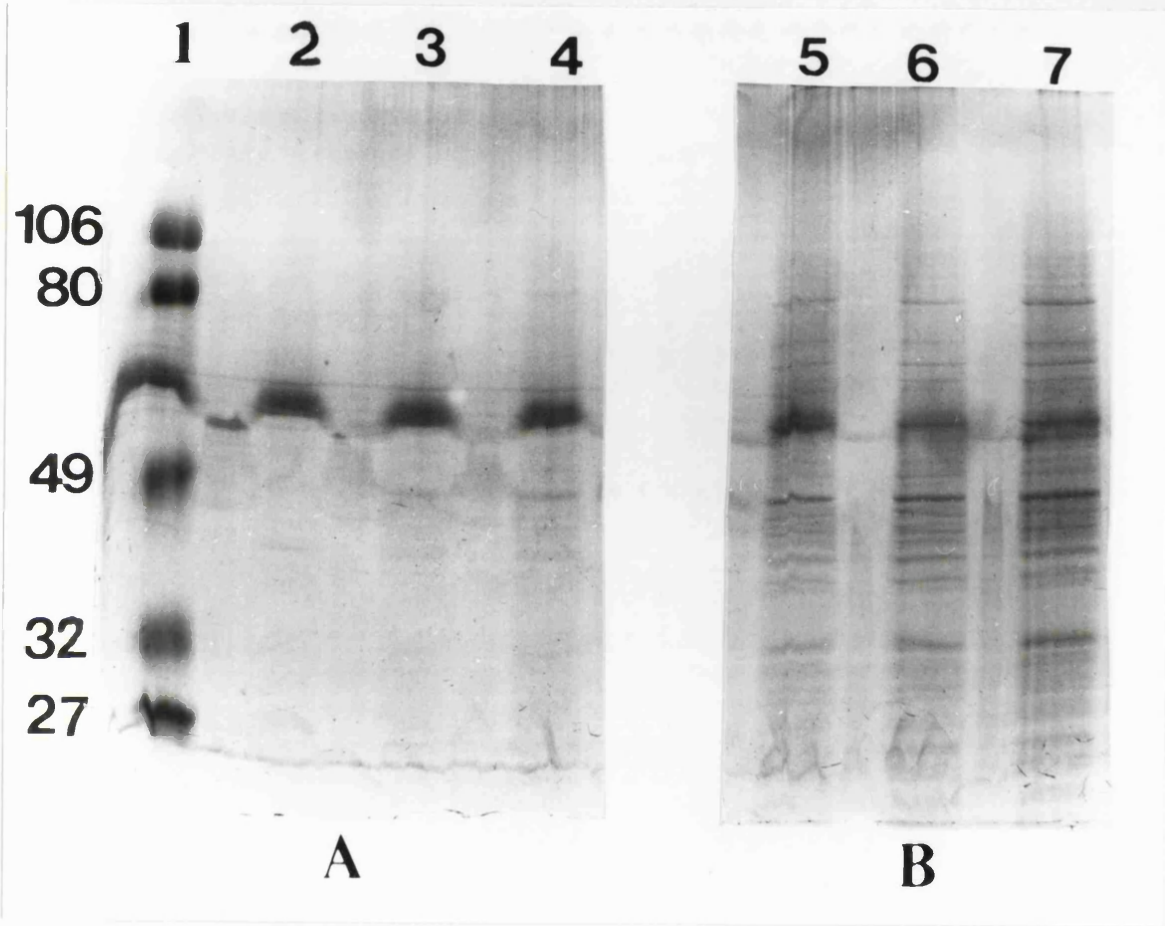

Figure 5.1 Efficiency of electrophoretic protein transfer. Staining of a 10% polyacrylamide gel with Coomassie blue R-250 before and after electrophoretic transfer to nitrocellulose membranes. Following electrophoresis, the gel was cut in half, where one half was directly stained and the other half was first subjected to protein transfer and then stained as described in *Materials and Methods* section 2.2.16. Panel A shows staining after transfer and panel B before transfer. Lane 1 represents the molecular weight markers (range 27-106 kDa). Lanes 2-7 represent different amounts of MA10 membrane proteins loaded. Lanes 2 & 5 = 25μg, lanes 3 & 6 = 50μg and lanes 4 & 7 = 100μg.

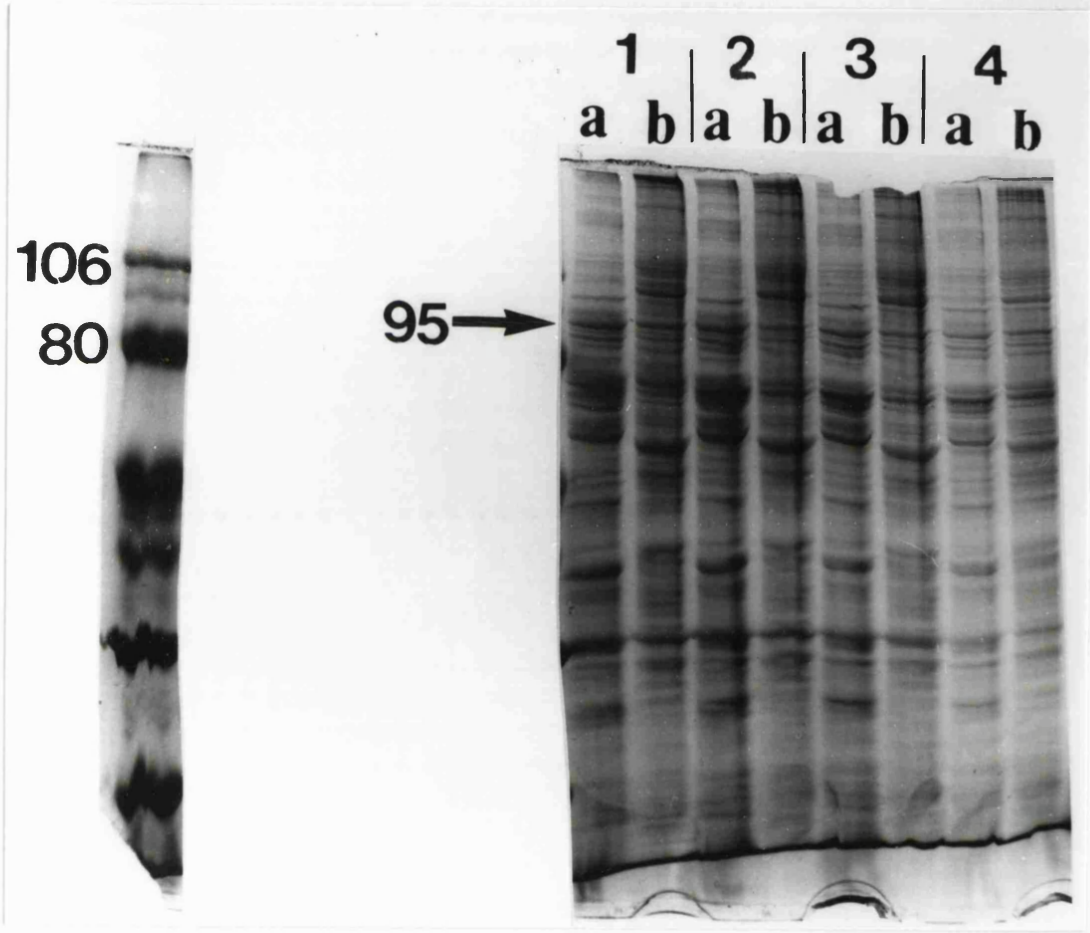

Figure 5.2 Protein staining pattern of MA10 and ovarian membrane proteins. Staining with Coomassie blue R-250 showing the protein pattern of both MA10 and ovarian membranes. Proteins were resolved on 10% polyacrylamide gels and then stained as described in *Materials and Methods* section 2.2.16. Molecular weight markers are shown on the left of the figure. The lanes marked "a" are MA10 cell membranes and "b" lanes are the ovarian membranes. Different amounts of protein were loaded. Lane 1a,b = 25μg, lane 2 a,b = 50μg, lane 3a,b = 75μg and lane 4a,b = 100μg. The protein band with a molecular mass of 95 kDa protein which may contain the LH/CG receptor is indicated.

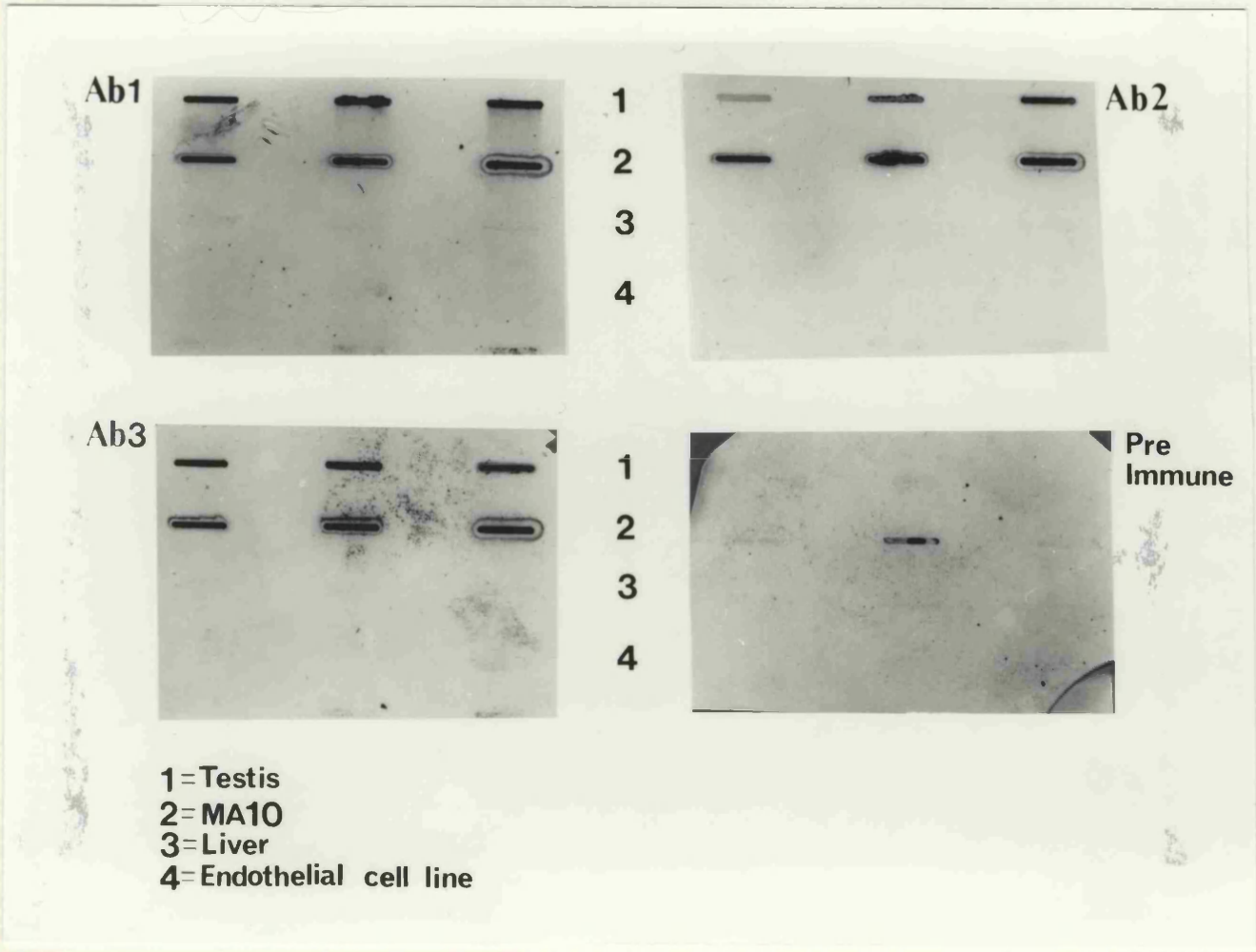

Figure 5.3 Immunoslot blotting analyses using testicular, MA10, liver and endothelial cell membranes. Immunoslot blotting with the three antibodies and pre-immune IgG against MA10 cell membranes (row 1), testicular membranes (row 2), liver membranes (row 3) and endothelial cell membranes (row 4). Each slot blot was carried out in triplicate with 100μg/lane and the amount of primary antibody (10μg/ml) bound, was detected as described in *Materials and Methods* section 2.2.17. Experiments were repeated three times and similar results were obtained.

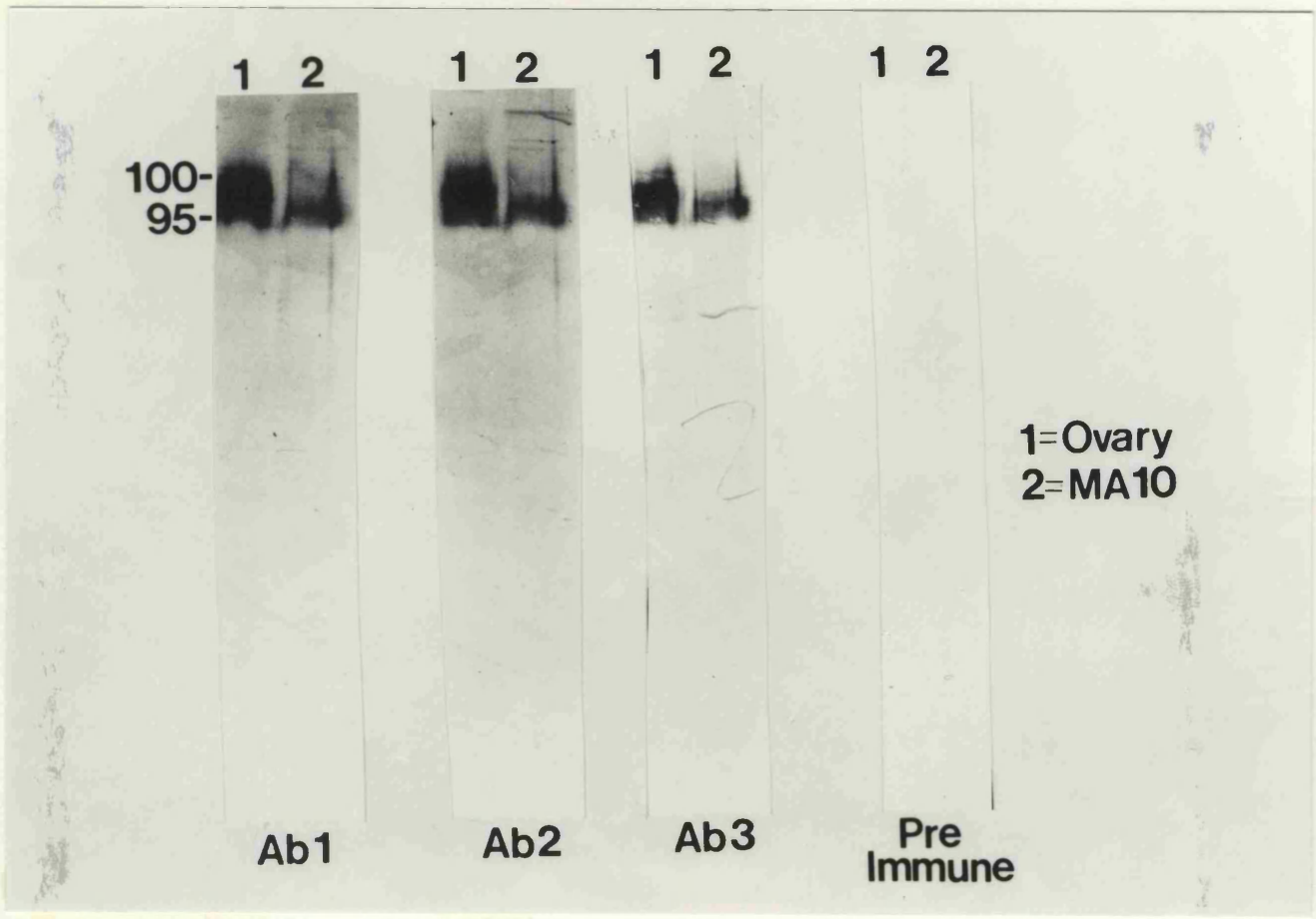

Figure 5.4 Western blotting using ovarian and MA10 cell membrane proteins. Western blotting of membranes obtained from superovulated rat ovarian cells (lane 1) and MA10 cells (lane 2) using the three LH receptor antibodies and pre-immune IgG. Membrane proteins (50-100μg) were resolved on 10% SDS-polyacrylamide gels and thentransblotted onto nitrocellulose membranes. Affinity purified primary antibody (20μg) or pre-immune IgG (20μg) was added and binding was detected as described in *Materials and Methods* section 2.2.17. The molecular weights (kDa) of the proteins identified by the antibodies are indicated on the left of the figure. Experiments were repeated four times with similar results for the ovarian membranes and the MA10 cells.

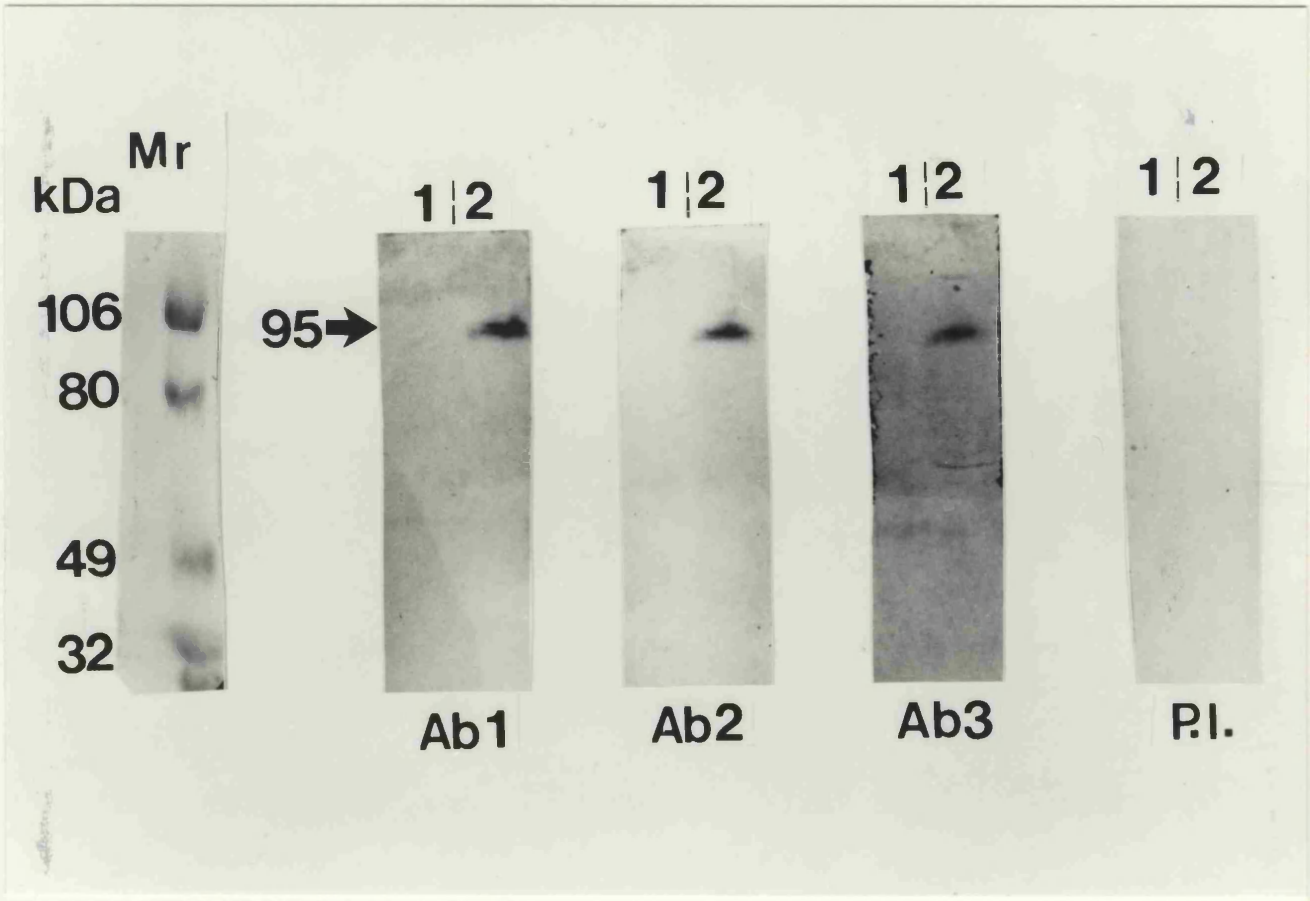

Figure 5.5 Western blotting using testicular and liver membrane proteins. Western blotting of membranes obtained from liver membranes (lane 1) and rat testis (lane 2) using the three LH receptor antibodies and pre-immune IgG. Membrane proteins (50-100μg) were resolved on 10% SDS-polyacrylamide gels and then transblotted onto nitrocellulose membranes. Affinity purified primary antibody (20μg) or pre-immune IgG (20μg) was added and binding was detected as described in *Materials and Methods* section 2.2.17. Pre-stained molecular weight markers are shown on the left of the figure. The 95 kDa protein identified by the antibodies is also indicated. Experiments were repeated four times with similar results for the testicular and liver membranes.

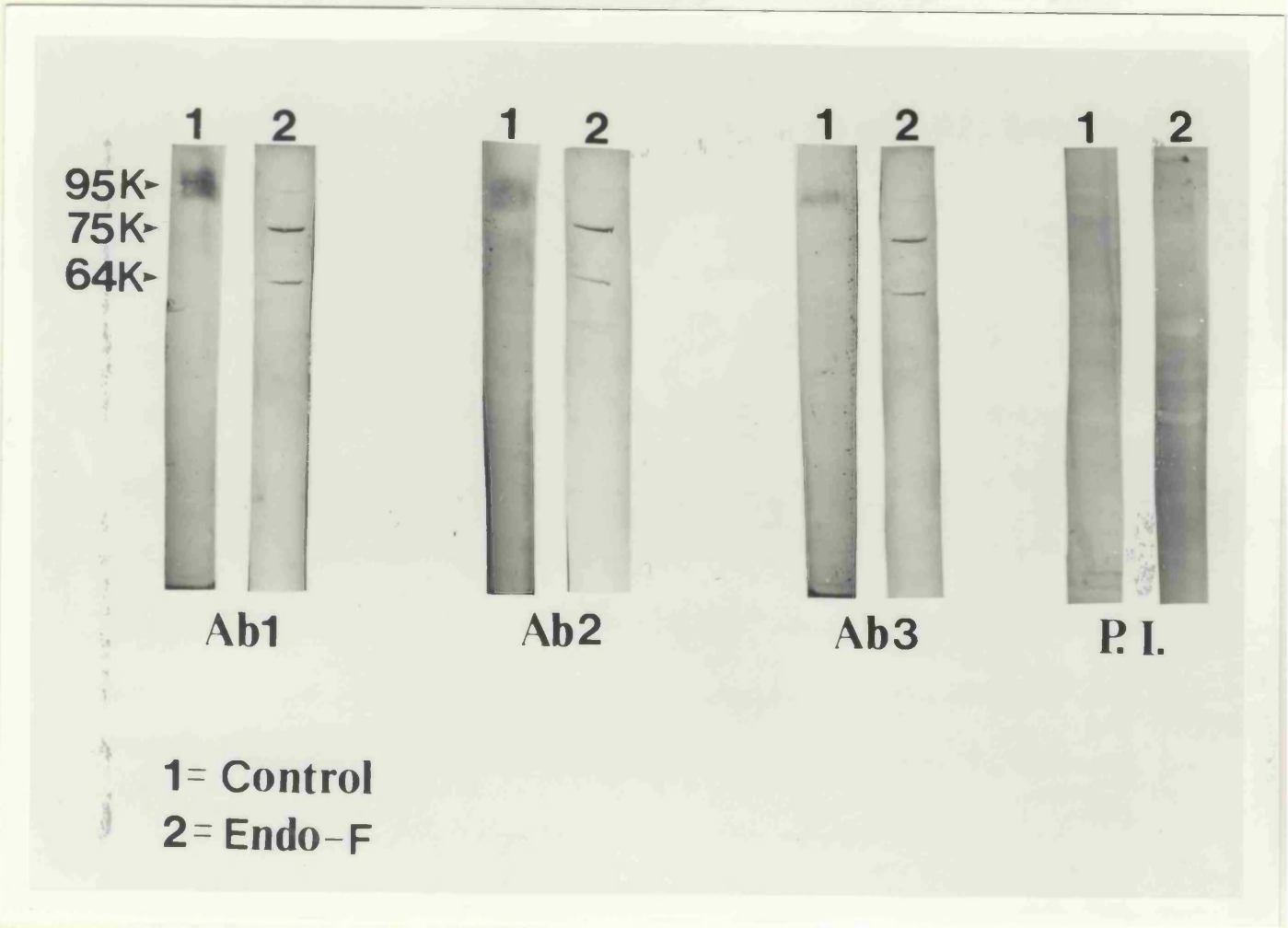

Figure 5.6 Western blotting with MA10 cell membrane proteins before and after deglycosylation. Western blotting of MA10 cell membranes in the absence (lane 1) or presence of Endoglycosidase F (lane 2), using the three LH receptor antibodies and pre-immune IgG. Membrane proteins (50-100μg) were resolved on 10% SDS-polyacrylamide gels and then transblotted onto nitrocellulose membranes. Affinity purified primary antibody (20μg) or pre-immune IgG (20μg) was added and binding was detected as described in *Materials and Methods* section 2.2.17. The molecular weights (kDa) of the three proteins identified by the antibodies are shown on the left of the figure. Experiments were repeated three times with similar results.

5.2.5 Ligand blotting

Triton X-100 extracts of pseudopregnant rat ovaries (see *Materials and Methods* section 2.2.12) were subjected to SDS-PAGE under non-reducing conditions and transferred electrophoretically to nitrocellulose paper. After blocking the free protein binding sites with a protein mixture, the nitrocellulose strips were incubated with ^{125}I-hCG (see *Materials and Methods*, section 2.2.22). Autoradiography revealed an intensely labelled 95 kDa protein band that was displaced by an excess of unlabelled hCG (fig.5.8, lane 2A,2B). When MA10 cell membranes were used, they also produced displaceable labelling of the 95 kDa band (fig. 5.9). These results demonstrate that the binding of ^{125}I-hCG to the 95 kDa protein is consistent with the binding obtained using the three antibodies. The binding is also specific to the known target cells for LH because no binding of the labelled hormone to protein blots of rat liver membranes was observed (fig.5.8, lane 1A,1B). These results demonstrate the similar sizes of both rat and murine LH/CG receptors and also provide strong evidence that the LH/CG receptor is a 95 kDa protein. When both the ovarian (fig. 5.10) and the MA10 (fig. 5.11) membrane protein samples were reduced by treating with 2% (v/v) mercaptoethanol and 4mM DTT prior to SDS-PAGE, no radioactive bands were observed indicating the existence of disulphide bonds in the LH/CG receptor that are necessary for the active hormone-binding conformation of the receptor. Again, the 95 kDa band was detected in the absence of reducing agents and was displaced by excess unlabelled hCG.

5.2.6 Immunocytochemistry

The three antibodies and pre-immune IgG were used at two different concentrations for staining rat testicular sections 2.5μg/ml and 5μg/ml (dilutions 1:400 and 1:200). Figures 5.12 - 5.15 clearly show staining in the interstitial space where the Leydig cells are found. Some non-specific staining was also observed around the connective tissue when the antibodies were used in high concentrations. Specific staining was only visible when antibodies 1 and 2 were used. No staining was seen with antibody 3 and pre-immune IgG. Similarly when MA10 cells were used (figs 5.16 and 5.17) pericellular staining was only observed with antibodies 1 and 2.

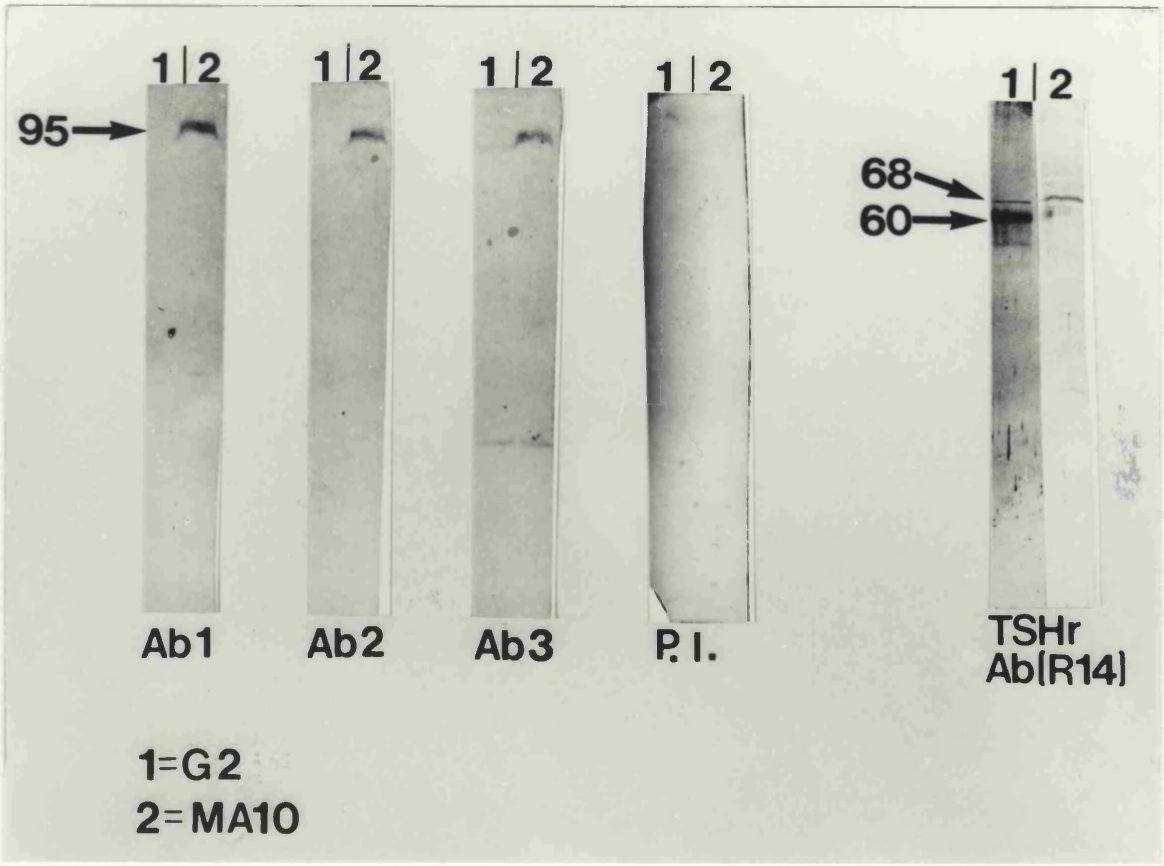

Figure 5.7 Western blotting using TSH receptor protein and MA10 membrane proteins. Western blot analyses of the extracellular region of hTSHr expressed in CHO cells (ExG2) (lane 1) and the MA10 cell membranes (lane 2) with antiserum R14 and the three LH receptor antibodies. Membrane proteins (50-100μg) were resolved on 10% SDS-polyacrylamide gels and then transblotted onto nitrocellulose membranes. Binding of the antibodies was detected as described in *Materials and Methods* section 2.2.17. The positions for the 95, 68 and 60 kDa molecular weights are also shown. Experiments were repeated twice with similar results.

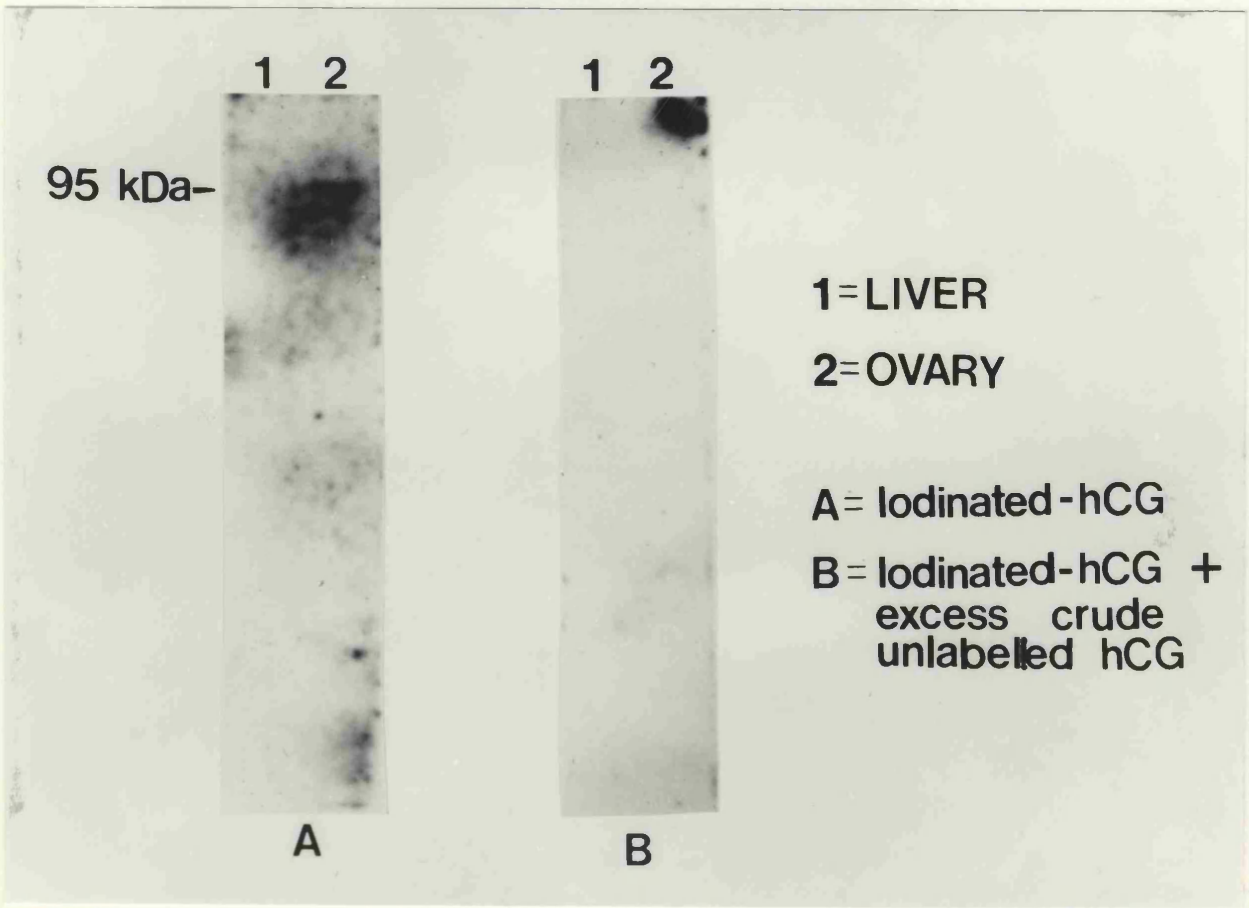

Figure 5.8 Ligand blotting using ovarian and liver membrane proteins. Ligand blotting of liver (lane 1) and ovarian membranes (lane 2). Membrane proteins (100µg/lane) were resolved on 10% polyacrylamide gels under non-reducing conditions and transblotted onto nitrocellulose membranes. Panel A was incubated with 1 x 10^6 cpm of ^{125}I-hCG. Panel B was incubated with ^{125}I-hCG in the presence of excess unlabelled crude hCG (10IU/ml) and then subjected to autoradiography as described in *Materials and Methods* section 2.2.22. Experiments were repeated three times with similar results.

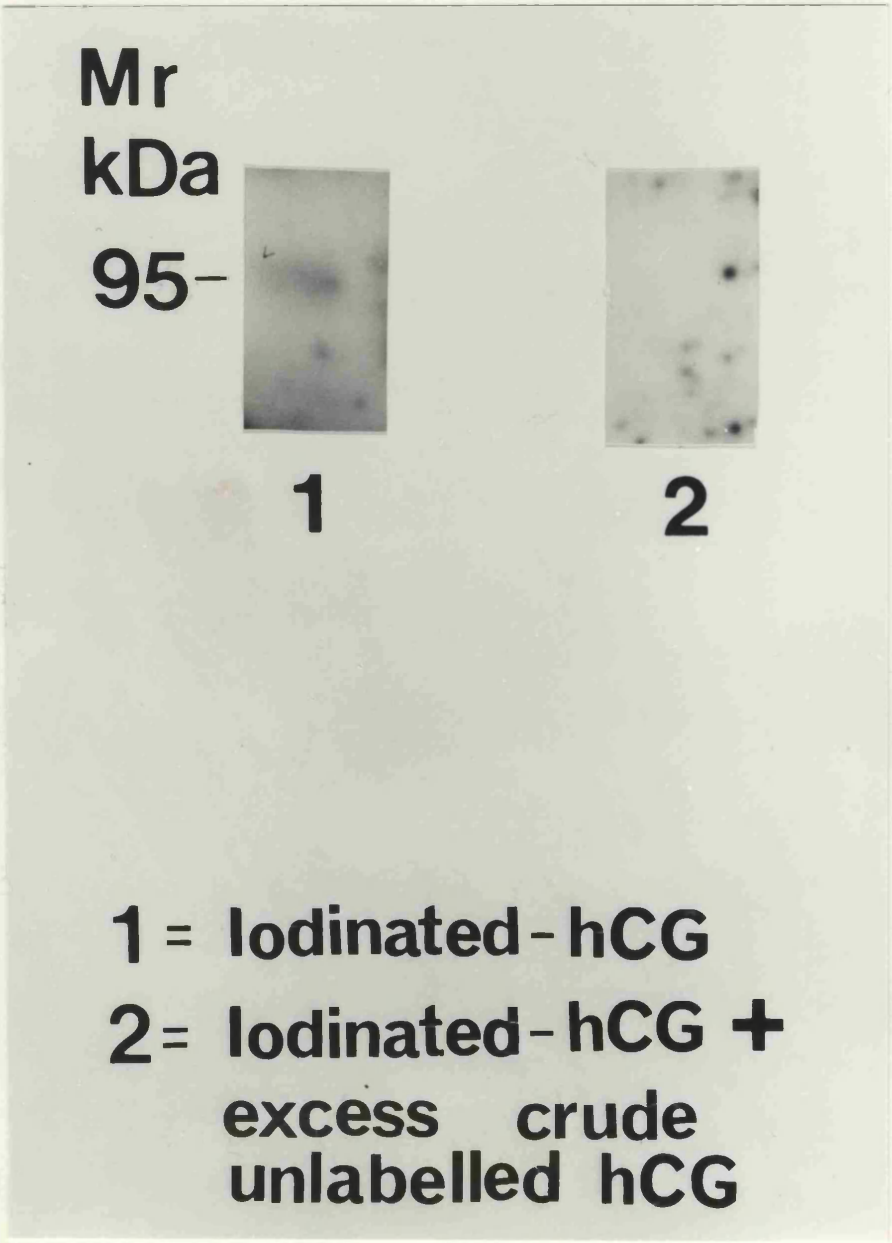

Figure 5.9 Ligand blotting using MA10 cell membrane proteins. Ligand blotting of MA10 cell membranes. Protein membrane samples (100μg/lane) were resolved on 10% polyacrylamide gels under non-reducing conditions and transblotted onto nitrocellulose membranes. Lane 1 was incubated with ^{125}I-hCG (1 x 10^6 cpm) and lane 2 with ^{125}I-hCG in the presence of an excess unlabelled crude hCG (10IU/ml) as described in *Materials and Methods* section 2.2.22. Experiments were repeated three times with similar results.

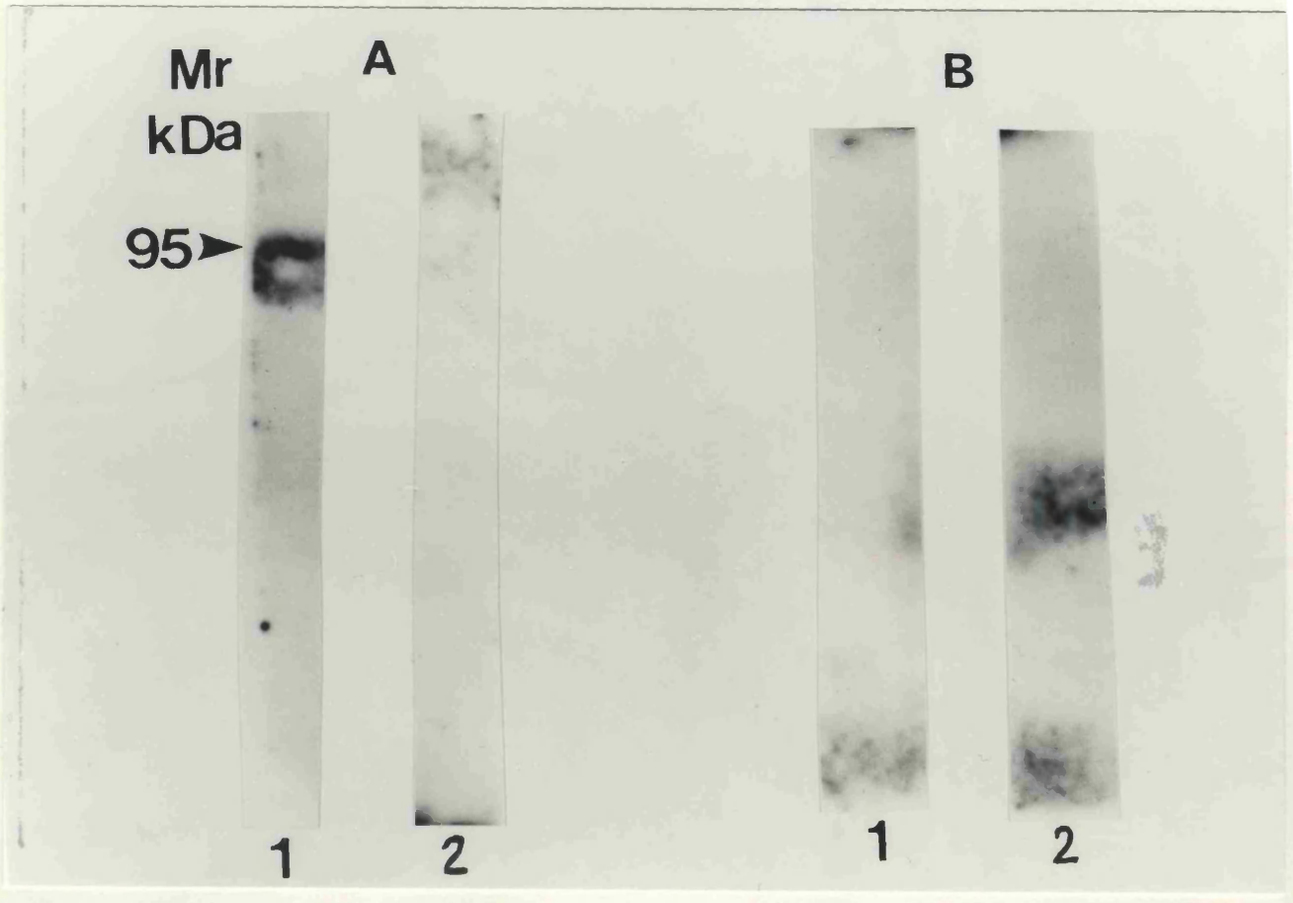

Figure 5.10 Ligand blotting using ovarian membrane proteins under reducing and non-reducing conditions. Ligand blotting analyses of ovarian membranes. Membrane protein samples (100μg/lane) were resolved on 10% gels and transblotted onto nitrocellulose membranes. Proteins in lanes 1 were incubated with ^{125}I-hCG (1 x 10^6 cpm) and lane 2 with ^{125}I-hCG in the presence of an excess unlabelled crude hCG (10IU/ml) as described in *Materials and Methods* section 2.2.22. Protein samples from panel A were under non-reducing conditions and proteins in panel B were reduced by treating with 2% (v/v) mercaptoethanol and 4mM DTT prior to SDS-PAGE. The molecular weight of the 95 kDa protein is shown on the left of the figure. Experiments were repeated three times with similar results.

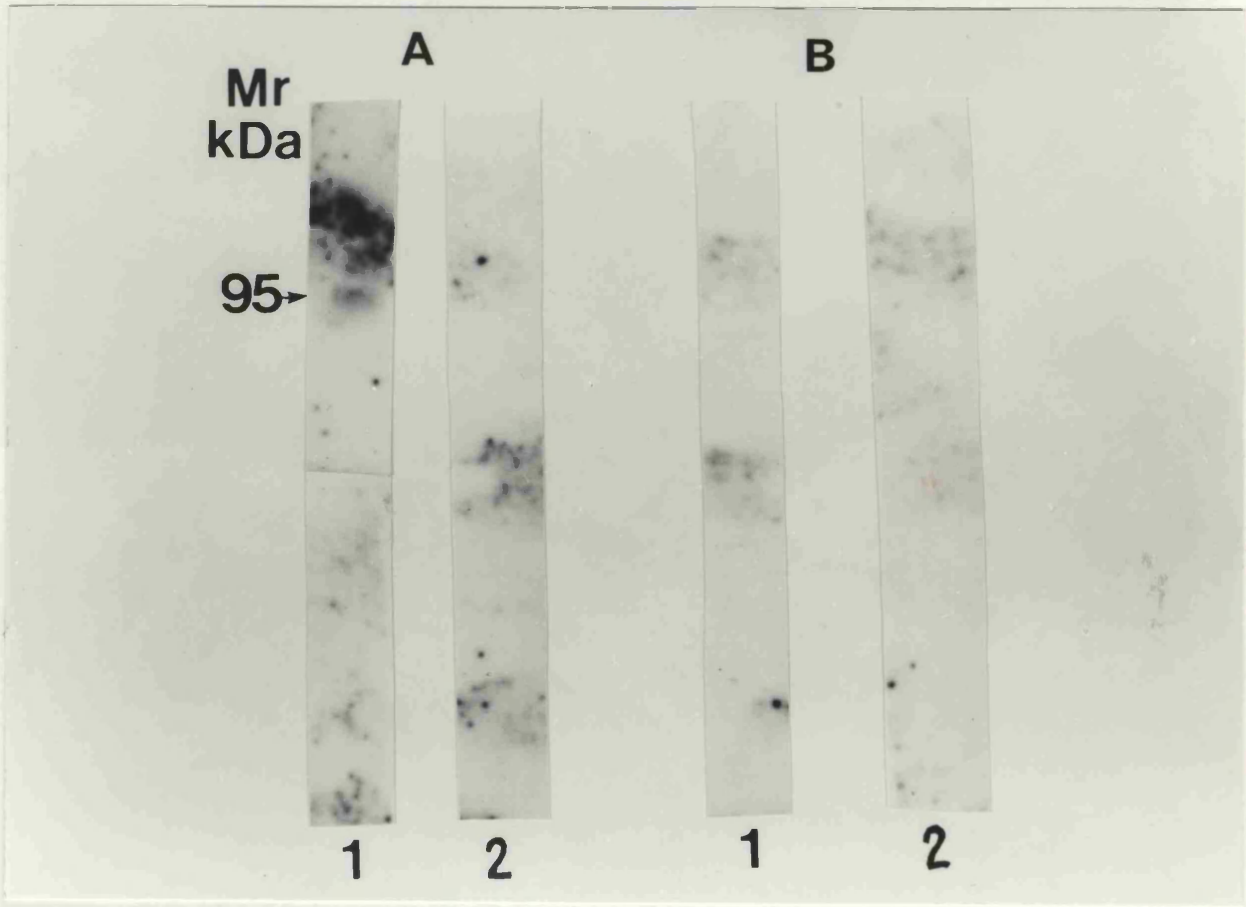

Figure 5.11 Ligand blotting using MA10 cell membrane proteins under reducing and non-reducing conditions. Ligand blotting analyses of MA10 cell membranes. Protein samples (100μg/lane) were resolved on 10% gels and transblotted onto nitrocellulose membranes. Proteins in lanes 1 were incubated with ^{125}I-hCG (1 x 10^6 cpm) and lane 2 with ^{125}I-hCG in the presence of an excess unlabelled crude hCG (10IU/ml) as described in *Materials and Methods* section 2.2.22. Protein samples from panel A were under non-reducing conditions and proteins in panel B were reduced by treating with 2% (v/v) mercaptoethanol and 4mM DTT prior to SDS-PAGE. The molecular weight of the 95 kDa protein is shown on the left of the figure. Experiments were repeated three times with similar results.

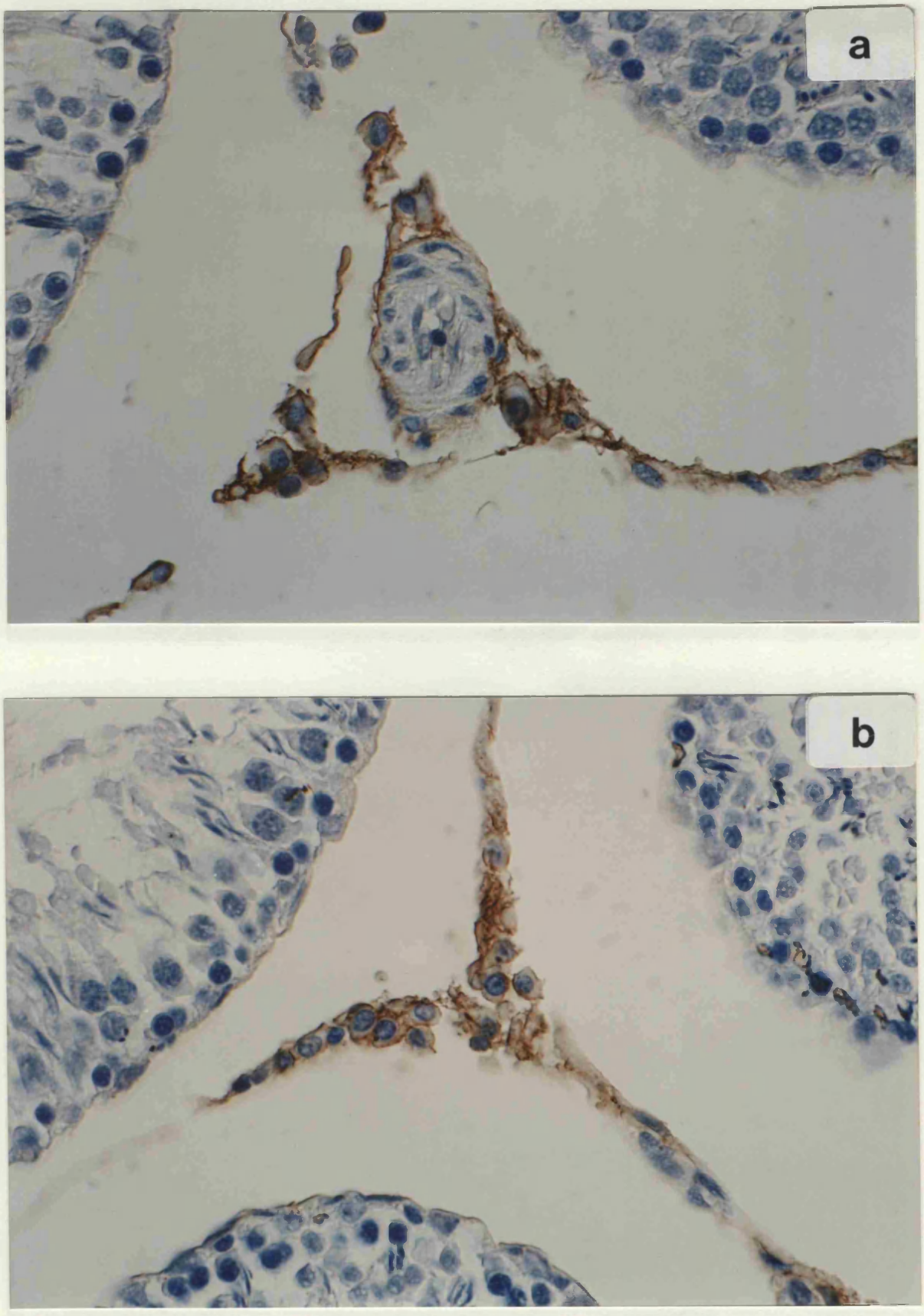

Figure 5.12a,b Immunocytochemistry on testicular sections using antibody 1. Immunocytochemistry on rat testicular sections using antibody 1 at (a) 5μg/ml (1:200 dilution) and (b) 2.5μg/ml (1:400 dilution). These are representative of 6 photomicrographs. The magnification is x40.

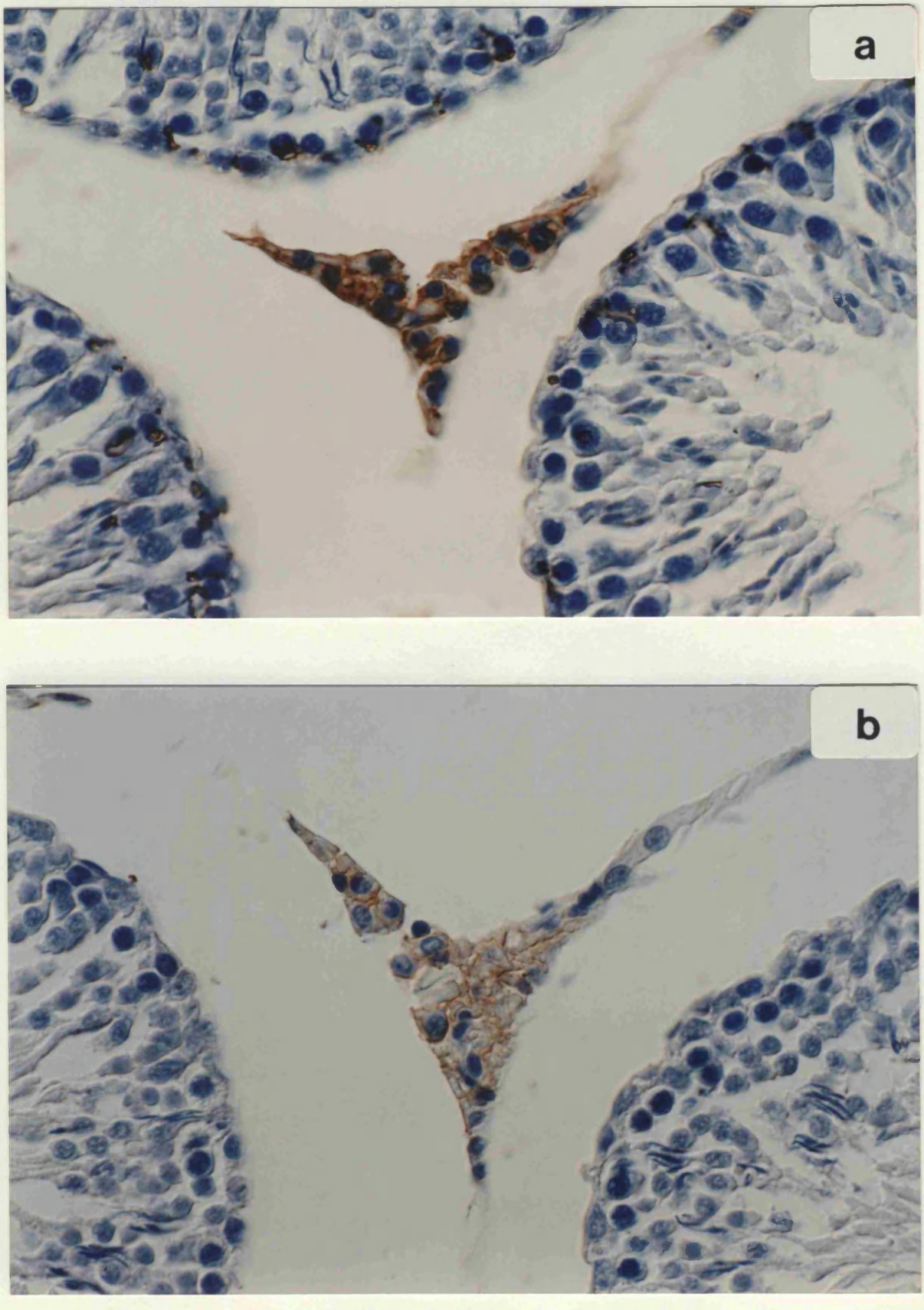

Figure 5.13a,b Immunocytochemistry on testicular sections using antibody 2. Immunocytochemistry on rat testicular sections using antibody 2 at (a) 5µg/ml (1:200 dilution) and (b) 2.5µg/ml (1:400 dilution). These are representative of 6 photomicrographs. The magnification is x40.

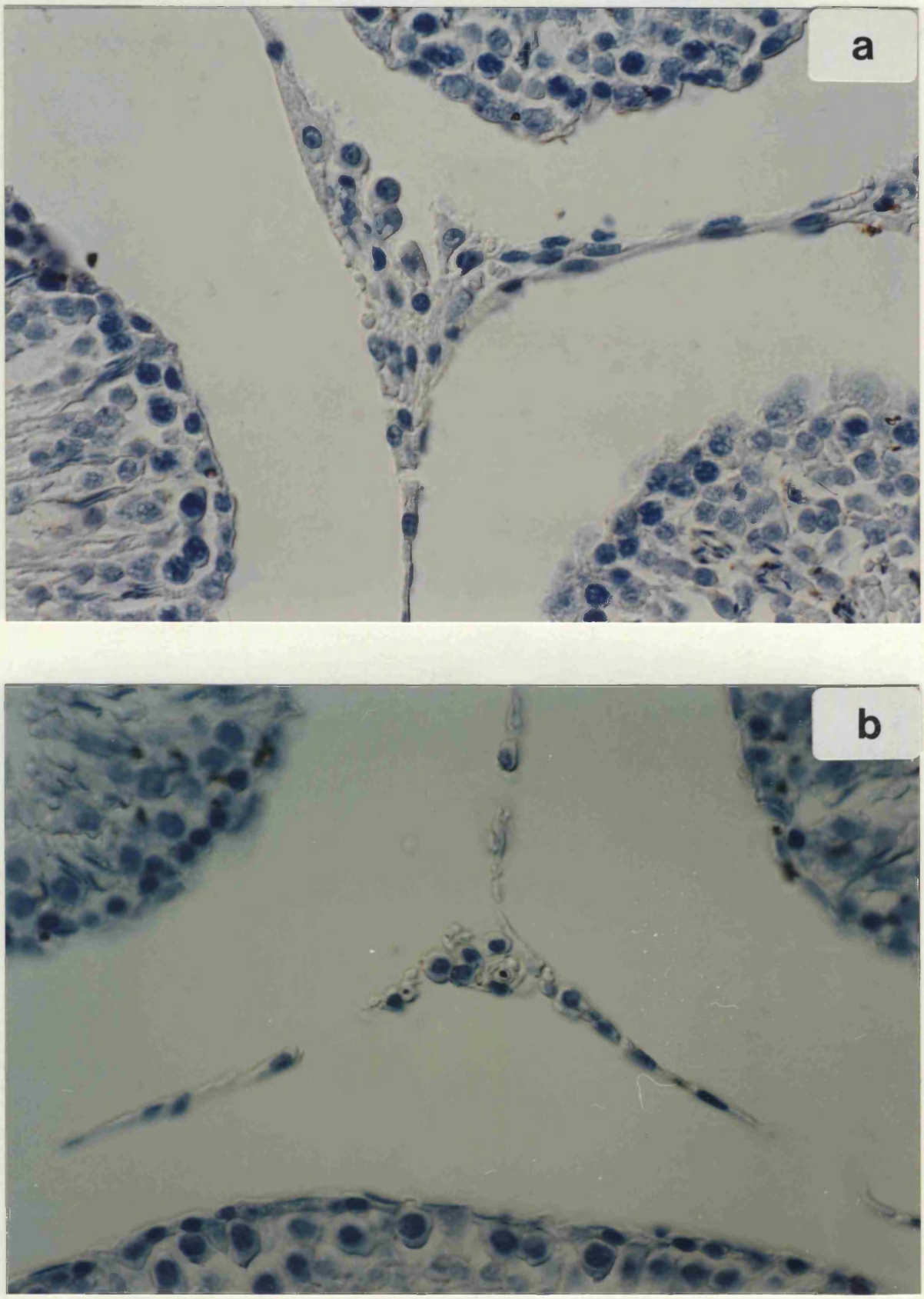

Figure 5.14a,b Immunocytochemistry on testicular sections using antibody 3. Immunocytochemistry on rat testicular sections using antibody 3 at (a) 5μg/ml (1:200 dilution) and (b) 2.5μg/ml (1:400 dilution). These are representative of 6 photomicrographs. The magnification is x40.

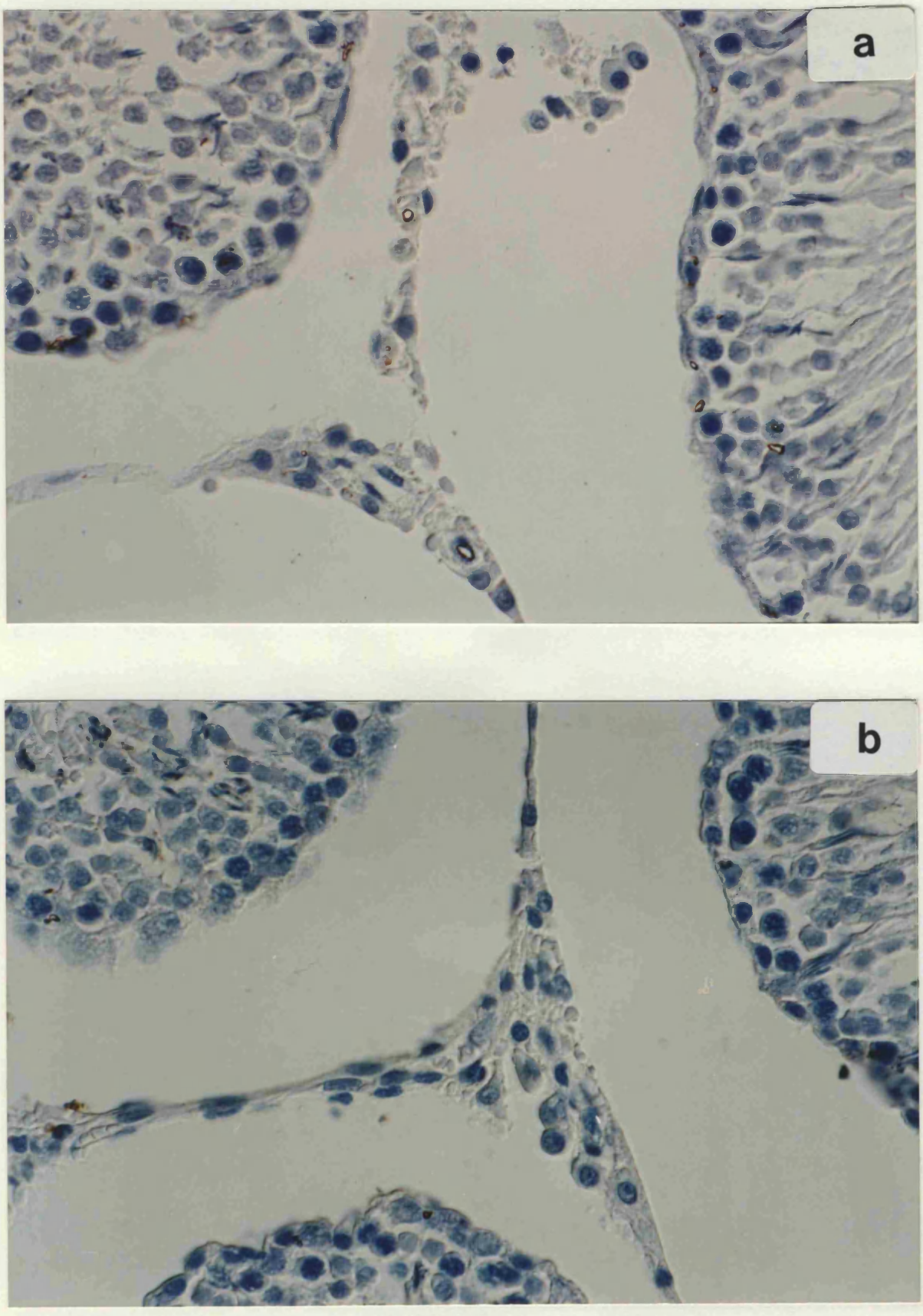

Figure 5.15a,b Immunocytochemistry on testicular sections using pre-immune IgG. Immunocytochemistry on rat testicular sections using pre-immune IgG at (a) 5μg/ml (1:200 dilution) and (b) 2.5μg/ml (1:400 dilution). These are representative of 6 photomicrographs. The magnification is x40.

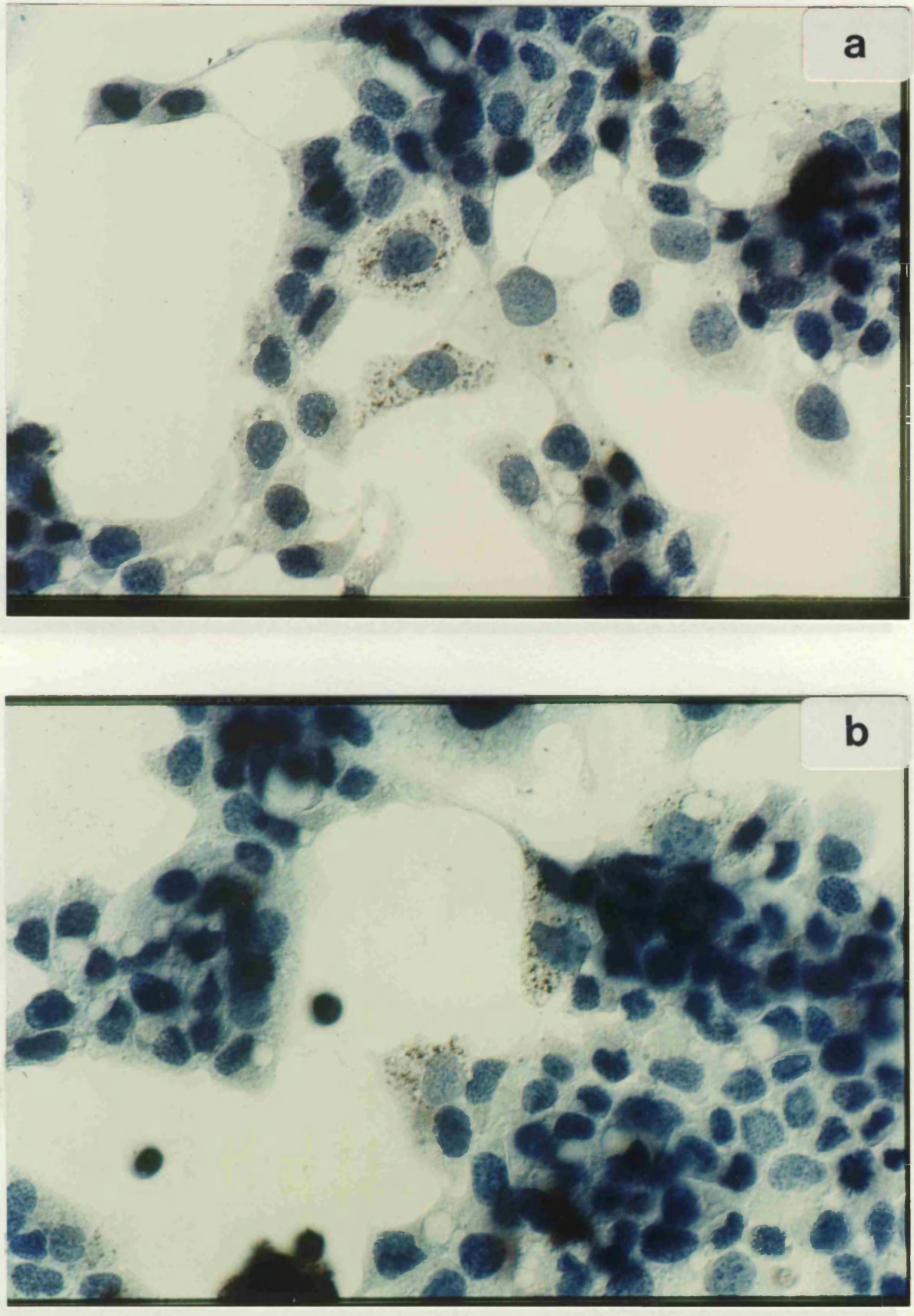

Figure 5.16a,b Immunocytochemistry on MA10 cells using antibody 1. Immunocytochemistry on MA10 cells using antibody 1 at (a) 5μg/ml (1:200 dilution) and (b) 2.5μg/ml (1:400 dilution). These are representative of 4 photomicrographs. The magnification is x40.

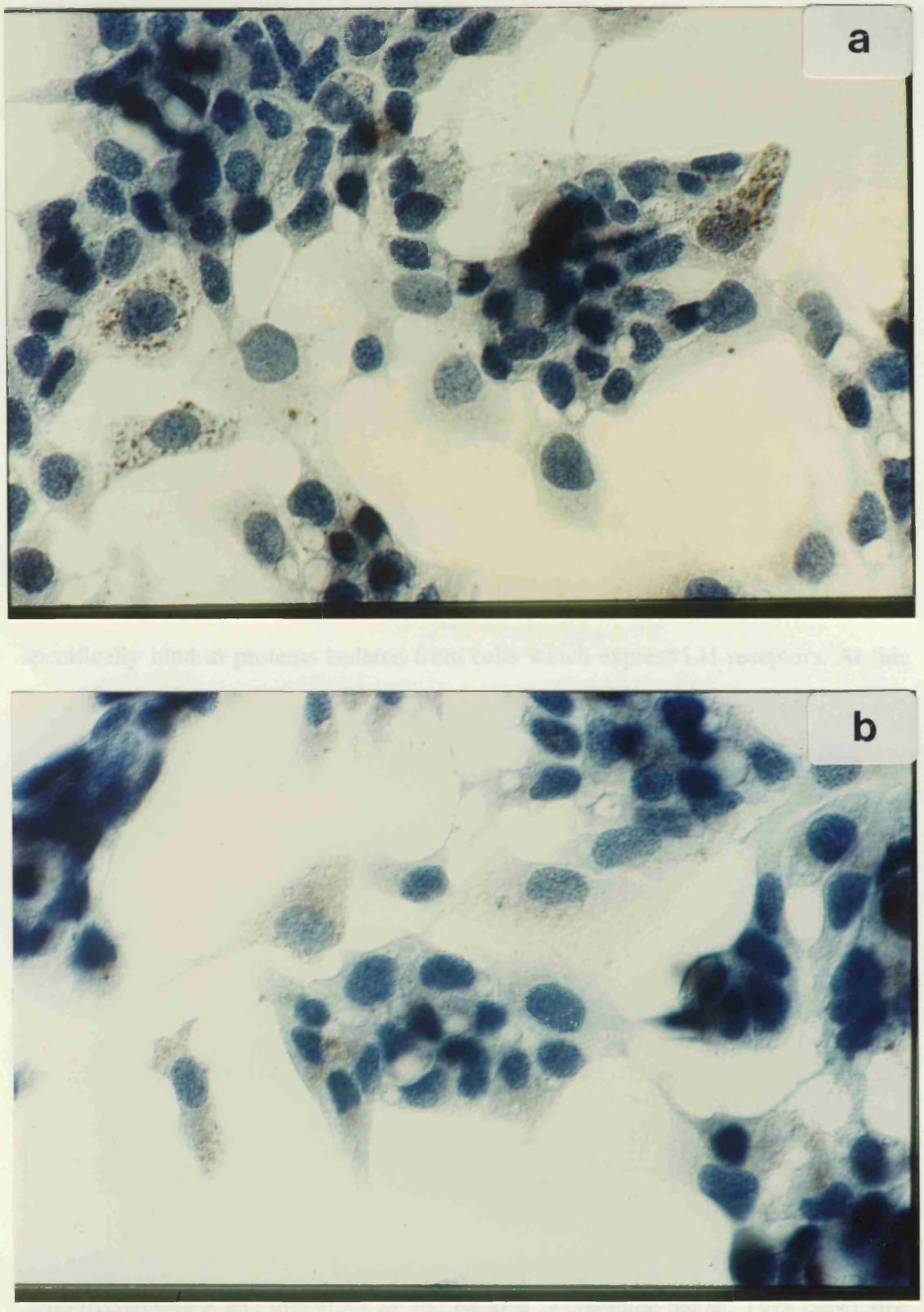

Figure 5.17a,b Immunocytochemistry on MA10 cells using antibody 2. Immunocytochemistry on MA10 cells using antibody 2 at (a) 5μg/ml (1:200 dilution) and (b) 2.5μg/ml (1:400 dilution). These are representative of 4 photomicrographs. The magnification is x40.

5.3 Discussion

Using a combination of Western, ligand and slot blotting analyses, the results described in this chapter clearly demonstrate that the antibodies raised in this study recognize a 95 kDa protein in MA10, testicular and ovarian membranes.

The slot blot analyses demonstrated that the three LH receptor antibodies (but not pre-immune IgG), recognized membrane proteins prepared from tissues known to express LH/CG receptors; i.e. ovarian tissues, MA10 cells and rat testis. There was no binding of these antibodies with tissues thought to be devoid of such receptors (e.g. membranes from endothelial cell lines and liver). This indicated that the antibodies specifically bind to proteins isolated from cells which express LH receptors. At this stage it was not evident that the antibodies are actually binding specifically to the LH receptor. Confirmation of this was provided by Western blots of superovulated rat ovaries, rat testis and mouse MA10 cells where only 95-100 kDa proteins (comparable in size to that reported for the LH receptor (see review Segaloff and Ascoli, 1993)) were recognised. The differences in mobility seen for the bands recognised by the antibodies on the Western blots of the rat ovarian, rat testicular and mouse MA10 cells are probably attributable to differences in the N-linked glycosylation pattern in the ovary and testis, as discussed previously by Minegishi *et al.* (1989). The two bands observed after treatment with endoglycosidase-F (75 and 64 kDa) confirmed the finding of Sojar and Bahl (1989) where they treated the rat ovarian LH receptor with endoglycosidase F and identified 73 and 64 kDa polypeptide bands. They concluded the presence of approximately 20% carbohydrate distributed in two or more N-linked carbohydrate chains. Endoglycosidase F is known to hydrolyse the glycosidic bond

structure adjacent to asparagine residues. The present results together with those of Sojar and Bahl (1989) indicate therefore that LH/CG receptors contain N-linked glycosidic chains in both the MA10 cells and the ovary. N-linked glycosylation may be responsible for the size difference in the LH/CG receptor from testis and ovaries, as demonstrated by the Western blot analyses. Sharp bands are obtained when rat testis and MA10 cell membranes are used, whereas the band obtained using rat ovarian membranes always appears broader and sometimes as a doublet. The Western blotting results also suggest that the antibodies recognise the receptor regardless of the sugar residues, i.e. the carbohydrate residues do not interfere with the binding of the antibodies and the fact that the C-terminal antibody (antibody 3) binds to the 64 kDa fragment confirms that the latter is not simply the extracellular domain of the receptor.

The specificity of the antibodies was also confirmed in the cross-reaction Western blot experiment using the hTSH receptor protein (ExG2) and antibody R14. The three LH receptor antibodies failed to identify the hTSH receptor extracellular protein with a molecular mass of 60 kDa but only bound a 95 kDa protein in the adjacent lane containing the MA10 cell membranes. Similarly antibody R14 bound specifically only to a 60 kDa protein from the ExG2 protein but not with the LH receptor found in the MA10 membranes. Pre-immune IgG did not bind to either of the membranes.

Additional evidence for the identity of the LH receptor as a 95 kDa protein was provided by its ability to bind ^{125}I-hCG in ligand blot experiments. ^{125}I-hCG bound to a 95 kDa protein in ovarian and MA10 cell membranes and was displaced by excess unlabelled hCG. No specific labelling was found when rat liver membranes were used.

The 95 kDa band was not apparent when the ovarian and MA10 membrane protein samples were treated with reducing agents (2% (v/v) mercaptoethanol and 4mM DTT), showing that integrity of receptor disulphide bonds is essential for the hormone-receptor interaction. This finding is consistent with earlier observations on the effect of reducing agents on the binding of ^{125}I-hCG on rat testicular particulate and soluble LH receptor (Dufau *et al.,* 1974). These disulphide bonds, however, are not required for antibody binding since all three antibodies recognize the 95 kDa protein under reducing conditions. The present demonstration of specific binding of the antibodies to a 95-100 kDa protein as well as the specific binding of ^{125}I-hCG by a 95 kDa protein from rodent ovaries and MA10 cells, support the view that the LH/CG receptor is represented by a 95-100 kDa membrane protein. These results are compatible with those of Rapoport (1984) and Rebois (1982) who have observed specific covalent cross-linking of radiolabelled hCG to 100 kDa proteins.

Immunocytochemistry data in the present study provided more evidence that the antibodies recognize the intact native LH receptor in rat testicular sections and MA10 cells. The staining observed in the testicular sections was uniform, whereas in the MA10 cells staining was sporadic. Kilgore and Stocco (1989) reported the isolation of a subclone of MA10 cells which secreted less than 10% of the hCG-stimulated steroid synthesized by the parent MA10 cells. The sporadic staining obtained with the MA10 cells in the immunocytochemical experiments reported in this study can therefore be explained by the low distribution of LH receptors in what might be an MA10 cell subclone. Certainly it would appear that LH receptors are expressed to a

higher level in some MA10 cells that others and that the expression of the receptor protein is highly localized on the cell surface.

Staining was only visible with the two N-terminal antibodies (antibodies 1 and 2), and it was located at the cell boundary whereas antibody 3 (C-terminal antibody) and pre-immune IgG failed to react. Since antibody 3 had access to the cytoplasmic region of the receptor in the testicular sections, and still was not able to bind, this suggests that antibody 3 may not be able to recognise epitopes in the intact LH receptor. This may be attributable to palmitoylation of the intracellular cysteine residues (C^{621}-C^{622}) and hence formation of a fourth cytoplasmic loop that may interfere with antibody binding. Antibody 3 was raised to a sequence overlapping one of the cysteine residues (C^{622}) involved in the membrane-anchoring site, hence, the antigenic site may be too close to the plasma membrane to allow antibody binding. From the Western blotting experiments it was evident that antibody 3 bound to the intact LH receptor after separation on SDS-gels. This same antibody, however, did not react in immunocytochemical experiments using rat testicular sections, supporting the existence of a fourth cytoplasmic loop.

These results prove that the antibodies raised in this study are not only useful on Western blotting analyses, but they can also be successfully applied to immunocytochemical techniques on both rat and mouse tissues. In conclusion, rat ovarian, rat testicular and mouse MA10 cell LH/CG receptors were identified as 95-100 kDa proteins by both Western blotting and ligand binding after size separation of

the proteins and transfer to nitrocellulose. These results also demonstrate the structural similarities between rat and murine LH/CG receptors.

CHAPTER 6

FUNCTIONAL STUDIES USING MA10 CELLS AND MA10 CELL PLASMA MEMBRANES

6.1 Introduction

In this chapter, experiments were designed to determine: **1.** if the LH receptor antibodies interfered with ^{125}I-hCG binding to cultured MA10 cells, and: **2.** if they displaced ^{125}I-hCG from MA10 cell membranes in ligand blot analyses. The ability of the antibodies to inhibit LH-stimulated cAMP and progesterone production in MA10 cells was also investigated. Different antibody concentrations were used (0-100μg/ml) and a range of LH doses (0-1000ng/ml). In addition, cAMP and progesterone production were measured in MA10 cells in the presence and absence of the three antibodies after stimulation with dbcAMP (0-100mM), cholera toxin (0-50μg/ml) and forskolin (0-100μM).

Plasma membranes can be prepared from tumour Leydig cells using a 2 phase (dextran-polyethylene glycol) centrifugation method, and still retain an adenylyl cyclase response when challenged with LH (Levi *et al.,* 1982). Guanosine 5'-[β,γ-imido]triphosphate (p(NH)ppG) is a non-hydrolysable analogue of GTP and activates the adenylyl cyclase through a guanosine nucleotide-dependant regulatory protein. Sodium fluoride (NaF) was shown to stimulate adenylyl cyclase activity in plasma membranes, but requires the presence of Mg^{2+} (concentration > 2mM) (Levi *et al.,* 1982). The stimulatory effect of NaF is absent in the G_s-deficient lymphoma cell clone (S49) membrane system and is restored upon readdition of pure G_s (Ross and Gilman, 1977; Ross *et al.*, 1978). Thus, G_s mediates not only stimulatory effects of guanine nucleotides, but also that of fluoride ion. The actions of NaF resemble those of the nonhydrolyzable GTP analogues in that they lead to a persistent activation of G_s and, as analyzed in detergent solutions with crude (Howlett and Gilman, 1980) and pure

(Northup *et al.*, 1983) G_s, it promotes the subunit dissociation of this protein. An analysis of the fate of the three subunits of G_s revealed that the $\alpha\beta\gamma$ complex dissociates under the influence of NaF to give α plus $\beta\gamma$. It is not known with which of these subunits fluoride interacts to activate and dissociate the G_s molecule. In the present study, MA10 cell plasma membranes were prepared by the 2 phase centrifugation method and adenylyl cyclase was assayed by measuring the rate of formation of cAMP from ATP using a cAMP RIA as described in *Materials and Methods* section 2.2.25.1 after treating the membranes with LH, p(NH)ppG, p(NH)ppG + LH and NaF. Western blotting experiments were also performed on these membranes (prepared by the 2 phase method) in order to confirm binding of the antibodies.

6.2 Results

6.2.1 The effects of the LH receptor anti-peptide antibodies on the binding of ^{125}I-hCG to intact MA10 cells

The cells were incubated for 1h at 37^{o}C in the presence and absence of the three antibodies at different concentrations and the degree of binding of ^{125}I-hCG was determined during incubation of the cells at 4^{o}C for 48h. At a concentration of 1μg/ml, antibody 1 inhibited specific ^{125}I-hCG binding to the cells by 76% compared to the binding measured in the presence of an equivalent concentration of pre-immune IgG. At the same concentration, neither antibody 2 nor 3 had any greater effect than the pre-immune IgG. The latter inhibited binding to a small but significant extent relative to the IgG-free control. At the higher concentration of 10μg/ml, both

antibodies 1 and 2 showed significant inhibition of binding relative to the pre-immune IgG controls, with antibody 1 showing the greater inhibition (92% compared with 67% inhibition with antibody 2). In contrast, the effect of antibody 3 at this concentration did not differ from that of the pre-immune control (fig. 6.1).

6.2.2 The effects of the LH receptor antibodies on the binding of ^{125}I-hCG to solubilized MA10 cell membranes in ligand blotting analyses

MA10 cell membrane proteins were resolved on 10% gels by PAGE under non-reducing conditions and then transblotted onto nitrocellulose membranes. The latter were then incubated with or without antibodies and unlabelled crude hCG in the presence of ^{125}I-hCG and then subjected to autoradiography. In the absence of antibodies (lane 1) the ^{125}I-hCG bound mainly to a 95 kDa band (fig. 6.2) and also to a second band with a mass of 80 kDa. The latter is more clearly seen in lane 5 which contained solubilized membranes incubated with antibody 3. The appearance of the 80 kDa band was not apparent in previous ligand blotting (or Western blotting) experiments carried out in chapter 5 and therefore can be attributed to the different membrane preparation that was used with a different batch of MA10 cells. Binding of ^{125}I-hCG to the 95 kDa band was completely displaced by excess unlabelled hCG (10IU/ml) (lane 2) and by antibody 1 (10μg/ml) (lane 3). Antibody 2 also partially inhibited binding when used at the same concentration (lane 4), but antibody 3 was without effect (lane 5).

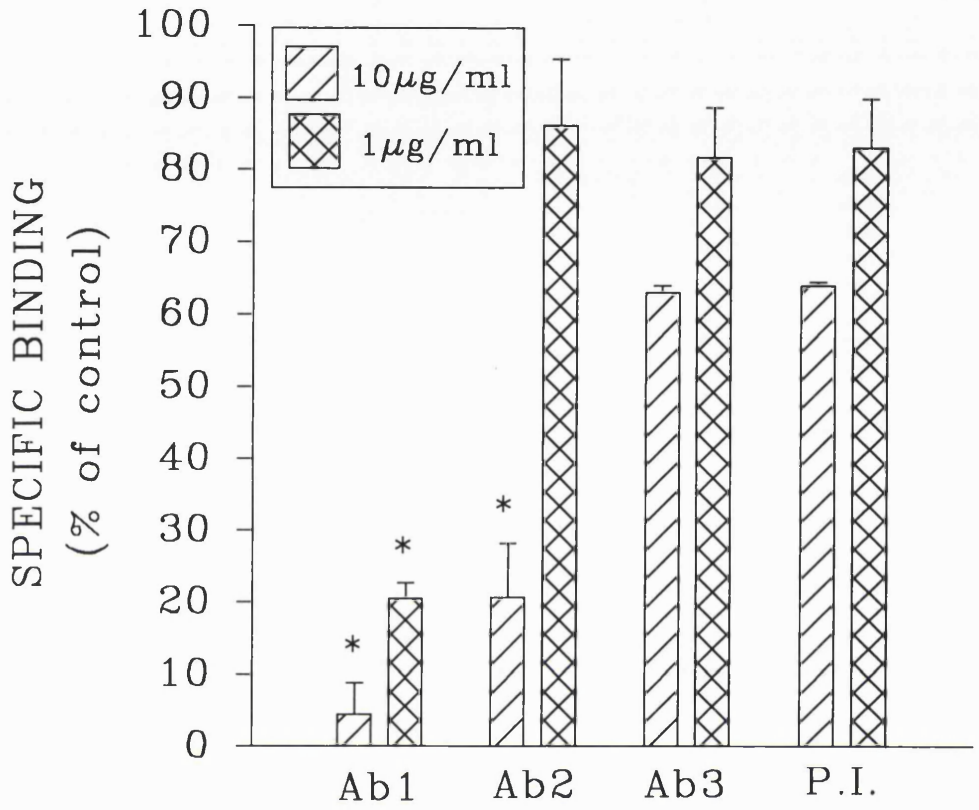

Figure 6.1 The effect of the LH receptor antibodies and pre-immune IgG on the specific binding of ^{125}I-hCG to MA10 cells. The effects of the three anti-peptide antibodies and pre-immune IgG on the binding of ^{125}I-hCG, relative to an IgG-free control were measured at the concentrations indicated. MA10 cells were plated at a density of 1 x 10^6 cells/well in 6 well plates and preincubated with either medium only, antibody or pre-immune IgG (at 1μg/ml and 10μg/ml) for 1h at 37^{O}C. ^{125}I-hCG binding was determined as described in *Materials and Methods* section 2.2.23. The 100% value represents binding in the absence of IgG (2580 ± 23 cpm). Non-specific binding, (approximately 10% of the total binding (235 ± 18 cpm)), was determined for each treatment by the addition of 300IU/ml unlabelled crude hCG. Values given are the mean + S.E.M. of three independent experiments, n=3 (triplicate determinations were carried out for each treatment in each experiment). * indicates a significant difference ($p<0.05$) between the effect of an antibody and that of its pre-immune control (unpaired *t*-tests).

6.2.3 Effect of the LH receptor antibodies on LH-stimulated cAMP and progesterone production in intact MA10 cells

Experiments were carried out using different antibody concentrations (0-100μg/ml) in order to determine the effects of different concentrations of the antibodies. The antibodies alone had no effect on basal progesterone production and cAMP accumulation (figs. 6.3 and 6.4). Figs 6.5 and 6.6 show that antibody 1 significantly inhibited LH-stimulated cAMP and progesterone production in a concentration-dependent manner. At concentrations of 1μg/ml and 10μg/ml, antibody 1 inhibited both of these responses completely. Antibody 2 only significantly inhibited the two responses at a concentration of 10μg/ml. Antibody 3 and pre-immune IgG had no effect when used at these concentrations. At concentrations of 100μg/ml, all three antibodies as well as pre-immune IgG had an effect on both the responses, which is probably attributable to the high IgG concentration used. On the basis of these results, an IgG concentration of 10μg/ml was chosen as the most appropriate for use in further investigation of the effects of the antibodies on LH action.

When different LH concentrations were used in similar studies, antibody 1 (10μg/ml) significantly inhibited the concentration-dependent stimulation of both cAMP and progesterone production by LH at concentrations of 10ng/ml or greater (fig. 6.7 and 6.8). The extent of inhibition by antibody 1 was relatively constant at about 79.5 ± 2.3% (mean ± S.E.M; n=4) for progesterone production and 64.5 ± 1.2 % (mean ± S.E.M; n=4) for cAMP accumulation for LH concentrations of 10-1000ng/ml. Antibody 2 also inhibited the two responses to LH at concentrations of 100 and 1000ng/ml. The degree of inhibition by antibody 2 was again relatively constant at

about 45.5 ± 1.5% (mean ± S.D; n=4) for progesterone and 28.2 ± 2.0% (mean ± S.D; n=4) for cAMP. In contrast, neither antibody 3 nor pre-immune IgG had a significant effect on progesterone or cAMP production at any LH concentration tested.

6.2.4 Effects of the LH receptor antibodies on dbcAMP-, cholera toxin- and forskolin- stimulated cAMP and progesterone production in intact MA10 cells

In these experiments, the cells were incubated with or without the antibodies and then stimulated with dbcAMP, cholera toxin and forskolin before measuring cAMP and progesterone production. The purpose of this study was mainly to establish if the inhibition observed with antibodies 1 and 2 is actually caused specifically through inhibition of receptor-hormone interaction and not through another non-specific effect. Fig. 6.9 illustrates dbcAMP-stimulated progesterone production in the presence and absence of the antibodies. There was no effect with any of the antibodies or with the pre-immune IgG on dbcAMP-stimulated progesterone production at dbcAMP concentrations of 0.01-100mM. Cholera toxin-stimulated progesterone production and cAMP accumulation were also examined at various cholera toxin concentrations (0.005-50μg/ml) and again there was no effect of addition of the three antibodies (figs. 6.10 and 6.11). Finally forskolin-stimulated progesterone production and cAMP accumulation were measured in these cells at various forskolin concentrations (0.01-100μM) and there was no inhibitory effect observed by any of the antibodies or the pre-immune IgG (figs. 6.12 and 6.13).

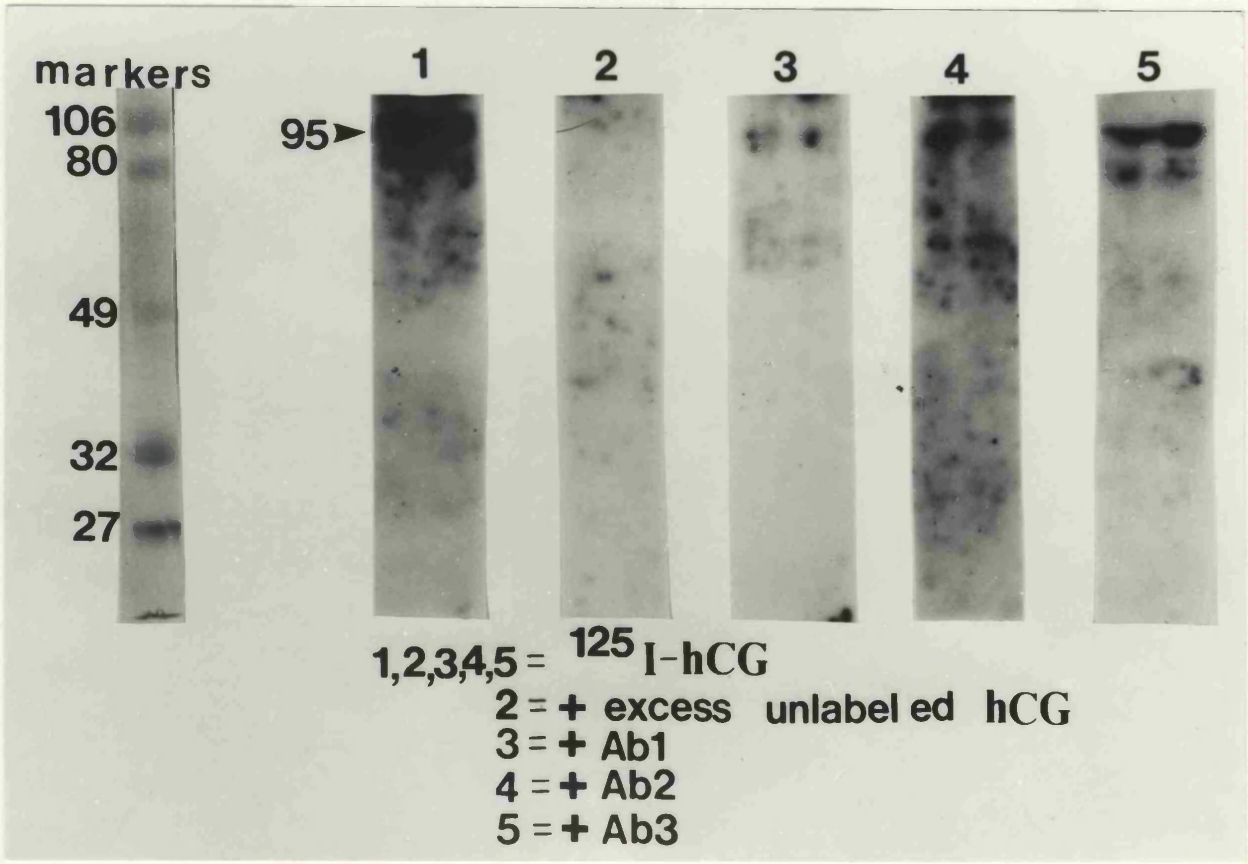

Figure 6.2 Competitive ligand blotting analyses. Ligand blotting of MA10 cell membranes (100μg/lane, duplicate lanes) with ^{125}I-hCG. Excess unlabelled hCG and the three antibodies were used to displace the binding of ^{125}I-hCG. Lane 1 = ^{125}I-hCG. Lane 2 = ^{125}I-hCG + unlabelled hCG (10IU/ml). Lane 3 = ^{125}I-hCG + antibody 1 (10μg/ml). Lane 4 = ^{125}I-hCG + antibody 2 (10μg/ml). Lane 5 = ^{125}I-hCG + antibody 3 (10μg/ml). Molecular weight markers are shown on the left of the figure. This experiment was repeated three times and gave similar results each time.

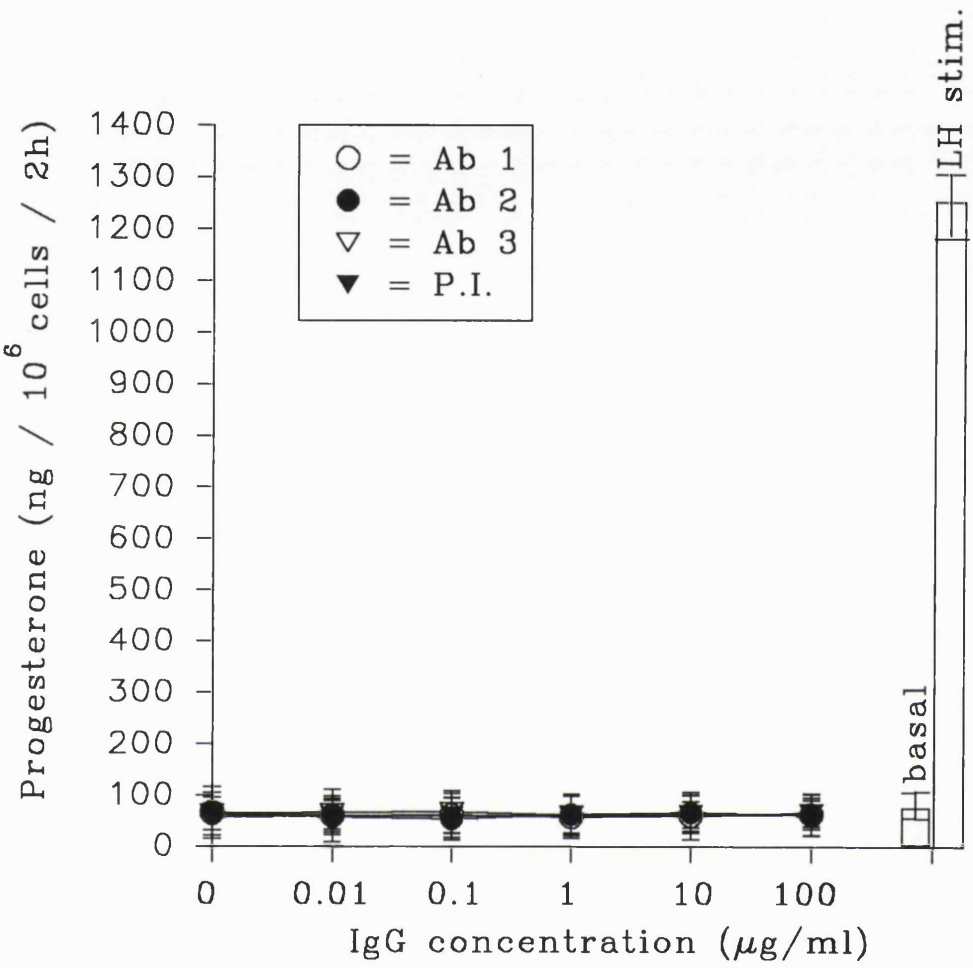

Figure 6.3 Effects of the antibodies and pre-immune IgG on progesterone production in MA10 cells. MA10 cells were plated at a density of $1x10^5$ cells/ml in 96 well plates and stimulated for 2h with the three antibodies or pre-immune IgG. Progesterone was measured by RIA as stated in *Materials and Methods* section 2.2.25.2. Values given are the mean ± S.E.M. of three independent experiments, n=3 (triplicate determinations for each treatment in each experiment). The open bars represent the values for basal and LH (1000ng/ml) stimulated progesterone production.

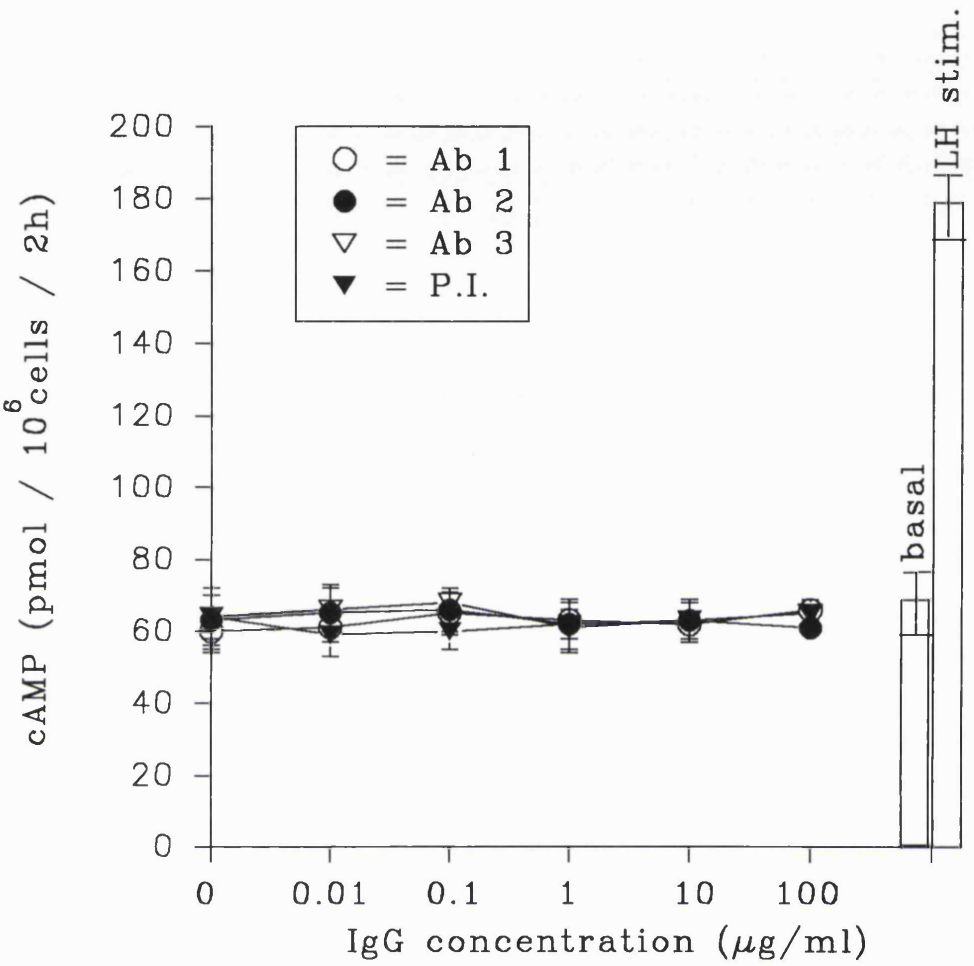

Figure 6.4 Effects of the antibodies and pre-immune IgG on cAMP accumulation in MA10 cells. MA10 cells were plated at a density of $1x10^5$ cells/ml in 96 well plates and stimulated for 2h with the three antibodies or pre-immune IgG. cAMP accumulation was measured by RIA as stated in *Materials and Methods* section 2.2.25.1. Values given are the mean ± S.E.M. of three independent experiments, n=3 (triplicate determinations for each treatment in each experiment). The open bars represent the values for basal and LH (1000ng/ml) stimulated cAMP accumulation.

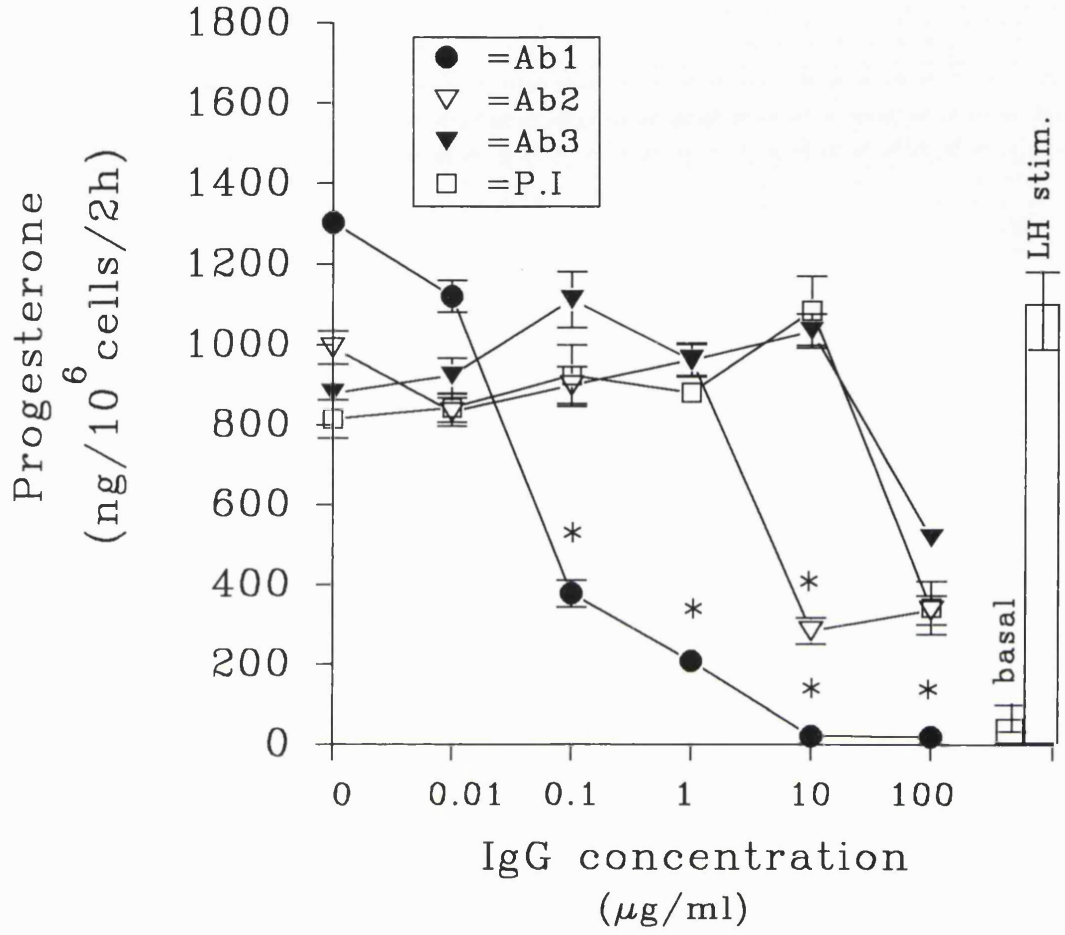

Figure 6.5 Concentration-dependent effects of the antibodies on LH-stimulated progesterone production by MA10 cells. MA10 cells were plated at a density of $1x10^5$ cells/ml in 96 well plates and preincubated for 1h with and without antibodies or pre-immune IgG at concentrations ranging from 0.01μg/ml-100μg/ml. The cells were then challenged for 2h with 10ng/ml LH. Reactions were stopped by the addition of perchloric acid and assayed for progesterone by RIA as stated in *Materials and Methods* section 2.2.25.2. The open bars show basal and maximal LH-stimulated values obtained in the absence of IgG. Values given are the mean ± S.E.M. of four independent experiments, n=4 (triplicate determinations for each treatment in each experiment). * indicates a significant difference ($p<0.05$) between the effect of an antibody and that of its pre-immune control (unpaired *t*-tests).

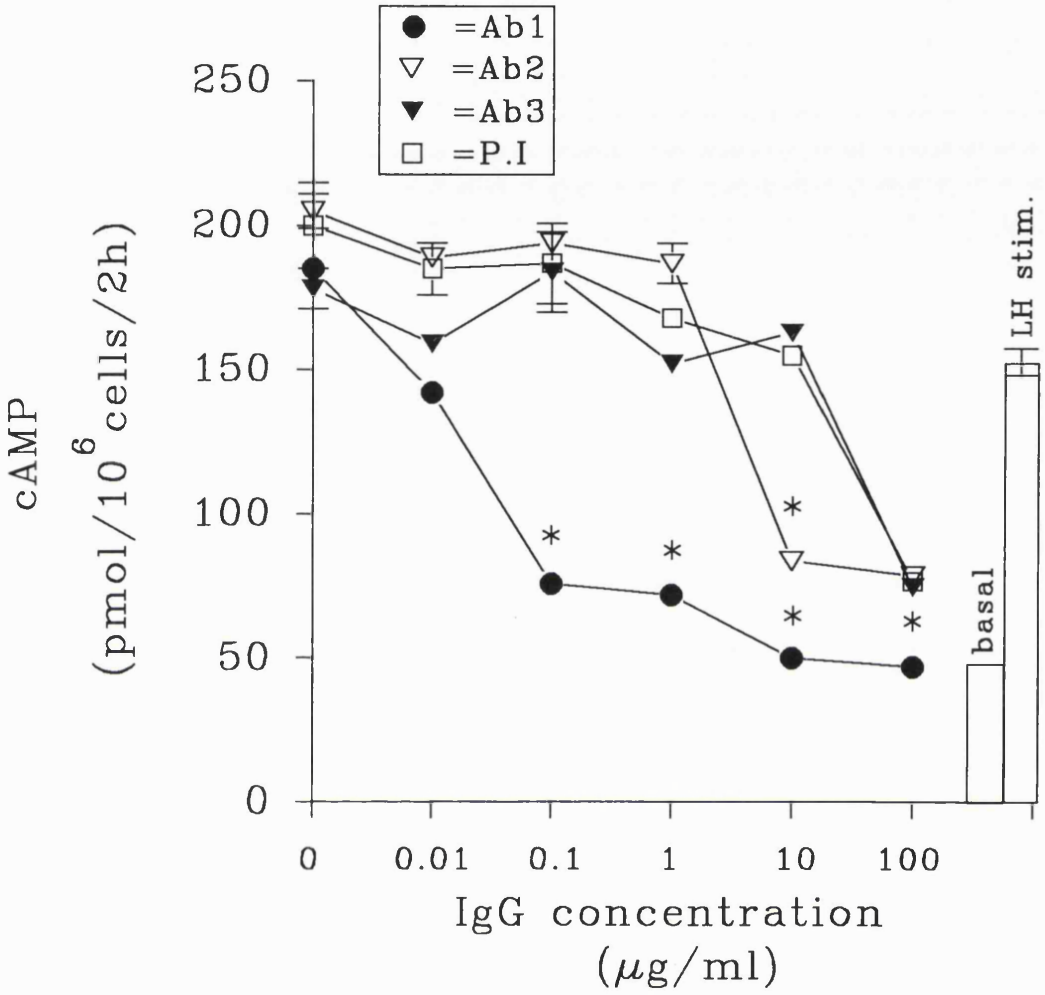

Figure 6.6 Concentration-dependent effects of the antibodies on LH-stimulated cAMP accumulation by MA10 cells. MA10 cells were plated at a density of $1x10^5$ cells/ml in 96 well plates and preincubated for 1h with and without antibodies or pre-immune IgG at concentrations ranging from 0.01μg/ml-100μg/ml. The cells were then challenged for 2h with 10ng/ml LH. Reactions were stopped by the addition of perchloric acid and assayed for cAMP RIA as stated in *Materials and Methods* section 2.2.25.1. The open bars show basal and maximal LH-stimulated values obtained in the absence of IgG. Values given are the mean ± S.E.M. of four independent experiments, n=4 (triplicate determinations for each treatment in each experiment). * indicates a significant difference ($p<0.05$) between the effect of an antibody and that of its pre-immune control (unpaired *t*-tests).

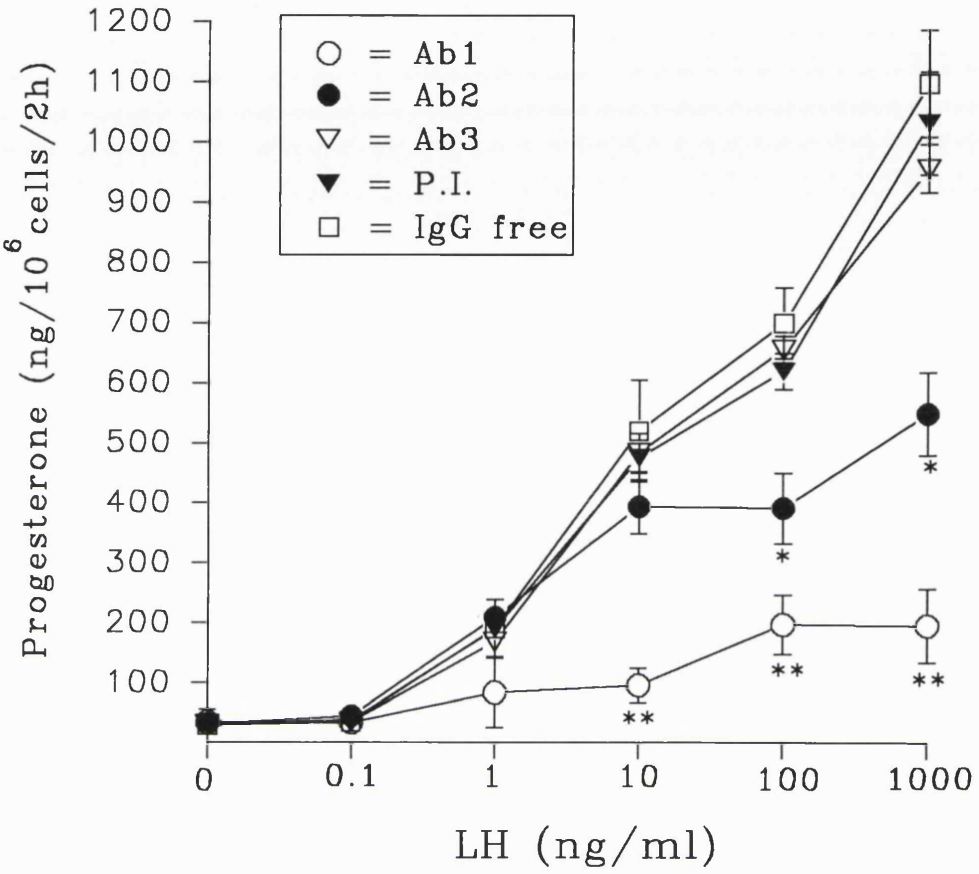

Figure 6.7 LH-stimulated progesterone production in the presence of the three antibodies, pre-immune IgG and IgG-free controls. MA10 cells were plated at a density of $1x10^5$ cells/ml in 96 well plates and preincubated for 1h with and without antibodies or pre-immune IgG at a concentration of 10μg/ml and then stimulated for 2h with a range of LH concentrations (0-1000ng/ml). Reactions were stopped by the addition of perchloric acid and assayed for progesterone by RIA as stated in *Materials and Methods* section 2.2.25.2. Values given are the mean ± S.E.M. of four independent experiments, n=4 (triplicate determinations for each treatment in each experiment). * indicates a significant difference ($p<0.05$) and ** a significant difference ($p<0.01$) between the effect of an antibody and that of its pre-immune control at the respective LH concentration (unpaired *t*-tests).

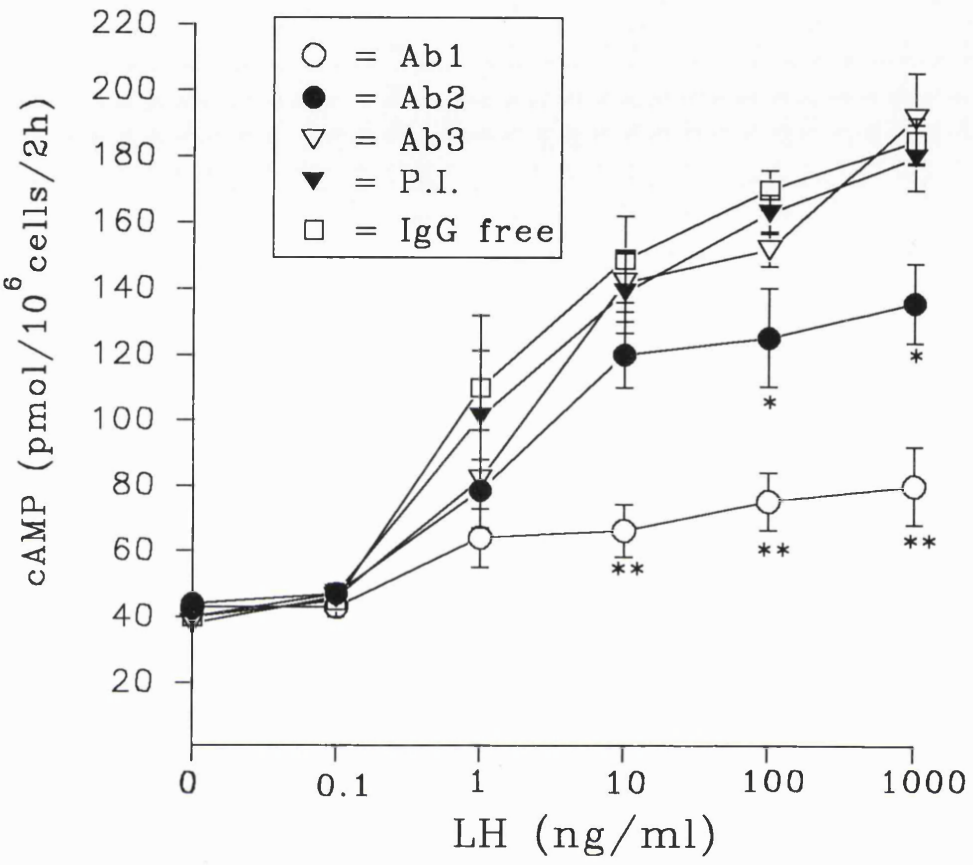

Figure 6.8 LH-stimulated cAMP accumulation in the presence of the three antibodies, pre-immune IgG and IgG-free controls. MA10 cells were plated at a density of $1x10^5$ cells/ml in 96 well plates and preincubated for 1h with and without antibodies or pre-immune IgG at a concentration of 10µg/ml and then stimulated for 2h with a range of LH concentrations (0-1000ng/ml). Reactions were stopped by the addition of perchloric acid and assayed for cAMP by RIA as stated in *Materials and Methods* section 2.2.25.1. Values given are the mean ± S.E.M. of four independent experiments, n=4 (triplicate determinations for each treatment in each experiment). * indicates a significant difference ($p<0.05$) and ** a significant difference ($p<0.01$) between the effect of an antibody and that of its pre-immune control at the respective LH concentration (unpaired *t*-tests).

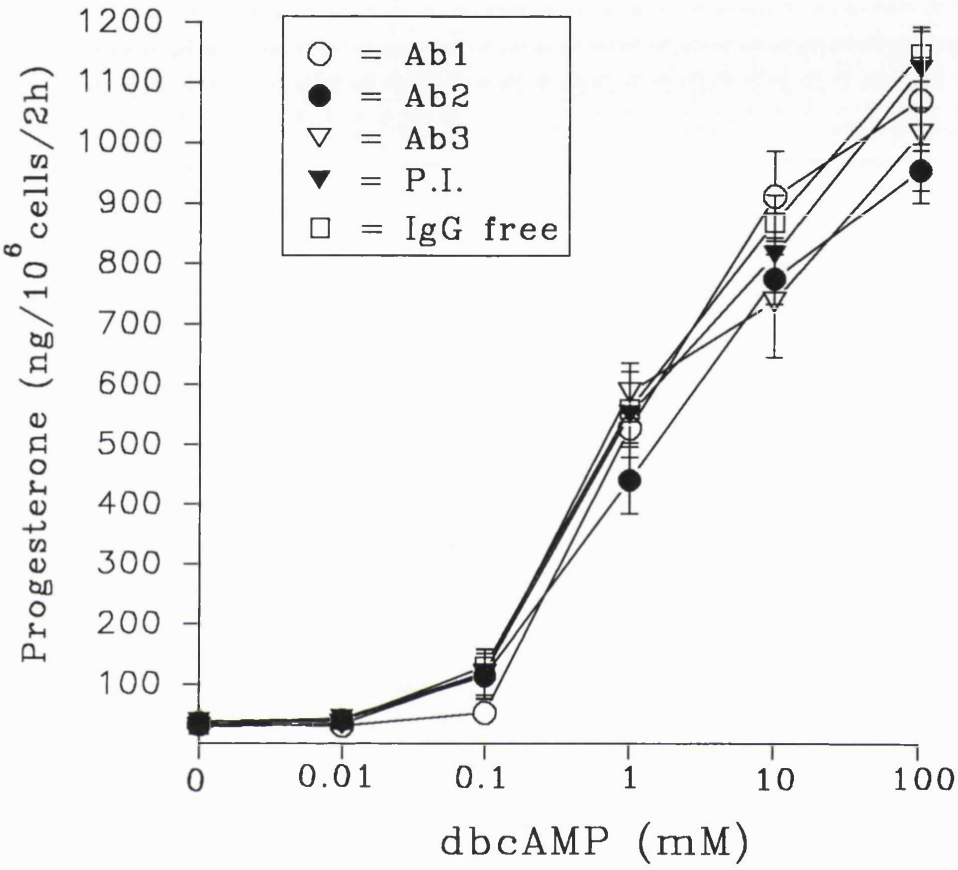

Figure 6.9 dbcAMP-stimulated progesterone production in the presence of the three antibodies, pre-immune IgG and IgG-free controls. MA10 cells were plated at a density of $1x10^5$ cells/ml in 96 well plates and preincubated for 1h with and without antibodies or pre-immune IgG at a concentration of 10μg/ml and then stimulated for 2h with a range of dbcAMP concentrations (0-100mM). Reactions were stopped by the addition of perchloric acid and assayed for progesterone by RIA as stated in *Materials and Methods* section 2.2.25.2. Values given are the mean ± S.E.M. of four independent experiments, n=4 (triplicate determinations for each treatment in each experiment). There was no significant statistical difference between the effect of an antibody and that of its pre-immune control (unpaired *t*-tests).

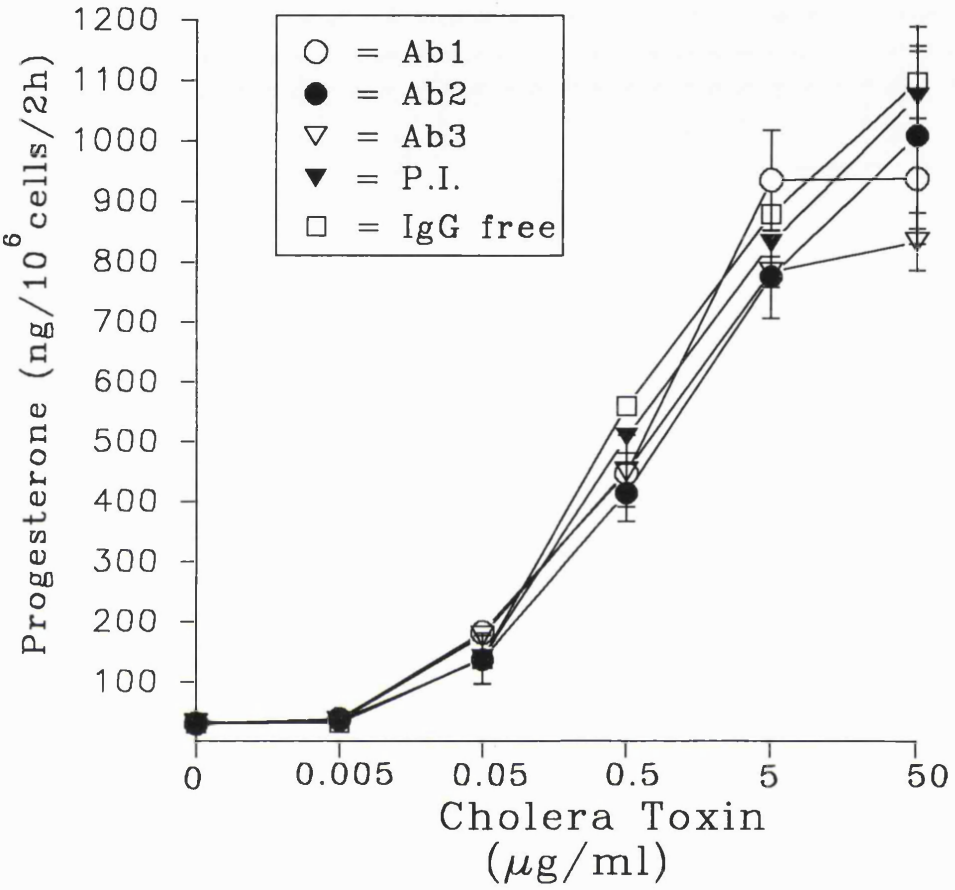

Figure 6.10 Cholera toxin-stimulated progesterone production in the presence of the three antibodies, pre-immune IgG and IgG-free controls. MA10 cells were plated at a density of $1x10^5$ cells/ml in 96 well plates and preincubated for 1h with and without antibodies or pre-immune IgG at a concentration of 10μg/ml and then stimulated for 2h with a range of cholera toxin concentrations (0-50μg/ml). Reactions were stopped by the addition of perchloric acid and assayed for progesterone by RIA as stated in *Materials and Methods* section 2.2.25.2. Values given are the mean ± S.E.M. of four independent experiments, n=4 (triplicate determinations for each treatment in each experiment). There was no significant statistical difference between the effect of an antibody and that of its pre-immune control (unpaired *t*-tests).

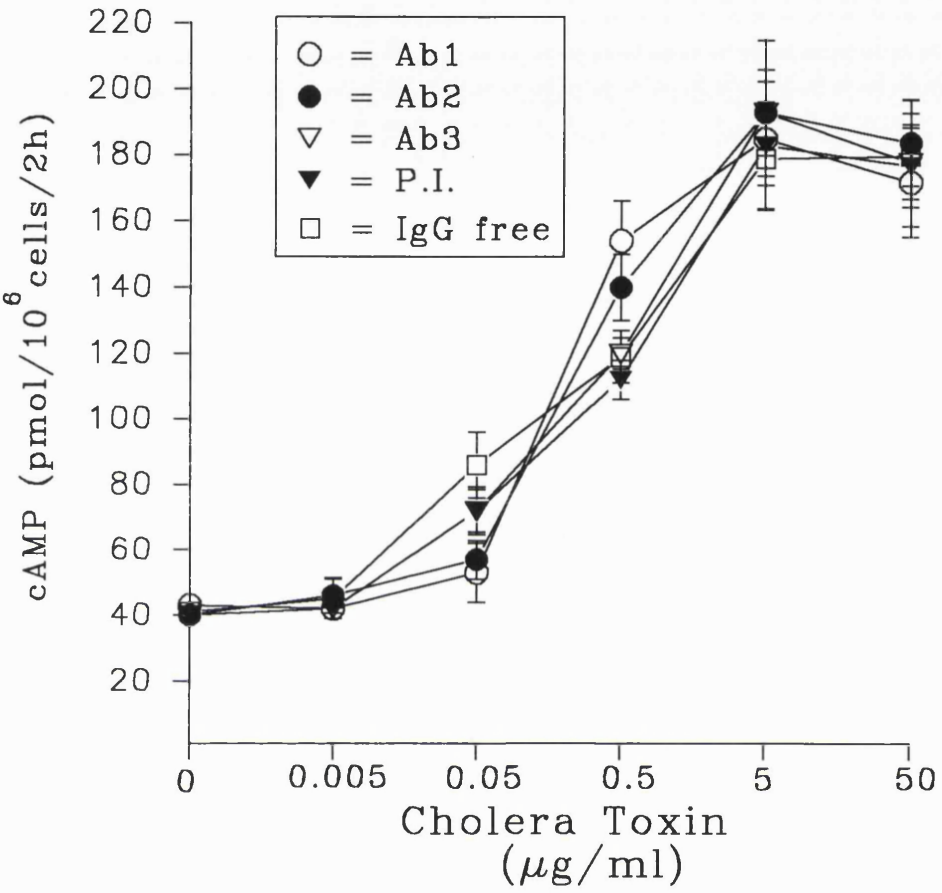

Figure 6.11 Cholera toxin-stimulated cAMP accumulation in the presence of the three antibodies, pre-immune IgG and IgG-free controls. MA10 cells were plated at a density of $1x10^5$ cells/ml in 96 well plates and preincubated for 1h with and without antibodies or pre-immune IgG at a concentration of 10μg/ml and then stimulated for 2h with a range of cholera toxin concentrations (0-50μg/ml). Reactions were stopped by the addition of perchloric acid and assayed for cAMP by RIA as stated in *Materials and Methods* section 2.2.25.1. Values given are the mean ± S.E.M. of four independent experiments, n=4 (triplicate determinations for each treatment in each experiment). There was no significant statistical difference between the effect of an antibody and that of its pre-immune control (unpaired *t*-tests).

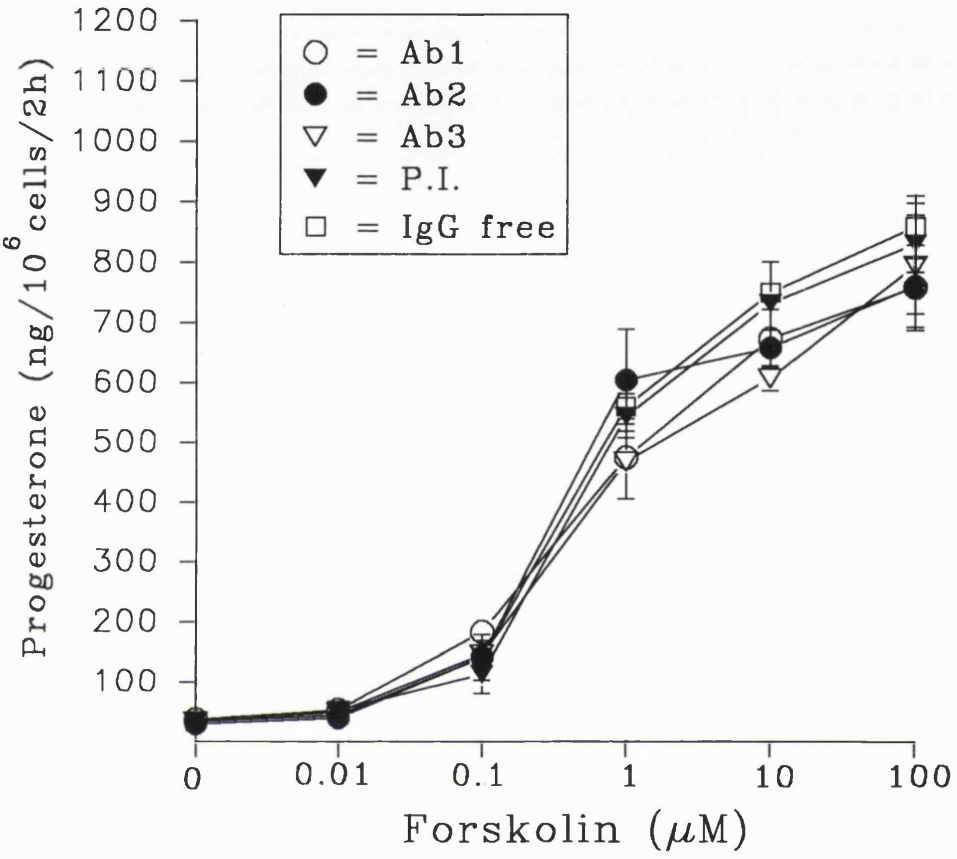

Figure 6.12 Forskolin-stimulated progesterone production in the presence of the three antibodies, pre-immune IgG and IgG-free controls. MA10 cells were plated at a density of $1x10^5$ cells/ml in 96 well plates and preincubated for 1h with and without antibodies or pre-immune IgG at a concentration of 10μg/ml and then stimulated for 2h with a range of forskolin concentrations (0-100μM). Reactions were stopped by the addition of perchloric acid and assayed for progesterone by RIA as stated in *Materials and Methods* section 2.2.25.2. Values given are the mean ± S.E.M. of four independent experiments, n=4 (triplicate determinations for each treatment in each experiment). There was no significant statistical difference between the effect of an antibody and that of its pre-immune control (unpaired *t*-tests).

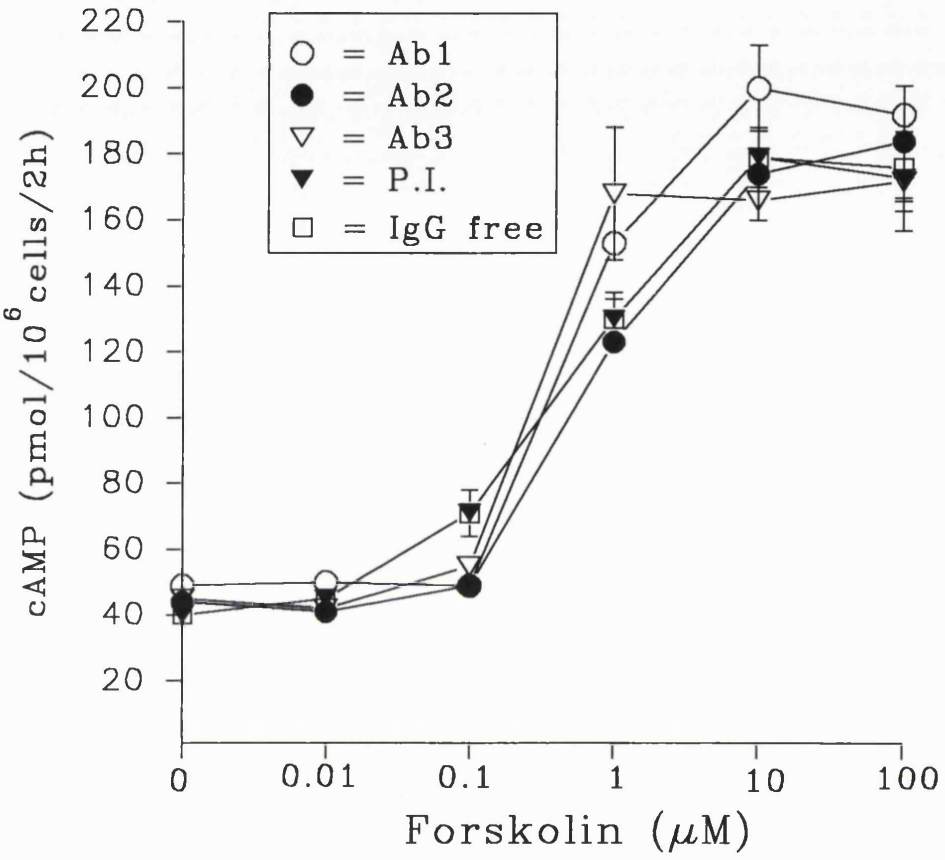

Figure 6.13 Forskolin-stimulated cAMP accumulation in the presence of the three antibodies, pre-immune IgG and IgG-free controls. MA10 cells were plated at a density of $1x10^5$ cells/ml in 96 well plates and preincubated for 1h with and without antibodies or pre-immune IgG at a concentration of 10μg/ml and then stimulated for 2h with a range of forskolin concentrations (0-100μM). Reactions were stopped by the addition of perchloric acid and assayed for cAMP by RIA as stated in *Materials and Methods* section 2.2.25.1. Values given are the mean ± S.E.M. of four independent experiments, n=4 (triplicate determinations for each treatment in each experiment). There was no significant statistical difference between the effect of an antibody and that of its pre-immune control (unpaired *t*-tests).

6.2.5 Effects of the LH receptor antibodies on adenylyl cyclase activity in MA10 plasma membranes stimulated with LH, p(NH)ppG, p(NH)ppG + LH and NaF in the presence of the antibodies.

In this set of experiments, the effects of the LH receptor antibodies on adenylyl cyclase activity in isolated plasma membranes from MA10 cells was investigated. MA10 cell plasma membranes were prepared by a 2 phase centrifugation method (see *Materials and Methods* section 2.2.13) which produces very pure plasma membranes (Brunnette & Till 1971). They were stimulated by LH, p(NH)ppG and fluoride ions, in the absence and presence of the three antibodies. In the absence of the antibodies the stimulation by LH was further enhanced by the presence of p(NH)ppG. LH + p(NH)ppG and fluoride ions produced the highest stimulation. In the presence of antibodies 1 and 2, there was an inhibition of cAMP accumulation with LH or LH + p(NH)ppG. There was no effect of the antibodies on the adenylyl cyclase response to NaF or p(NH)ppG alone. Inhibition of LH action by antibody 1 ($p<0.01$) was statistically more significant than antibody 2 ($p<0.05$) when compared to the controls. Antibody 3 and pre-immune IgG had no effect on the adenylyl cyclase response to any of the treatments (fig. 6.14).

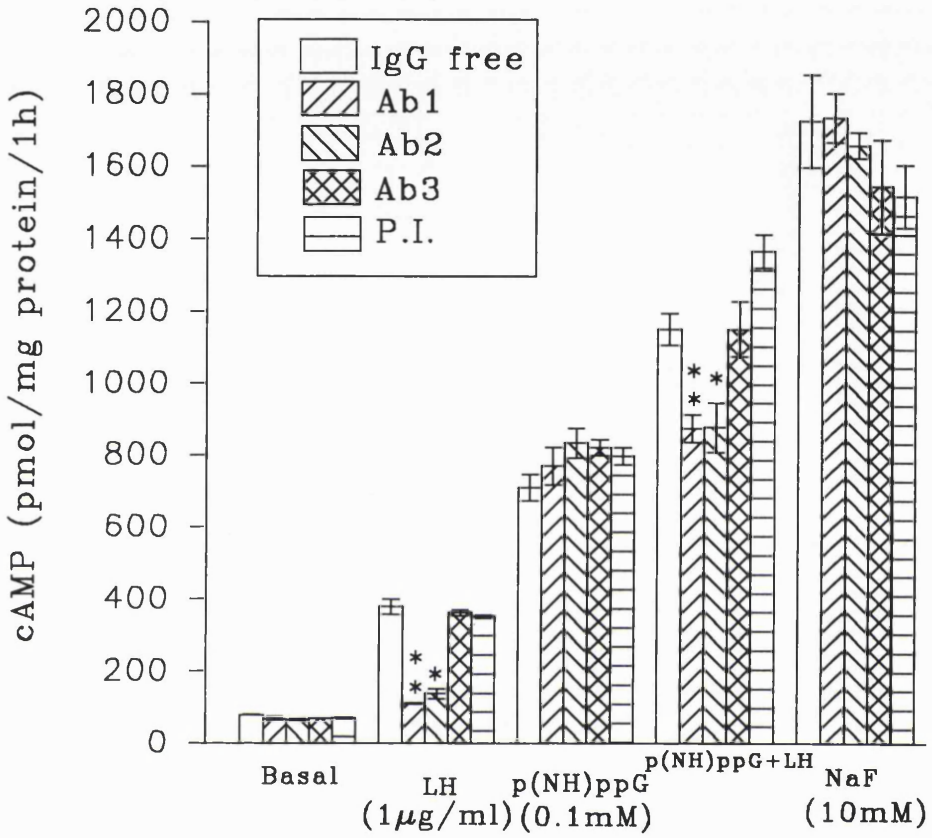

Figure 6.14 Adenylyl cyclase activation in isolated MA10 cell plasma membranes. Plasma membranes were prepared by the 2 phase method as described in *Materials and Methods* section 2.2.13 and incubated for 1h with or without the three antibodies and pre-immune IgG. The membranes were further incubated with either LH (1μg/ml), p(NH)ppG (0.1mM), p(NH)ppG (0.1mM) + LH (1μg/ml) or NaF (10mM). The cAMP produced was measured as described in *Materials and Methods* section 2.2.25.1. Results represent the mean ± S.E.M. of three independent experiments, n=3 (triplicate determinations for each treatment in each experiment). * indicates a significant difference ($p<0.05$) and ** a significant difference ($p<0.01$) between the effect of an antibody and that of its pre-immune control (unpaired *t*-tests).

6.2.6 Western blotting analyses using the MA10 cell plasma membranes

A Western blot was performed under non-denaturing conditions to determine if the three antibodies do actually recognize the receptor found in the plasma membranes prepared by the 2 phase method and to establish whether the effects seen with the antibodies in the preceding study are actually due to antibody-receptor binding rather than a non-specific effect. Fig. 6.15 shows the result of this study. Antibodies 1, 2 and 3 all recognized the 95 kDa protein from plasma membranes prepared by this method. Pre-immune IgG failed to react with this protein. The signal (binding of antibody to the receptor protein) obtained in this experiment was weaker than the binding usually obtained in Western blots from chapter 4. This can be explained by the fact that the method used to prepare the membranes varied. Traditionally, when preparing membrane receptor proteins for Western blot analyses, detergents are used to extract the receptor from the membrane (see *Materials and Methods* section 2.2.12). The 2 phase method used in this experiment did not involve the use of such detergents, hence making it more difficult for the receptor to electrophorese.

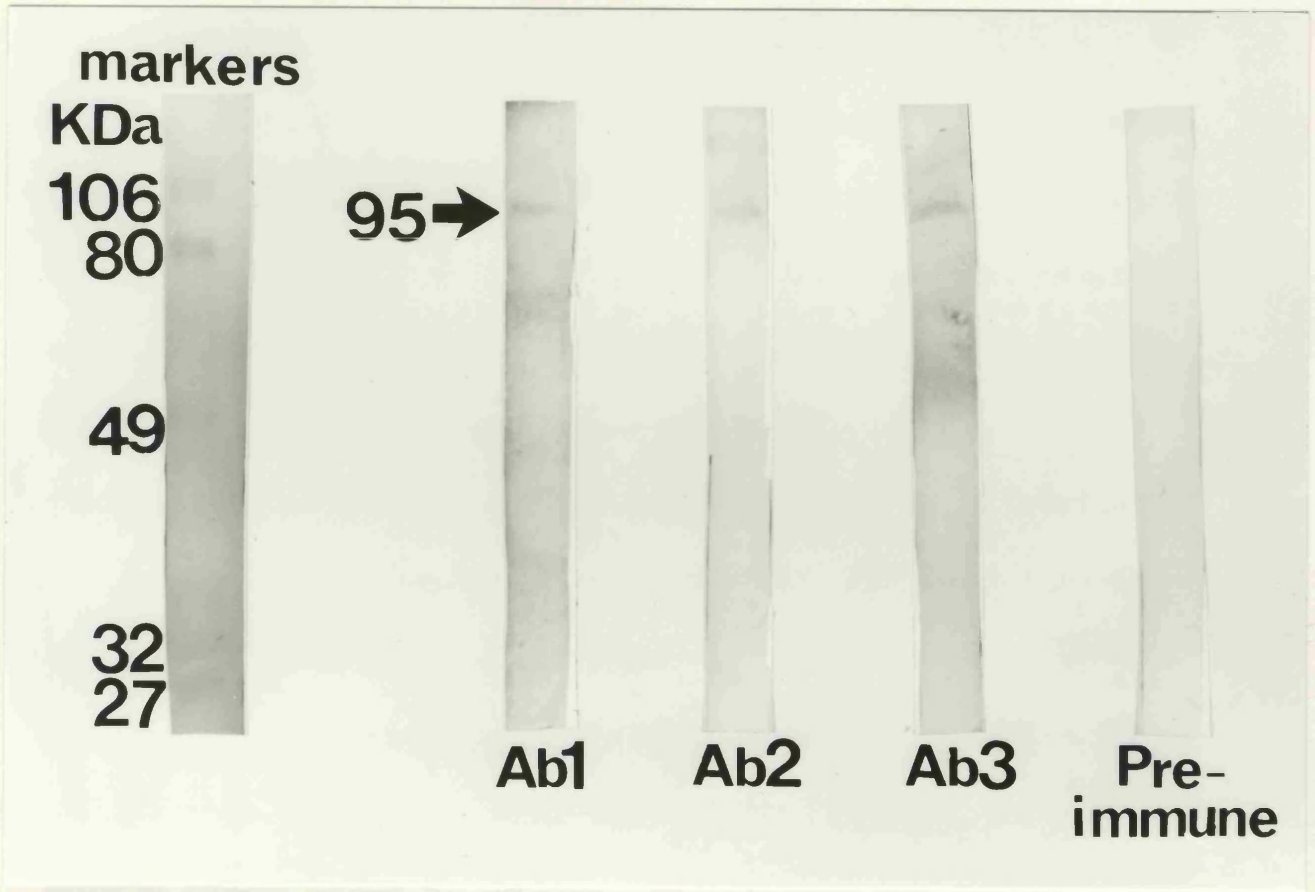

Figure 6.15 Western blotting using the three LH receptor antibodies and isolated MA10 cell plasma membranes. Western blotting of MA10 cell plasma membranes prepared by a 2 phase centrifugation method as described in *Materials and Methods* section 2.2.13. Plasma membranes (50μg) were resolved on 10% polyacrylamide gels under non-denaturing conditions and transblotted onto nitrocellulose membranes. Affinity purified antibodies (20μg) or pre-immune IgG (20μg) was added and binding was determined as described in *Materials and Methods* section 2.2.17. Pre-stained molecular weight markers are shown on the left of the figure. The 95 kDa molecular weight protein identified by the three antibodies is also indicated. Experiments were repeated three times with similar results.

6.3 Discussion

The results presented in this chapter clearly demonstrate the differential effect of the three antibodies on the binding of ^{125}I-hCG and signal transduction stimulated by various ligands both in intact MA10 cells and isolated plasma membranes.

The ability of antibodies 1 and 2 to inhibit ^{125}I-hCG binding in intact MA10 cells is consistent with the fact that the N-terminus of the LH receptor includes the ligand binding domain. Similarly, the lack of effect of antibody 3 on the responses of intact cells is consistent with the C-terminal region of the receptor being intracellular. The observation that the C-terminal antibody and pre-immune IgG have a small effect on ^{125}I-hCG binding indicates non-specific protein interference between the ligand and the receptor. In the competitive ligand blot analyses the 95 kDa protein identified in solubilised MA10 cell membranes by ^{125}I-hCG was completely displaced by excess unlabelled hCG indicating that this protein band is hormone specific. The binding of ^{125}I-hCG to this band was also displaced by antibody 1 (10μg/ml) and to a lesser extent by antibody 2 (10μg/ml). Antibody 3 when used at the same concentration had no effect on ^{125}I-hCG binding. This observation shows that binding of the two N-terminal antibodies to the membrane proteins prevents ^{125}I-hCG from binding. This could be due to steric hindrance since the antibodies are large molecules. However, the Western blotting experiments detailed in chapter 5 showed that antibody 3 is also capable of binding to these membrane proteins. Since binding of antibody 3 did not prevent ^{125}I-hCG from binding, antibody 3 must bind to a region of the receptor which is not involved in ligand binding. Furthermore, the effects of antibodies 1 and 2 are unlikely to be attributable to steric hindrance.

Antibody 1 inhibited both LH-stimulated cAMP accumulation and steroidogenesis to a greater extent than the inhibition observed with antibody 2, and since both of these antibodies were raised to peptide regions within the N-terminus of the LH receptor, these results illustrate that these antibodies, and especially antibody 1, bind to a region of the N-terminus that affects both ligand binding and signal transduction. There was no inhibition with any of the antibodies when dbcAMP, cholera toxin and forskolin were used, suggesting that the inhibition of LH action is occurring at the ligand-receptor step rather than a post receptor step. The results of the study by Roche *et al.* (1992) indicate that the binding regions for LH on the LH receptor is located at aa residues 21 - 38, 102 - 115, 253 - 272 and 573 - 583. Therefore, neither antibodies 1 or 2 would be predicted to interact with these regions of the N-terminus. However, antibody 1 was raised to a peptide sequence (aa residues 48-65) which is closer to the putative region involved in LH β-subunit ligand binding (aa residues 21-38) than the peptide sequence used to raise antibody 2 (aa residues 187-206). Therefore, it is possible that the binding of antibody 1 decreases ligand binding and action via steric hind rance and/or induction of conformational changes in the hormone binding domain. Hence the effects seen by antibody 1 are greater than those seen using antibody 2. It is also possible that antibody 1 might have a higher affinity for the receptor than antibody 2.

In the experiments using the MA10 cell plasma membranes prepared by the 2 phase method, it was demonstrated that these membranes retain their ability to respond to LH in terms of cAMP production and that this activity can be further increased in the presence of p(NH)ppG which amplified the response to LH. Antibodies 1 and 2, but

not antibody 3, inhibited the LH responses in these plasma membranes indicating, once again, that antibodies 1 and 2 are actually binding to the LH receptor hence blocking further signalling. The lack of effect seen by antibody 3 may have been due to its inability to bind to the receptor successfully when the receptor is in its native state. The experiments carried out in chapter 5 in which it was demonstrated that antibody 3 does bind to the LH receptor (95 kDa band in the Western blotting) were carried out with denatured LH receptor protein. Also in the studies with intact cells the C-terminus would not have been accessible to antibody 3. However, the experiments described in this chapter in which Western blotting was carried out on plasma membranes under non-denaturing conditions suggest that antibody 3 (as well as antibodies 1 and 2) is able to bind to the LH receptor in its native state. The binding of all three antibodies in this experiment was not as strong as binding of the antibodies to solubilized membranes seen in the previous chapters. This can be explained due to the difficulty in electrophoresing membranes with the retention of associated proteins and/or lipids, hence the weak signal obtained. Thus, the results with isolated MA10 cell membranes suggest that antibody 3 is unable to inhibit stimulation due to the fact that its binding to the C-terminus of the receptor does not interfere with ligand binding.

Palmitoylation of the receptor in the isolated membranes would reduce the chances of antibody 3 binding to the receptor. On the assumption that antibody 3 binds to the C-terminal sequence (C^{622}-A^{636}), it demonstrates that this particular region does not appear to interfere with the receptor's ability to couple to the G_s. Future work is required however to confirm these potentially important results. In particular direct

binding studies need to be carried out using labelled antibodies to determine their affinities for the LH receptor.

CHAPTER 7

LH RECEPTOR BINDING PROTEINS

7.1 Introduction

Several studies have investigated whether a LH/CG binding protein might exist. Tsai-Morris *et al.* (1990) reported the synthesis and secretion of a LH/CG binding protein. These workers demonstrated secretion of LH/CG binding sites into the medium by COS-1 cells expressing a LH/CG receptor cDNA with a 266bp deletion. They concluded that this soluble form of the LH/CG receptor may be of importance in modulating extracellular hormone concentrations by reducing the concentration of free LH available for interaction with membrane receptors. Another study by Xie *et al.* (1990), in which a mutated construct of the rat luteal LH/CG receptor encoding for only the extracellular domain was transfected in human kidney 293 cells demonstrated that high affinity binding sites for LH could only be detected in the solubilized cells and were not secreted into the culture medium. This study raised the possibility that a LH/CG binding protein, consisting of the extracellular domain of the receptor might be expressed by gonadal cells but may remain trapped intracellularly. West and Cooke (1991), concluded that the LH receptors are regulated by proteolysis at the plasma membrane in both mouse and rat Leydig cells. Furthermore, they showed that truncation of the LH receptor in the mouse Leydig cells is involved in down-regulation, whereas in the rat, truncation of the LH receptor is a continuous process. These workers also concluded that the cleaved receptor is not acting as a serum binding protein.

In this chapter, the concept of proteolysis (discussed previously in *Chapter 1* section 1.5.8) was further investigated firstly by assessing ^{125}I-hCG binding to MA10 cells in the presence and absence of protease inhibitors and secondly, using the LH receptor

specific anti-peptide antibodies in order to identify and prove the existence of any proteolytic fragments released into the incubation medium. Using the antibodies to the extracellular domain of the LH/CG receptor and an antibody against the cytoplasmic region, experiments were designed to identify these proteins in MA10 cell conditioned medium after triggering proteolysis of the receptor by inhibiting internalization.

7.2 Results

In order to study proteolysis of the LH/CG receptor, it was important to repeat some of the experiments carried out by West and Cooke (1991). To achieve desensitization, MA10 cells were incubated for 2h with LH (1000ng/ml) (Freeman and Ascoli 1982; Dix *et al.*,1982). In addition, cells were incubated with NaN_3 (5mM) (with and without LH) for 2h to inhibit internalization as shown by West and Cooke (1991). Cell surface bound hormone was removed by washing the cells with glycine-buffered saline (pH 3.0) (Ascoli, 1982) before the surface binding of ^{125}I-hCG was determined. West and Cooke (1991) found that a 2h incubation of MA10 cells in the presence of LH or NaN_3 caused a 50% loss in binding of ^{125}I-hCG. Preincubating the MA10 cells with a cocktail of protease inhibitors (leupeptin 100μM, PMSF 10μM, aprotinin 900KIU/ml) for 30min before the addition of LH or NaN_3, prevented the LH-induced decrease in specific ^{125}I-hCG binding. This observation was also made when the horse serum in the medium was replaced by 0.1% (w/v) BSA, demonstrating that the protease activity inhibited by the cocktail was not derived from the serum. This experiment was repeated under conditions identical to those described by West and Cooke (1991) and fig. 7.1 confirmed that there was a 40% loss of surface binding after treating with LH and NaN_3. However, the presence of the cocktail of protease inhibitors did not prevent the loss of binding sites.

A Western blotting experiment was carried out in an attempt to identify any proteolytic fragments released from the MA10 cells into the incubation medium. Cultured MA10 cells were treated with 5mM NaN_3 to inhibit receptor internalization and hence render the receptor susceptible to proteolysis as suggested by West and

Cooke (1991). The medium was collected and the proteins were concentrated using the centriprep-30 concentrators. Proteins were then electrophoresed on SDS gels along with MA10 cell membrane proteins as controls. Figure 7.2 shows the results obtained using all three antibodies and pre-immune IgG. The 95 kDa protein detected by all three antibodies in the MA10 cell membrane protein lane is consistent with the band obtained previously. In the lane containing the MA10 cell conditioned medium, no fragments were detected by any of the antibodies. Pre-immune IgG was clear in both lanes.

Since the results obtained with the Western blotting were negative, slot blot experiments were also carried out. The latter does not involve denaturing the proteins of interest and the SDS-gel separation and electroblotting steps that might cause problems are avoided. This experiment was designed according to the observations by West and Cooke (1991) who found that it was easier to detect proteolytic fragments when the ligand was bound to the receptor first before proteolysis had occurred. The slot blot experiment used in this study contained proteins from concentrated MA10 cell medium after treating the cells with NaN_3 either with or without the antibodies raised in this study pre-bound to the receptor. It was shown in previous chapters that both N-terminal antibodies bind to the intact LH/CG receptor expressed in MA10 cells and the theory behind the design of this experiment was to determine if the proteolytic fragment was easier to identify by binding the antibody first before treating with NaN_3, or by treating with NaN_3 and then using the antibodies to bind to the fragments released into the medium. Two lanes as positive controls were also used containing ovarian and MA10 cell membrane proteins. The result in fig. 7.3 shows positive

recognition with all three antibodies but not with pre-immune IgG with the ovarian and MA10 membrane controls (rows 1 and 2). Irrespective of whether or not the antibodies were bound to the receptor prior to NaN_3 treatment (Rows 3 and 4 respectively), all three antibodies did not recognize proteins in the MA10 cell conditioned medium. In all rows, binding of the secondary goat-anti-rabbit alkaline phosphatase antibody did not differ between the three primary antibodies and the IgG controls.

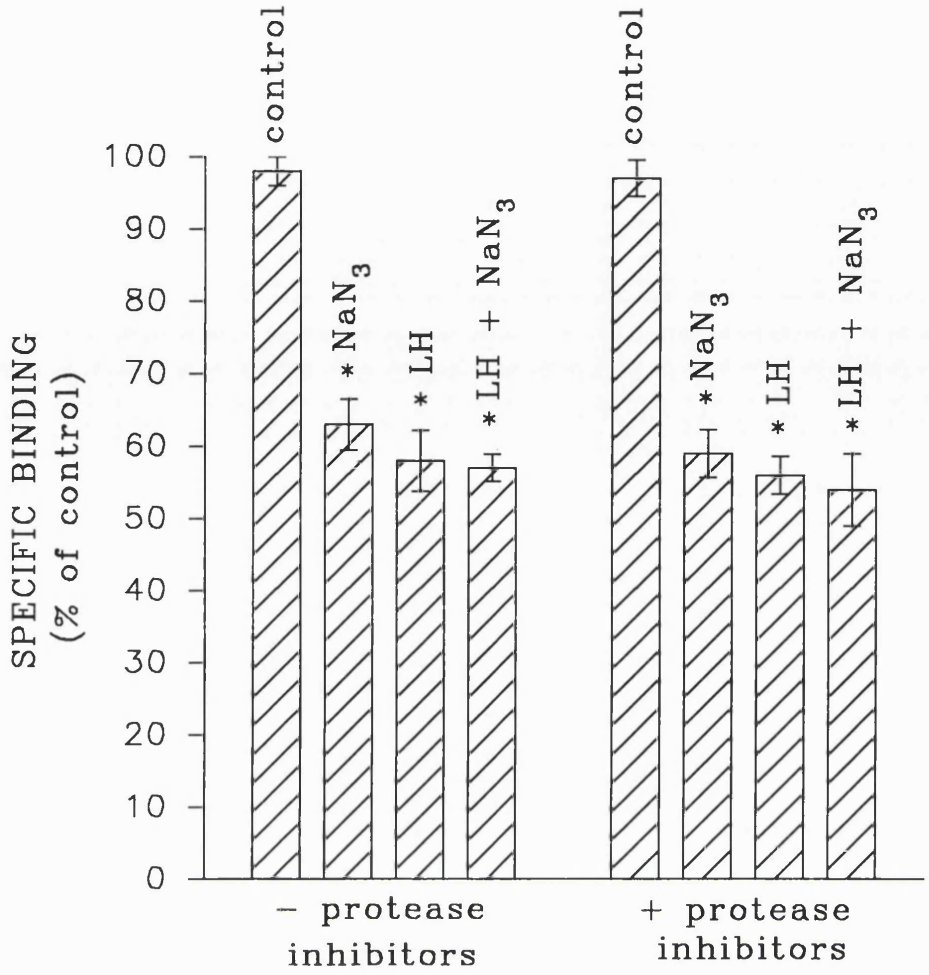

Figure 7.1 ^{125}I-hCG binding to MA10 cells in the presence/absence of protease inhibitors. MA10 cells were plated at a density of $1x10^6$ cells/well, and preincubated ± protease inhibitors (leupeptin 100μM, PMSF 10μM, aprotinin 900KIU/ml) for 30 min at 37°C. The cells were further incubated in the absence of protease inhibitors for 2h (either without hormone, with NaN_3 (5mM), with LH (1000ng/ml), or NaN_3 (5mM) + LH (1000ng/ml) or in the presence of protease inhibitors for 2h (either without hormone, with NaN_3 (5mM), with LH (1000ng/ml), or NaN_3 (5mM) + LH (1000ng/ml). After 2h the cells were placed on ice and washed twice with 50mM glycine-buffered saline (pH 3.0) before ^{125}I-hCG binding was determined as described in *Materials and Methods* section 2.2.23. The 100% values represents binding in the absence of LH or NaN_3 (2860 ± 33 cpm with protease inhibitors, 2923 ± 41 cpm without protease inhibitors). Non-specific binding, approximately 10% of the total binding (291 ± 28 cpm with protease inhibitors, 269 ± 12 cpm without protease inhibitors), was determined for each treatment by the addition of 300IU/ml unlabelled crude hCG. Values given are the mean ± S.E.M. of three independent experiments, n=3 (triplicate determinations for each treatment in each experiment). * indicates a significant difference ($p<0.05$) for each treatment compared to its control (unpaired *t*-tests).

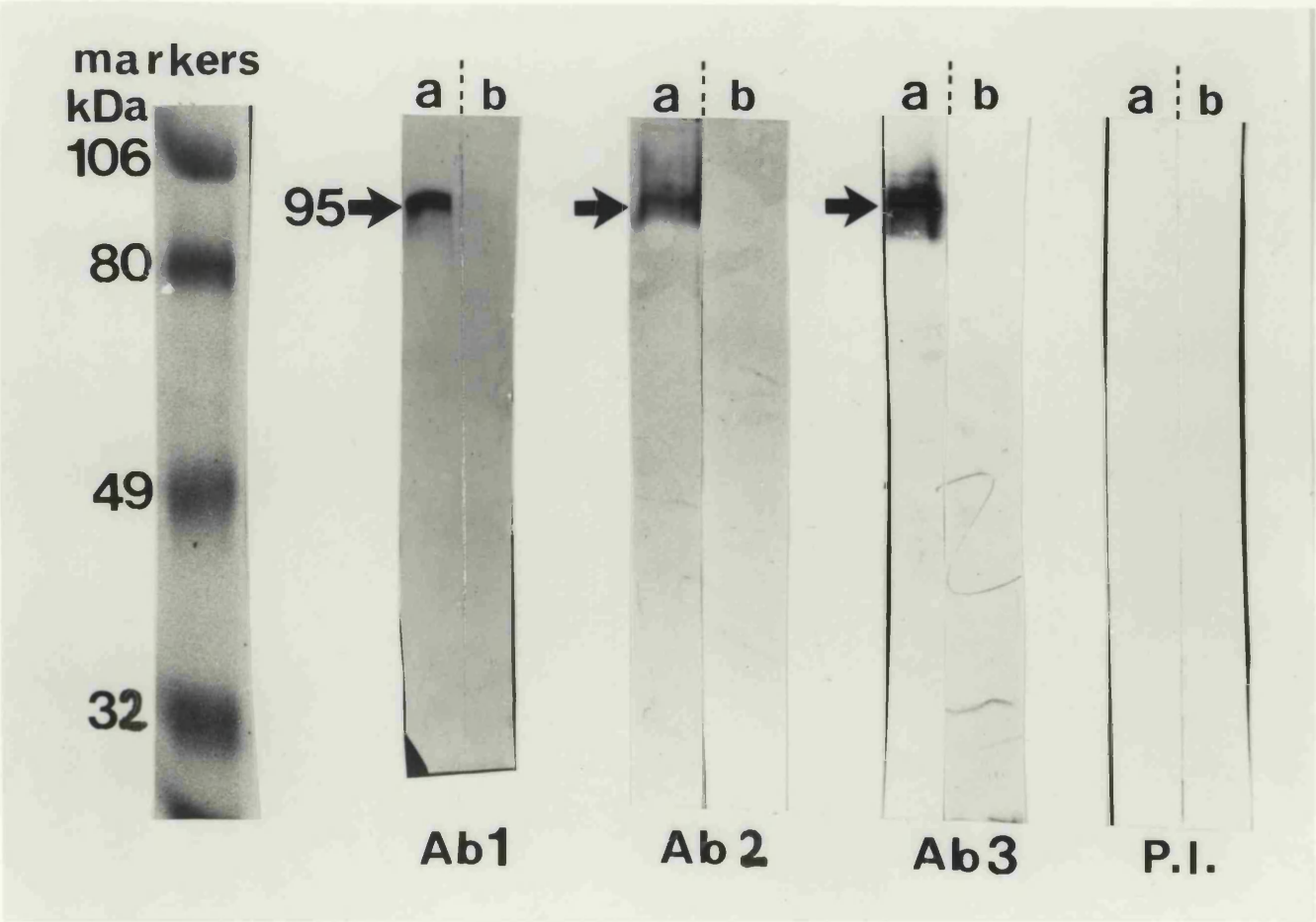

Figure 7.2 Western blot analyses using MA10 cell membrane proteins and NaN_3 treated cell conditioned medium against the three antibodies and pre-immune IgG. Lane a = MA10 cell membrane proteins and lane b = MA10 cell conditioned medium following NaN_3 treatment. Proteins from lane a (solubilized MA10 cell membranes) were prepared as described in *Materials and Methods* section 2.2.12. The conditioned medium was collected from cells treated with NaN_3 for 2h and then concentrated using the centriprep-30 concentrators. Membrane proteins and medium were both run on 10% SDS-gels and then transblotted onto nitrocellulose membranes. Affinity purified antibodies and pre-immune IgG (20μg) was then added, and binding was determined as described in *Materials and Methods* section 2.2.17. Pre-stained molecular weight markers are shown on the left of the figure. Experiments were repeated three times with similar results.

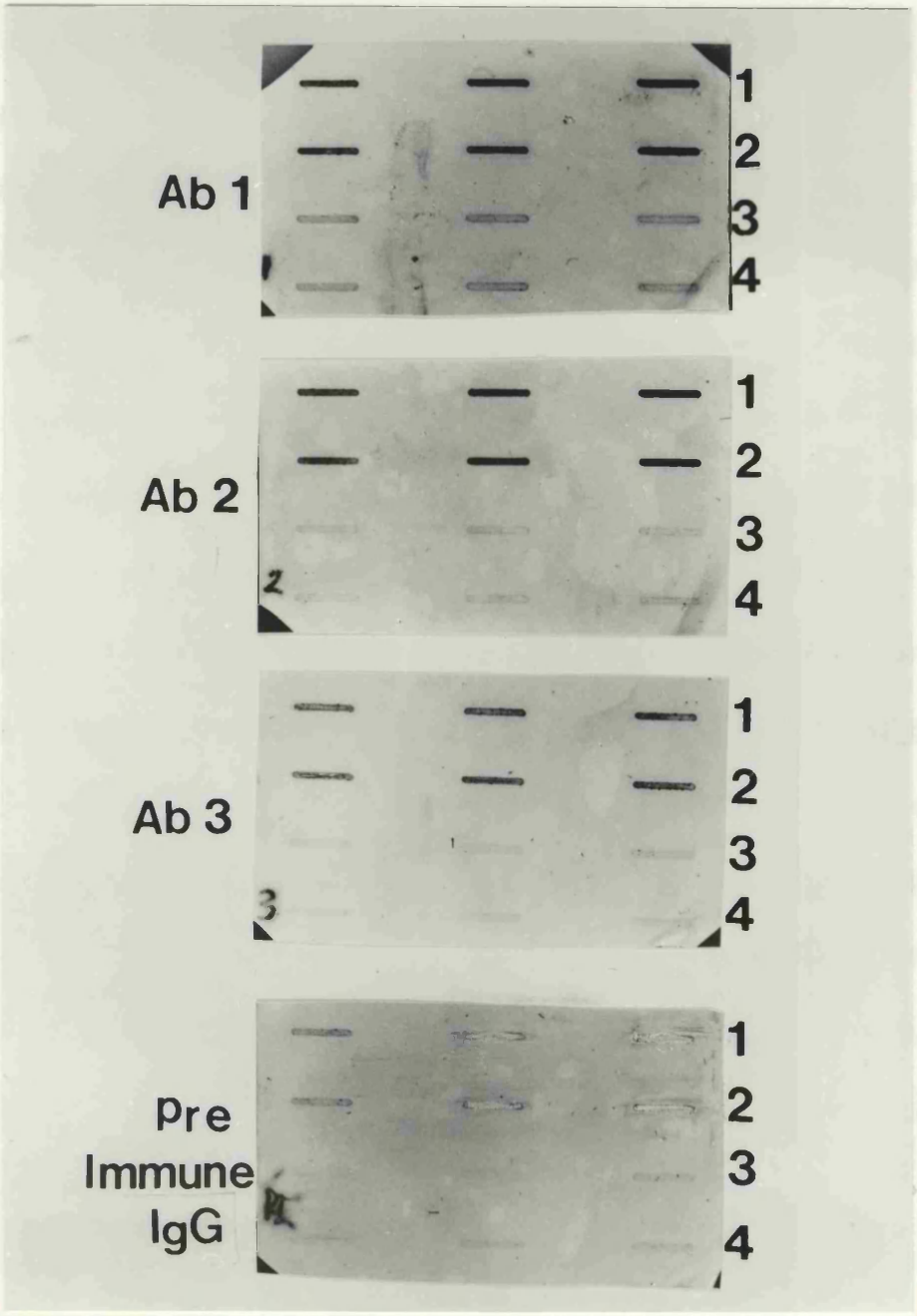

Figure 7.3 Slot blot analyses using the three antibodies and pre-immune IgG in order to identify proteolysis of the receptor in MA10 cells. Rows 1 and 2 contained triplicate lanes of ovarian (lane 1) and MA10 (lane 2) cell membranes (100μg protein/slot) prepared as described in *Materials and Methods* section 2.2.12. Row 3 also contained triplicate samples of MA10 cell conditioned medium (100μg protein/slot) from incubations in which the antibodies (10μg/ml) were allowed to bind to the receptor for 2h before treating for another 2h with 5mM NaN_3. The medium was collected, concentrated and blotted directly onto the nitrocellulose membranes where the secondary goat-anti-rabbit alkaline phosphatase antibody was allowed to bind as described in *Materials and Methods* section 2.2.17. Row 4 contained 100 μg protein/slot in triplicate lanes of MA10 cell incubation medium after treating with NaN_3 for 2h. In this case, the medium was collected, concentrated and blotted onto the nitrocellulose membranes and then the primary and secondary antibodies were allowed to bind. Experiments were repeated three times with similar results.

7.3 Discussion

It was found that treating MA10 cells for 2h with LH, NaN_3 or LH + NaN_3 caused a 40% decrease in ^{125}I-hCG binding compared to the controls. This effect was also observed by West and Cooke (1991) where a 50% loss of cell surface receptors was reported. They also found that this loss was prevented by the addition of protease inhibitors. However, in the current study, the addition of the protease inhibitors did not have such an effect. The experiment was repeated three times, each time using a new batch of cells and new protease inhibitors. The difference between cell surface ^{125}I-hCG binding in the presence of protease inhibitors was not statistically significant compared to the binding obtained in the absence of the inhibitors in each of these three experiments. Treating the cells with NaN_3 caused only a small decrease in the viability of the cells (from 99% to 85%) and this was also observed by West and Cooke (1991). Such a small decrease in viability would not be expected to have a deleterious effect on the detection of ^{125}I-hCG binding, and the loss in cell viability was taken into account before the specific binding was evaluated.

The Western blotting experiment was carried out in order to try and use the antibodies raised in this study to investigate the existence of proteolytic fragments of the LH receptor and to identify the size of these fragments that were reported previously to be proteolytically cleaved from the LH receptor. However, no extracellular fragments were identified with any of the antibodies. Antibody 3 (C-terminal antibody) was not expected to react with any proteins in the medium since the fragments released were proposed to be cleaved from the extracellular domain of the LH/CG receptor. However, antibodies 1 and 2 were both raised to short peptide sequences in the

extracellular domain of the receptor, but still failed to recognize any putative proteolytic fragments. Since protease inhibitors were added to the medium after the incubations with LH and NaN_3 were carried out, this rules out the possibility that any proteolytic fragments formed were further degraded by proteases. All three antibodies reacted with the control lane containing MA10 cell membranes, giving a 95 kDa band as expected. The pre-immune IgG failed to recognize any proteins. Another attempt to identify these fragments was also carried out using slot blotting analysis. The advantage of this method over the Western blotting is that the technique is a lot faster, and factors such as separation of proteins on SDS gels and electroblotting that can give rise to several problems are avoided.

It was previously argued by West and Cooke (1991) that in order to identify the proteolytic fragments it was easier and necessary to bind the radioligand to the receptor first before inducing proteolysis. In this study possible proteolysis was investigated firstly by allowing the antibodies to react with the receptor prior treatment with NaN_3, and secondly by using the antibodies to look in the medium after NaN_3 treatment. Both methods failed to identify any proteins in the incubation medium of MA10 cells. In contrast, all three antibodies (but not pre-immune IgG) in the same experiment reacted positively with lanes containing ovarian and MA10 cell membranes. Pre-binding the antibody to the receptor, however, did not cause the same effect as pre-binding the ligand hence suggesting that the proteolytic effects seen previously were ligand-induced. Thus, from this study it might be concluded that in order to achieve proteolysis of the LH receptor the ligand has to be pre-bound as described previously by West and Cooke (1991).

The results presented in this chapter do not support the concept that the LH/CG receptors are regulated by proteolysis or that the extracellular domain of the receptor can be cleaved and act as a serum binding protein. This was concluded after using the three antibodies raised in this study which were found to be powerful and specific enough to identify intact, denatured and reduced LH/CG receptors from both rat and mouse testicular and ovarian tissues as well as the MA10 cells but still failed to identify any receptor fragments in cultured media.

CHAPTER 8

GENERAL DISCUSSION

8.1 Discussion

In order to investigate the structure / activity relationships and regulation of the LH/CG receptor, the overall aim of this project was to raise and characterize polyclonal anti-peptide antibodies against sequences within both the extracellular and cytoplasmic domains of the LH/CG receptor. Antibodies to the LH/CG receptor have been available for a number of years (see *Chapter 1* section 1.6). However, several problems regarding the specificity and titre of these antibodies limited their use to certain tissues of certain species. In this study, the use of synthetic peptides in the production of antibodies which recognize the intact LH/CG receptors in both its native and denatured states in testicular and ovarian tissues from mice and rats as well as the MA10 cells were successful.

The results obtained in this study demonstrated that the anti-peptide strategy used to raise polyclonal antibodies was successful in that all three antibodies raised were peptide specific and that affinity purification of the antibodies resulted in an increase in the titre of the purified antibodies over the crude antiserum. In the dot blotting analyses all three antibodies recognized a protein(s) from solubilized superovulated rat ovarian membranes but failed to react with liver membranes. This gave an indication that the antibodies are binding to protein(s) of interest that are only specific to the ovarian membranes with it being consistent of being the LH/CG receptor. Further studies revealed that all three antibodies (but not pre-immune IgG) bound to solubilized protein(s) prepared from rat testis and MA10 cells in slot blots. Tissues which are thought not to express the LH/CG receptors failed to react with any of the antibodies. Although a number of certain other non-gonadal tissues were shown to

express LH/CG receptor mRNA or even express the protein (see *Chapter 1* section 1.5.3), using the antibodies raised in the present study, there was no evidence to suggest the expression of LH/CG receptor protein in liver and endothelial cell membranes. If these tissues do actually express LH/CG mRNA and protein, then the failure of the antibodies to bind could be explained by the lower abundance of the protein present in these tissues compared to gonadal tissues or simply by the fact that these non-gonadal tissues, although capable of expressing the mRNA for the LH/CG receptor, fail to translate the message to protein. Another way of checking the specificity of the LH receptor anti-peptide antibodies was in a Western blotting cross-reaction study using a hTSH extracellular receptor recombinant protein (ExG2) and an anti-ExG2 antibody (R14). This, once again, proved that the three LH receptor antibodies are specific for the LH receptor.

In order to identify the size of the proteins recognized by the antibodies, Western blotting was performed on different membrane samples. When membranes from superovulated rat ovaries, rat testis and MA10 cells were used, in all cases, a 95-100 kDa band was observed with all three antibodies which confirmed the previously reported size of the receptor (see reviews Ascoli and Segaloff 1989; Segaloff and Ascoli 1993). The affinity purified rat testicular LH receptor had previously been reported by Dufau *et al.* (1975) to yield a single component of 90 kDa on SDS-gels. Metsikko and Rajaniemi (1980; 1982), using rat ovarian receptors purified by immunoaffinity chromatography and direct affinity chromatography, isolated a 90-110 kDa protein by SDS-gel electrophoresis. The mature LH/CG receptor is a single polypeptide of 674 amino acids with a predicted molecular mass of 75 kDa (see

review Segaloff and Ascoli 1993). Approximately half of the amino acids are present in the hydrophilic extracellular domain, which contains six potential N-linked glycosylation sites. Although it is now clear that the LH/CG receptor is a glycoprotein (Minegishi *et al.*, 1989; Petaja-Repo *et al.*, 1991), it is not currently known which of these potential N-linked glycosylation sites are coupled to carbohydrate. The glycoprotein nature of the receptor, however, is presumably responsible for the difference between the molecular mass predicted from the open reading frame of the cDNA and the apparent mass (i.e. 95-100 kDa) deduced from SDS-PAGE. Two types of bands were obtained depending upon whether ovarian or testicular membranes were used. The band appeared to be broader or sometimes double in the case of the ovarian membranes, whereas with testicular proteins, the band appeared sharper. The different N-linked glycosylation pattern of the receptors in these membranes is a possible explanation for this observation (Minegishi *et al.*, 1989). Deglycosylation of the MA10 cell membrane LH/CG receptor resulted in two smaller bands of 64 and 75 kDa which were in agreement with the findings of Sojar and Bahl (1989) on the deglycosylation of the ovarian receptor. The results obtained in the present study also confirmed that the carbohydrate residues on the receptor did not interfere with antibody binding since antibody binding was unaffected by endoglycosidase F treatment. In addition, the fact that all three antibodies (N- and C-terminal antibodies) recognized both 64 and 75 kDa proteins confirms that both the N- and C-termini as well as the connecting transmembrane domains were present following deglycosylation. The 75 kDa band could therefore represent the fully deglycosylated LH receptor since the relative molecular mass agrees with that predicted from the open reading frame (see review Segaloff and Ascoli 1993). The 64 kDa band must therefore be a truncated

deglycosylated form of the receptor. This cleaved receptor form probably has part(s) of the N- or C-termini missing hence accounting for the lower molecular mass. Since all three antibodies are still capable of binding the 64 kDa protein then it appears that the majority of the receptor is intact (at least aa residues 48-636). Segaloff and Ascoli (1993) suggested that two cytoplasmic clusters of basic amino acid residues (K^{623}-R^{625} and R^{630}-K^{632}) could represent potential tryptic cleavage sites. Since antibody 3 is still able to bind to the 64 kDa protein, the present study suggests that the receptor is probably cleaved after amino acid residue 632 hence still allowing antibody 3 to recognise at least part of its epitope.

The appearance of a 95 kDa band was also apparent in MA10 and ovarian ligand blotting experiments where ^{125}I-hCG bound to this band in a displaceable manner. The relative significance of the receptor disulphide bonds for antibody versus hormone binding was also established in these studies. It was concluded from Western blotting (under reducing conditions) that these bonds are not necessary for antibody-receptor binding, but the ligand blotting experiments under reduced and non-reduced conditions established that the presence of these disulphide bonds is essential for hormone binding, confirming the conclusions of Dufau *et al.* (1974).

The immunocytochemistry data was an additional visible proof that the antibodies raised in this study were functional. The extracellular antibodies (antibodies 1 and 2) stained MA10 cells and the interstitial Leydig cells of rat testicular sections and the staining was located pericellularly. This was an important finding as it proved, once again, using a different technique, that the antibodies were able to recognize the intact

native LH receptor as well as demonstrating the potential use of these antibodies in immunocytochemical investigation of LH receptor expression. The cytoplasmic antibody (antibody 3) and pre-immune IgG failed to react. Failure of antibody 3 to react raised the possibility that the receptor is palmitoylated at the cytoplasmic C-terminal cysteine residues (as discussed previously by Kawate and Menon (1994) and Zhu *et al.* (1995)) supporting the formation of the fourth intracellular loop and hence the steric interactions involved, preventing this antibody from binding or alternatively masking of the antibody 3 antigenic site on the receptor. Evidence from the present study, therefore, supported the fourth cytoplasmic loop theory and is consistent with the existence of this loop when the receptor is in its native state in the plasma membrane. This proposed structural organization of the LH/CG receptor is shown diagrammatically in fig 8.1, which also shows the location of the antibody binding sites on the receptor. In this figure, the close proximity of antibody 1 and LH β-subunit binding regions is also apparent, which might offer a possible explanation as to why antibody 1 might be more potent than antibody 2 in inhibiting ligand binding (discussed later). Using the antibodies raised in this study it was concluded in chapters 4 and 5 that rat ovarian, rat testicular and mouse tumour Leydig cell LH/CG receptors migrate as 95-100 kDa bands on SDS-PAGE gels, hence demonstrating the similarity in size of the LH/CG receptors in these two different rodent species and between the sexes.

In chapter 6, the differential effect of the three antibodies on the function of the LH/CG receptor was determined. Only the two extracellular antibodies (antibodies 1 and 2) inhibited the binding of ^{125}I-hCG to MA10 cells. This is consistent with the

view that the N-terminus of the LH receptor includes the hormone binding domain. This was also demonstrated in competitive ligand blot analyses where ^{125}I-hCG binding to a 95 kDa protein band was displaced not only by excess unlabelled hCG, but also with antibody 1 and to a lesser extent, antibody 2. In these studies, antibody 1 was more potent than antibody 2 when used at the same concentration. This observation could be explained because antibody 1 would be expected to bind to a region which is closer to the putative binding domain of LH for the β-subunit on the receptor. Roche *et al.* (1992) identified the binding region of the LH β-subunit to be around aa residues 21-38 in the N-terminal sequence of the LH/CG receptor. Antibody 1 (raised against aa residues 48-65) is closer to this domain than antibody 2 (raised against aa residues 187-206) (fig. 8.1). However, further work needs to be carried out to study the relative affinities of all three antibodies for the receptor. The predictions made at this stage, while giving a good indication of the location of the hormone-receptor binding site, are based simply on the receptors primary structure. Future work needs to be carried out on the receptor's secondary structure to give more accurate interpretations of these data.

Although capable of blocking hormone binding, the antibodies when added alone had no stimulatory effects on receptor signal transduction. Antibody 1 and to a lesser extent antibody 2, inhibited LH-stimulated signal transduction. Both cAMP accumulation and steroidogenesis were inhibited when the cells were preincubated with the N-terminal antibodies prior to stimulation with LH. This inhibition was not apparent when dbcAMP, cholera toxin and forskolin were used to stimulate progesterone production and cAMP accumulation. From these experiments, it was

concluded that the inhibition of LH action is actually occurring at the ligand-receptor step. In order to study the possible effects of the intracellular antibody (antibody 3) on signal transduction, MA10 cell plasma membranes were used and adenylyl cyclase activity was measured after stimulation with LH. Antibodies 1 and 2 inhibited the LH responses in these plasma membranes as expected, presumably by binding to the receptor and blocking the LH-receptor interaction. Antibody 3 had no effect on signal transduction, although binding of all three antibodies to these plasma membranes was confirmed by Western blotting analyses. Assuming that antibody 3 binds to the plasma membranes regardless of the presence of the fourth cytoplasmic loop (as it did in the Western blots) then this finding, suggests, that antibody 3 binding does not interfere with signal transduction via the adenylyl cyclase pathway and that the particular region of the C-terminus to which the antibody 3 was raised is not involved in LH receptor-G_s coupling. This is in agreement with the results obtained with adrenergic receptors where an extended third cytoplasmic loop (rather than the carboxy terminus) seems to be required for specific interactions with G_s (Kobilka *et al.*, 1988; O'Dowd *et al.*, 1989; Strader *et al.*, 1989; Cheung *et al.*, 1991). Furthermore, Grasso *et al.* (1995) demonstrated that for the rat testicular FSH receptor, the third cytoplasmic intracellular loop (aa residues 533-555) acts as an antagonist of FSH receptor-mediated G-protein activation and inhibits FSH receptor mediated signal transduction in intact Sertoli cells. Likewise, Malek *et al.* (1993) demonstrated the existence of two specific sites in the third inner loop of the dopamine D_2 receptor which are involved in functional G-protein-mediated coupling to adenylyl cyclase. Peptides synthesized against these two sites were found not only to attenuate dopaminergic adenylyl cyclase inhibition in membranes, but also had the ability to activate directly GTPase activity in membranes.

From the above findings it is not surprising that binding of the C-terminal antibody to the cytoplasmic domain of the LH/CG receptor does not interfere with G-protein activation. Although the LH/CG receptor does not appear to have an extended third cytoplasmic loop, or significant sequence homology with the G-protein-coupling domain of the β-adrenergic receptors (McFarland *et al.,* 1989; Loosfelt *et al.,* 1989), presumably the third cytoplasmic loop of the LH/CG receptor might somehow be involved in G-protein coupling.

One of the main reasons for raising these anti-peptide antibodies to both the extracellular and cytoplasmic domains of the LH/CG receptor was to study possible proteolytic cleavage of the receptor and hence identify any fragments released that can act as binding proteins. A 1.2 kb mRNA transcript encoding for the N-terminus of the LH receptor (see review Segaloff *et al.,* 1990) is expressed in the testis. Therefore, truncated LH/CG receptor extracellular domains might be translated and secreted from Leydig cells with the potential to act as extracellular binding proteins that would bind available LH in the interstitium and decrease the concentration of free LH, hence preventing desensitization and down-regulation of the LH/CG receptors. However, from the results presented in chapter 7 it was concluded that the extracellular domain of the LH/CG receptor in MA10 cells is unlikely to act as a potential binding protein, since no such N-terminal fragments were detected by the antibodies. Furthermore, since previous workers (West and Cooke, 1991) showed that LH receptors are regulated by proteolysis of their extracellular domain, it can be now concluded from the present study that this proteolysis is hormone-induced since the antibodies cannot mimic this effect (figs. 8.2 - 8.4).

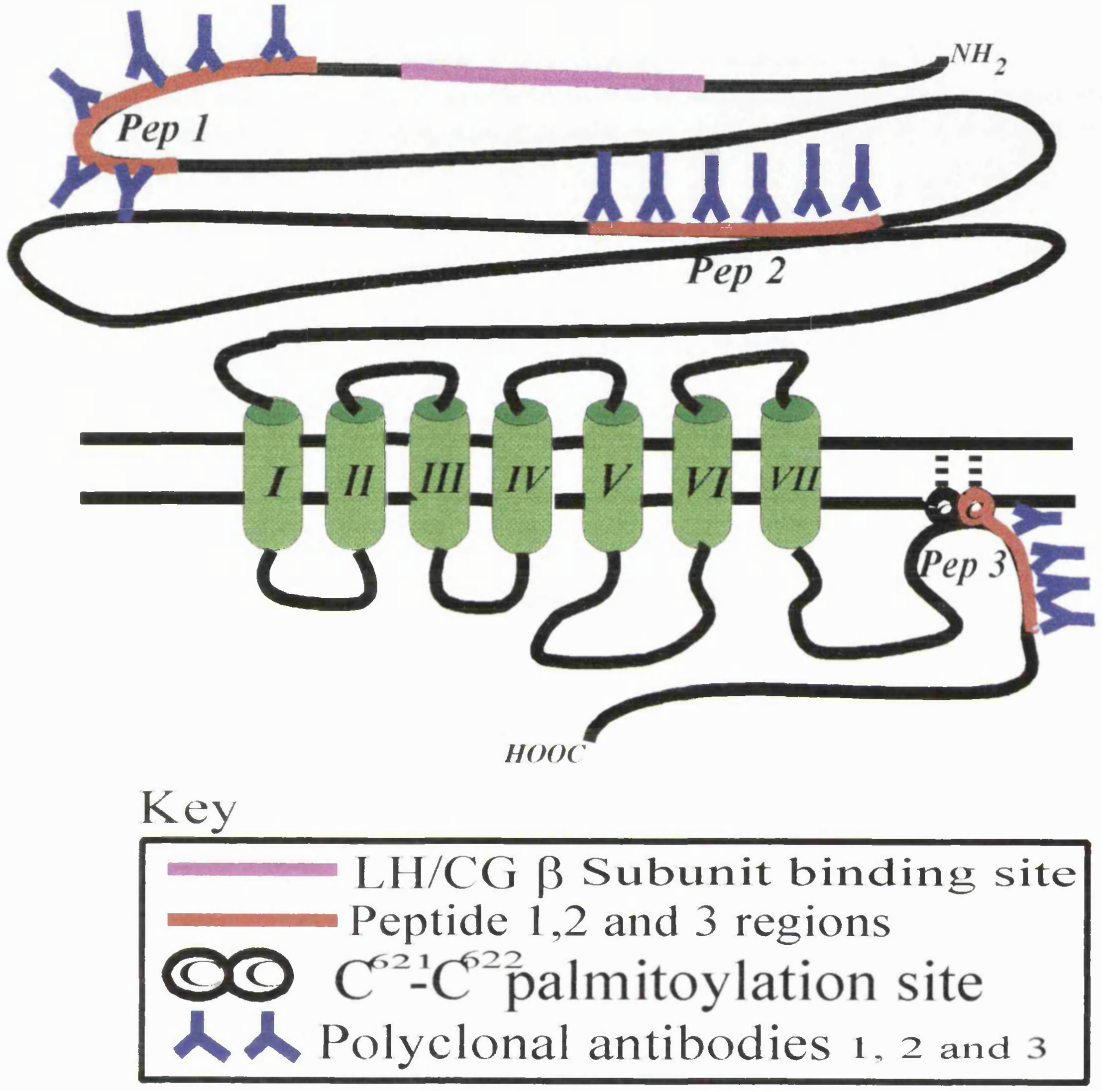

Figure 8.1 Proposed structural organization of the LH/CG receptor. Palmitoylated Cys^{621}-Cys^{622} residues may anchor the amino-terminal portion of the cytoplasmic domain to the plasma membrane, thereby creating a fourth intracellular loop. Transmembrane domains are indicated in Roman numerals. The locations of the three peptides as well as the β-subunit binding site for LH are also indicated.

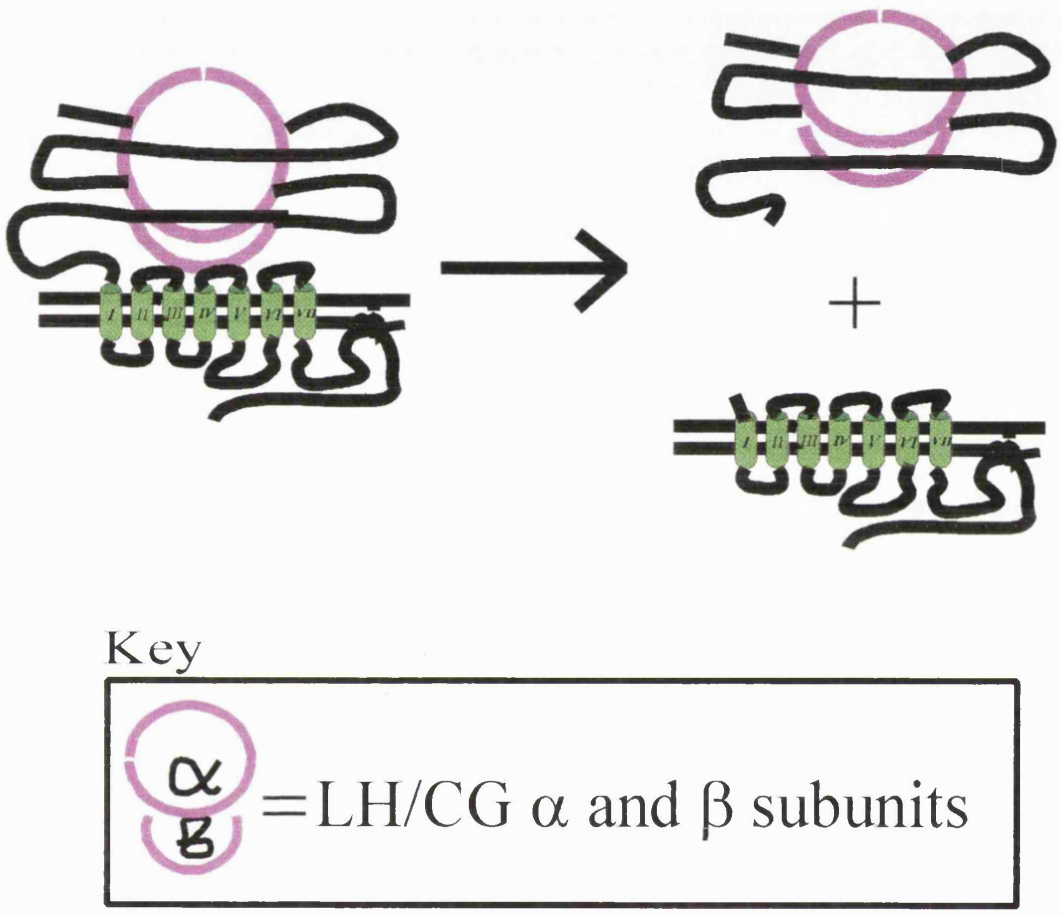

Figure 8.2 Ligand induced proteolysis of the LH receptor. In order for proteolysis to occur the ligand has to be pre-bound to the receptor (West and Cooke, 1991).

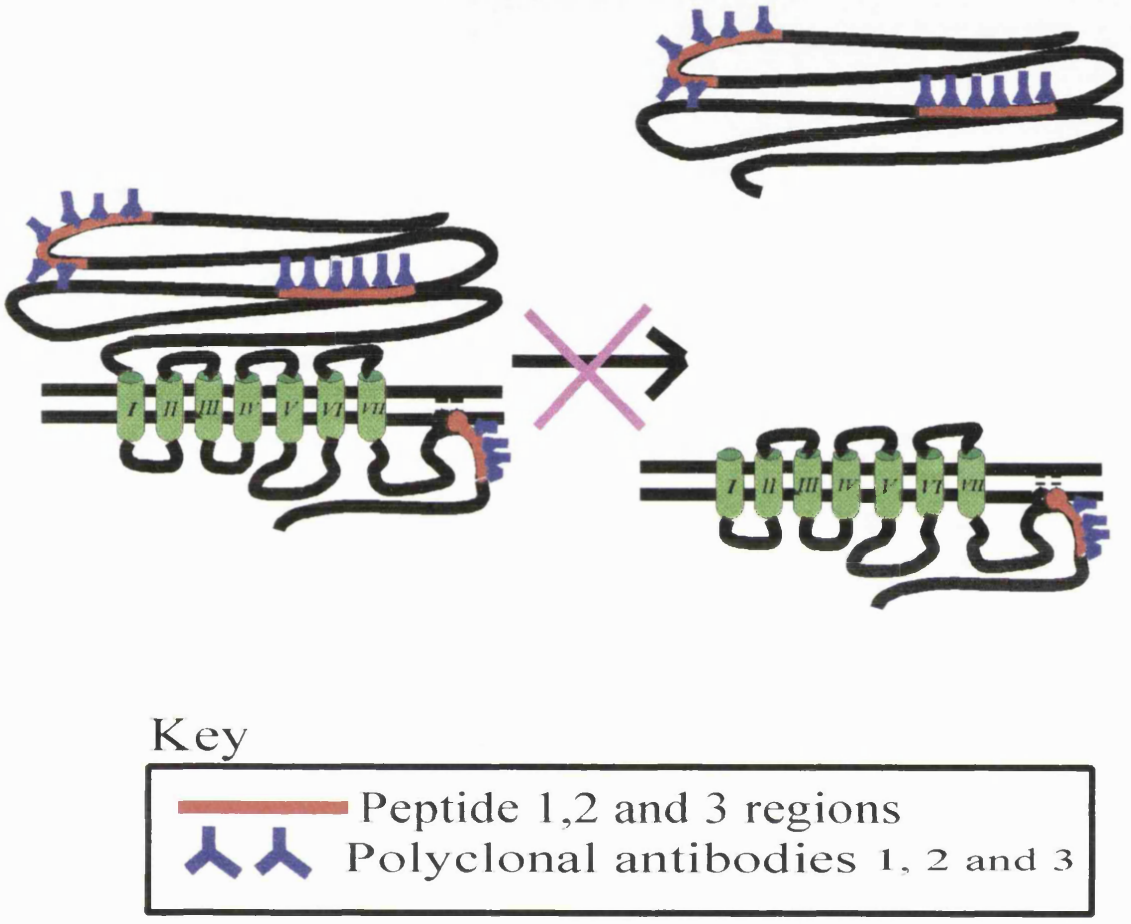

Figure 8.3 Antibody induced proteolysis. Pre-binding the antibodies to the receptor cannot mimic ligand-induced proteolysis.

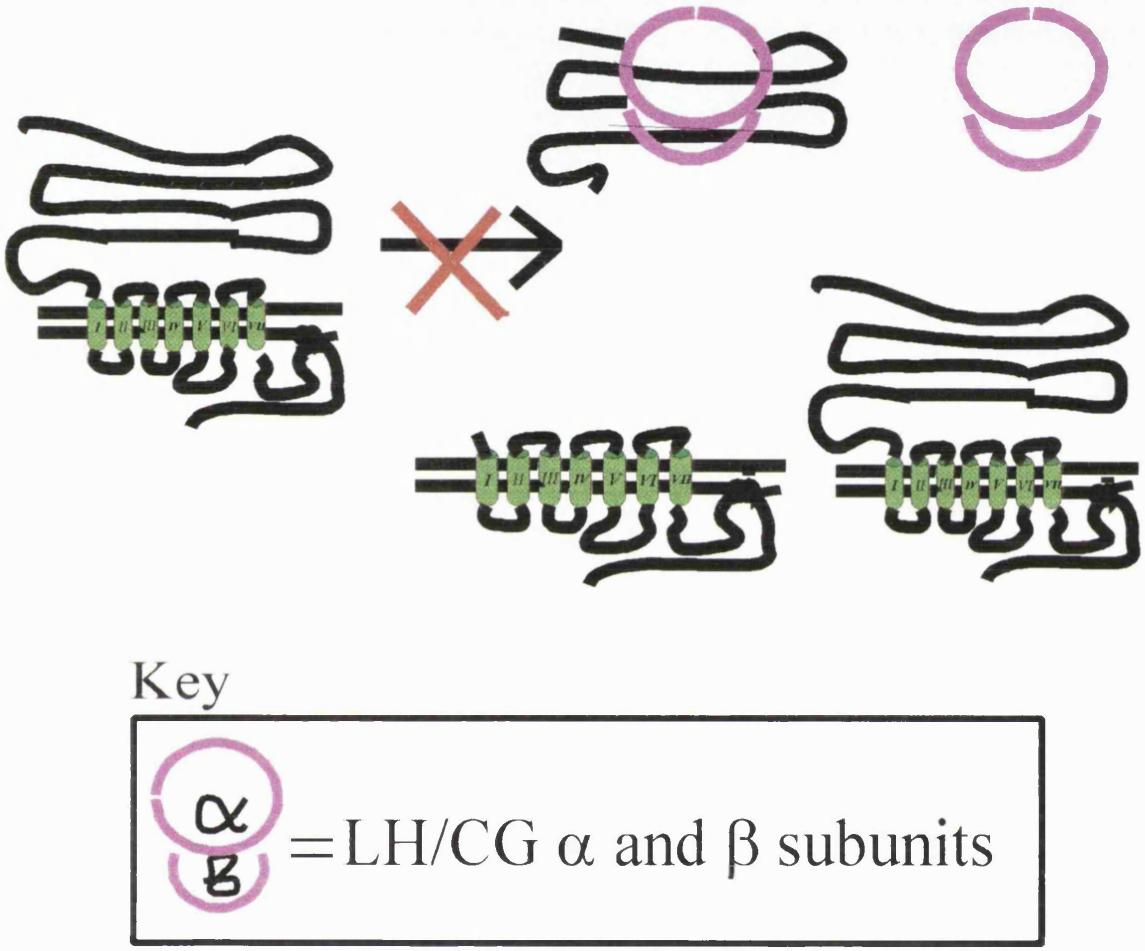

Figure 8.4 Involvement of the N-terminal fragment as an LH binding protein. The N-terminal extracellular fragment of the LH receptor cannot be cleaved and exists as a regulatory binding protein.

8.2 Future work

Having powerful antibodies for a specific protein can have an enormous advantage in the field of biochemistry. Although the anti-peptide antibodies raised in this study were extensively characterized, further work needs to be carried out on the binding affinity of these antibodies for the LH receptor. This can be achieved by iodinating the antibodies and performing direct binding studies on cells. This would explain whether the differences seen between antibodies 1 and 2 in inhibiting ligand binding is due to the different antibody affinities for the receptor, rather than a relative difference in the antibodies for the epitope regions in determining the LH binding. Furthermore, direct labelling of the antibodies, together with receptor-secondary structure studies, could help identify the binding sites on the receptor.

The successful production of site-specific antibodies which recognize the LH/CG receptors in their intact, denatured and native conformations has made available immunological probes for further investigations of the presence of LH/CG receptors in various tissues. Since many workers have identified mRNA encoding the LH/CG receptor in different tissues (rat prostates, Tao *et al.,* 1995; brain, Lei *et al.,* 1993; endometrial and myometrial blood vessels, Lei *et al.,* 1992; uterine arteries, Toth *et al.,* 1994; lymphocytes, Lin *et al.,* 1995), it is important to see if the mRNA is actually translated into either full or truncated proteins; the antibodies raised against the N- and C-termini of the LH receptor can be used for this purpose.

The LH receptor anti-peptide antibodies raised in this study can be used in Western blotting experiments, to investigate gel shifts which arise as a result of receptor

phosphorylation. Phosphorylated receptors will migrate as heavier proteins compared to non-phosphorylated proteins. This can be detected on gels using the three LH receptor antibodies, thus avoiding the use of radioactivity.

The antibodies can also be used to construct immunoaffinity columns in order to purify LH/CG receptors from various tissues and species. Since all three antibodies recognize the intact LH/CG receptor, three consecutive immunoaffinity columns can be used to obtain highly purified receptor protein.

The adaptation of the antisense inhibitory strategy carried out by West and Cooke (1991) provided a method to study different functional regions of the LH/CG receptor. The results obtained with this strategy were based on a theoretical generation of truncated proteins. Since no direct evidence was provided to support this hypothesis, the use of the antibodies raised in the current study to identify the putative truncated proteins will give an accurate interpretation of the former results in different species.

APPENDIX

SUPPLEMENTARY METHODS

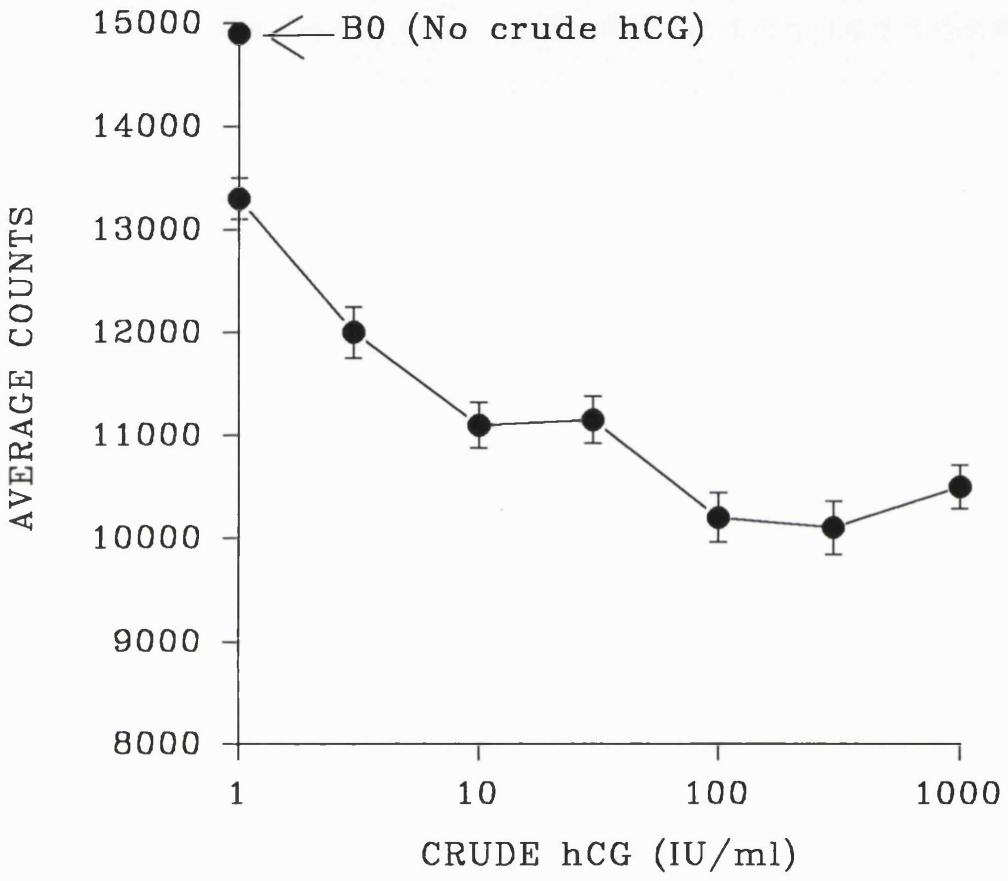

Figure A1.1 Binding study to determine NSB value using crude hCG (CR-127). Crude rat Leydig cells were prepared as described in *Materials and Methods* section 2.2.2. ^{125}I-hCG binding was determined as described in *Materials and Methods* section 2.2.21 in the presence of increasing amounts of crude hCG (1-1000IU/ml).

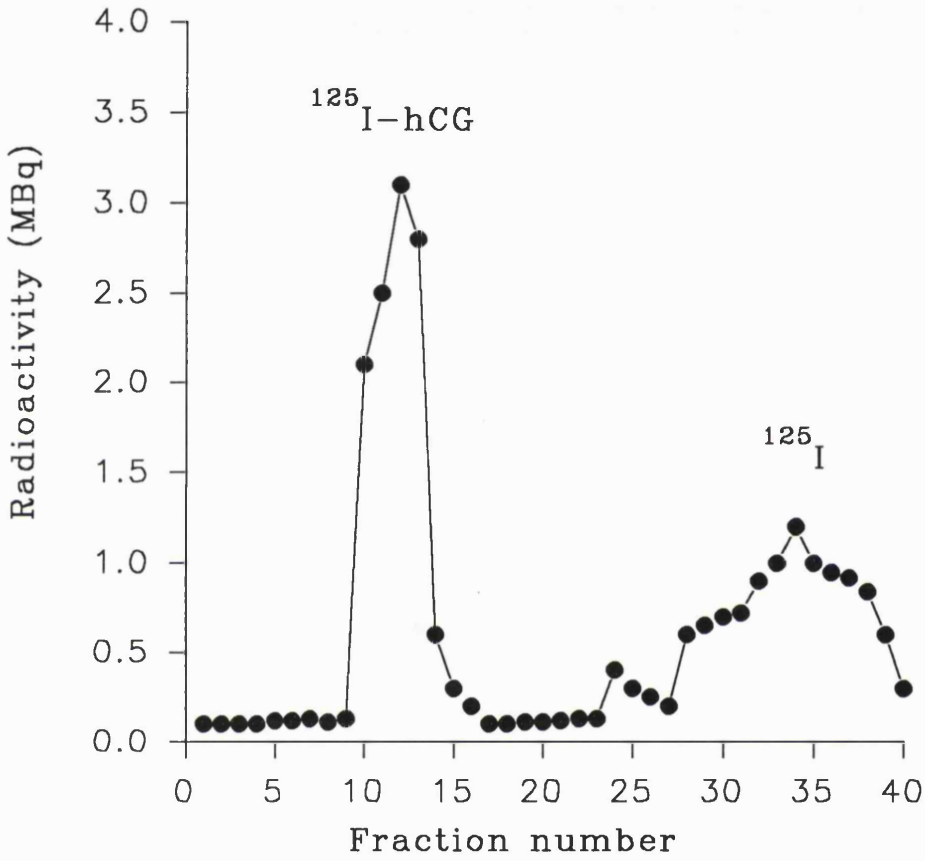

Figure A1.2 Profile obtained for the iodination of hCG. Typical elution profile of free ^{125}I and ^{125}I-labelled hCG from a PD-10 Sephadex G-25M column.

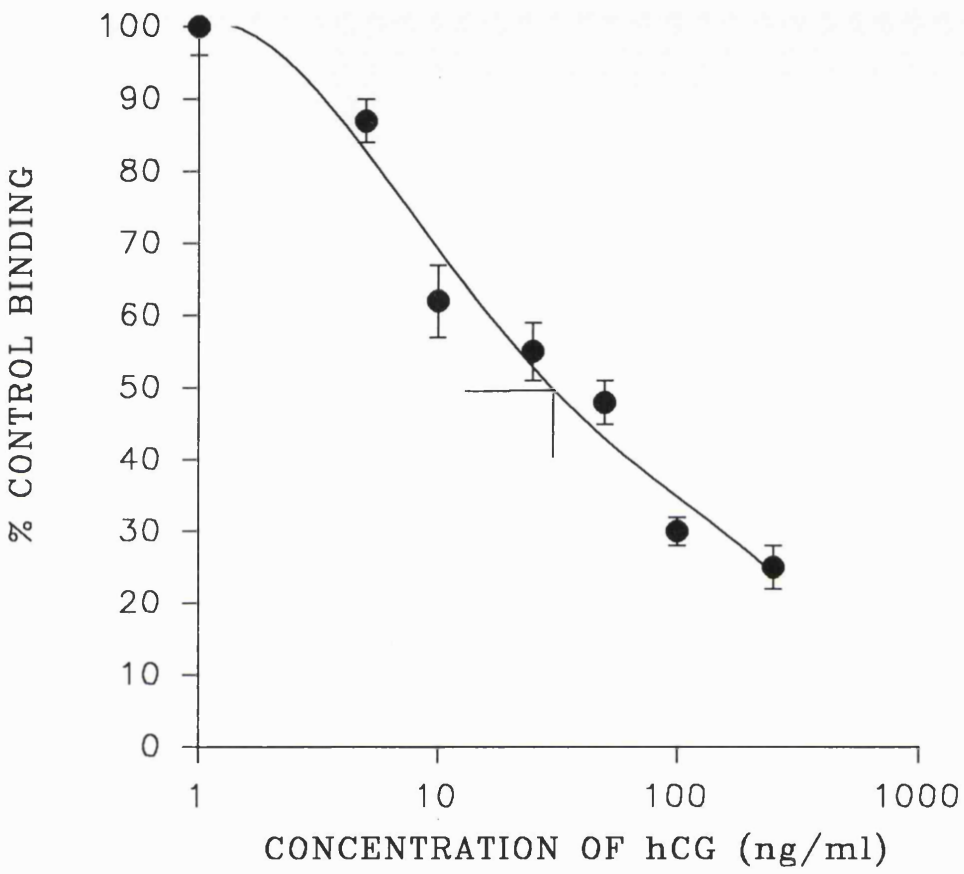

Figure A1.3 Characterization of iodinated hCG. Competitive binding curve for the characterization of ^{125}I-labelled hCG using increasing concentrations of unlabelled hCG (CR 127).

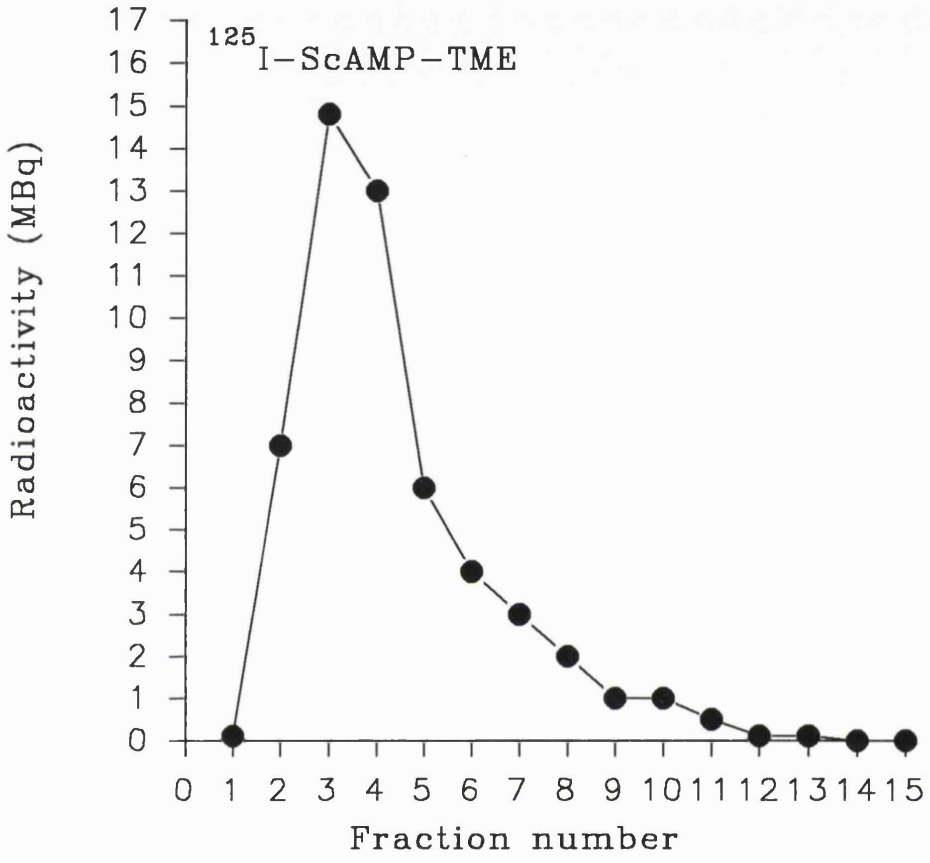

Figure A1.4 Profile obtained for the iodination of cAMP. Typical profile of separation of free ^{125}I and ^{125}I-ScAMP-TME using a C_{18} Sep-pak (Waters) column.

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Characterization of site-directed antibodies to the LH receptor in functionally active gonadal cells and their differential effects on LH-stimulated signal transduction in Leydig tumour (MA10) cells ☆

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Abstract

In this study site-directed antibodies have been used to investigate the structure/activity relationships of the LH receptor in functionally active gonadal cells. Polyclonal antibodies were raised in rabbits against synthetic peptides corresponding to regions within both the extracellular N-terminal domain (antibodies 1 and 2 against residues 48–65 and 187–206, respectively) and the cytoplasmic C-terminal domain (antibody 3 against residues 622–636) of the LH receptor. Following affinity purification by chromatography on columns of immobilised peptides the antibodies were demonstrated to be peptide specific both by ELISA and by dot-blotting assays. On Western blots of membranes proteins prepared from superovulated rat ovaries, mouse Leydig tumour (MA10) cells, and rat testes, all three antibodies recognised a single broad band of apparent M_r 95 000–100 000 corresponding to the putative LH receptor. The protein of apparent M_r 95 000–100 000 also bound ^{125}I-hCG on ligand blots, and binding was displaced by excess unlabelled hCG. The binding of ^{125}I-hCG in the ligand blots was completely inhibited by excess unlabelled hCG. The two N-terminal antibodies (antibodies 1 and 2 (10 μg/ml)) also inhibited ^{125}I-hCG binding to a greater extent than the C-terminal antibody (antibody 3 (10 μg/ml)). Antibody 1 (1 and 10 μg/ml) also potently inhibited the binding of ^{125}I-hCG to MA10 cells. A lesser but still significant inhibition of binding was produced by antibody 2 (with 10 μg/ml), whereas at the concentrations tested antibody 3 exerted no greater inhibition than that yielded by pre-immune IgG. At 0.1 μg/ml antibody 1 significantly inhibited and at 10 μg/ml completely inhibited LH-stimulated cAMP and progesterone production by MA10 cells. With antibody 2, 10 μg/ml was required to give a significant inhibition, whereas neither antibody 3 nor pre-immune IgG had a significant effect. The antibodies had no effect on cAMP or progesterone production when added to the MA10 cells in the absence of LH. These results indicate that binding of antibody 1 and, to a lesser extent, antibody 2 interferes with ligand binding which consequently affects signal transduction. In view of the ability of the antibodies to recognise the LH receptors both in the ovary and the testis and in more than one rodent species, and their greater apparent potency than previously available antisera, the anti-peptide antibodies raised in the present study will therefore be useful to study LH receptors in normal, functionally active gonadal cells.

Keywords: Anti-peptide antibody; Luteinizing hormone (LH) receptor; Mouse tumour Leydig cell (MA10); Steroidogenesis; Cyclic AMP; hCG binding

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1. Introduction

The luteinizing hormone/chorionic gonadotrophin (LH/CG) receptor is present on testicular Leydig cells and on ovarian theca-interstitial cells, mature granulosa cells and cells of the corpus luteum. In both the testis and the ovary, the LH/CG receptor recognizes the pituitary glycoprotein hormone LH, and has the capacity to bind the related glycoprotein, human CG (hCG). The signal transduction mechanism involves binding LH to the LH receptor in the plasma membrane of these gonadal cells which activates a guanosine triphosphate (GTP)-binding protein (Gs) with subsequent stimulation of adenylate cyclase. The resulting increase in cAMP activates the cAMP-dependent protein kinase (Rommerts and Cooke, 1988). The primary consequence of this response is an increase in the synthesis and secretion of steroid hormones.

The LH receptor has been cloned and sequenced from rat ovaries (McFarland et al., 1989), porcine Leydig cells (Loosfelt et al., 1989), as well as human (Minegishi et al., 1990) and mouse (Gudermann et al., 1992) ovarian tissue. It consists of a 341-residue extracellular domain displaying an internal repeat structure characteristic of members of the leucine-rich glycoprotein family, and a 333-residue membrane-spanning region which displays a low sequence similarity with other members of the GTP binding protein coupled receptor family, and a 68-residue cytoplasmic domain. From the amino acid sequence of the rat luteal LH receptor, and by analogy with the topography of other G-protein-coupled receptors, it was postulated that the LH receptor exists in the plasma membrane, with the amino terminal hydrophillic domain being extracellular and the carboxyl-terminal domain spanning the plasma membrane seven times, ending with a short cytoplasmic tail. This hypothesis was confirmed using immunofluorescence techniques with antibodies directed to peptide sequences in the amino terminal (residues 194–206) and the carboxyl terminal sequences (residues 661–674) of the LH receptor (Rodriguez and Segaloff, 1990).

The antibodies to the LH receptor that have been raised previously have been limited in their application mainly to either luteal cells from superovulated ovaries or cells transfected with the cloned LH receptor (i.e., cells with abnormally high levels of expression of the LH receptor). The relatively low abundance of the LH receptor in normal gonadal cells and/or the low titre of the available LH receptor antibodies have precluded the use of these antibodies to study receptor regulation and function in normal gonadal cells. Also LH receptor antibodies raised to the intact receptor show high species and cell specificity.

The aim of the present study was therefore to raise antibodies against peptides from the N- and C-terminal regions of the LH/CG receptor of a sufficiently high titre and specificity, in order that they may be used to investigate the structure/activity relationship of the LH receptor in functionally active gonadal cells.

2. Materials and methods

2.1. Animals

Highly luteinized ovaries were obtained from 21-day-old rats after injection with 50 IU PMSG (Intervet labs, Cambridge, U.K.) followed 56 h later with 25 IU hCG (Serono labs, Herts, U.K.) (Parlow, 1958). Animals were sacrificed by cervical dislocation 7 days after hCG administration.

2.2. Leydig tumour cells

Stock cultures of Leydig tumour (MA10) cells (a gift from Dr. M. Ascoli of the University of Iowa, IA, U.S.A.) were maintained in Waymouth's MB752/1 medium + 15% horse serum (Life Technologies Ltd, Scotland, U.K.) according to the method of Ascoli (1981). Experimental cultures were plated at a density of 1×10^5 cells/ml in Waymouth's medium and used 3 days after subculture. The growth medium was replaced on alternate days in culture. Cell numbers were determined using a haemocytometer and viability of the cells by the exclusion of 0.4% (v/v) Trypan Blue dye (Jeejeebhoy et al., 1975).

2.3. Synthesis of LH receptor peptides

Peptides corresponding to residues 48–65 and 187–206 (N-terminal peptides) and to residues 622–636 (C-terminal peptide) of the rat ovarian LH receptor were synthesized according to the method of Atherton and Sheppard (Atherton and Sheppard, 1995) using N^{α}-fluorenylmethoxycarbonyl amino acid pentafluorophenyl esters (Milligen, Watford, Herts, U.K.). A cysteine residue was attached to the C-terminus of the N-terminal peptides, and to the N-terminus of the C-terminal peptide to facilitate coupling to ovalbumin (Milligen, U.K.). The peptides were cleaved from the support using 95% (v/v) trifluoroacetic acid (Sigma, Poole, Dorset, U.K.) solution in distilled water (containing 2.5% (v/v) 1,2-ethanedithiol (Sigma, U.K.) and 2.5% (w/v) phenol) and then washed with diethylether (BDH, Dorset, U.K.). HPLC analysis of the peptides on a C_{18} column using gradients of acetonitrile in 0.1% (v/v) trifluoroacetic acid indicated that they were at least 80% pure. The purity and identity of the peptides was confirmed by amino acid analysis following their hy-

drolysis in 5 M HCl at 110°C for 24 h (data not shown) and so they were used without further purification.

2.4. Production of LH receptor peptide antibodies

Peptides were coupled to ovalbumin using maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce, Chester, U.K.), essentially as described by LaRochelle et al. (1985). Antisera against the conjugates were then raised in Dutch half lop female rabbits (starting weights, 2 kg) as follows. The conjugates (160 μg protein) in 0.5 ml 10 mM sodium phosphate, 150 mM NaCl, pH 7.2, were emulsified with 1.5 ml complete Freund's Adjuvant and then injected intradermally at multiple sites along the back. Additional injections of antigen (100 μg) in incomplete Freund's adjuvant were made after 10 and 12 weeks and then the animals were bled (test bleed 40 ml) after another 2 weeks. Antisera were treated at 56°C for 30 min to inactivate complement and then stored at −70°C. Control (pre-immune) sera were obtained from the rabbits before the first injection.

The antibodies were affinity purified by passing the antisera through columns of synthetic peptides immobilised on Sulfolink™ gel (Pierce, U.K.), followed by elution of the specifically bound IgG with 5 M $MgCl_2$ as described by Baldwin (1994). Using this procedure, 0.5–1.0 mg/ml serum of each affinity-purified antibody was obtained.

2.5. ELISA for screening anti-receptor peptide serum

Nunc 96-well microtiter plates (Nunclon, Denmark) were coated by addition to each well of 20 ng synthetic peptides in coating buffer (50 mM sodium carbonate buffer, pH 9.6) followed by drying down overnight in an evacuated desiccator at room temperature. The wells were then washed five times with 200 μl wash buffer (phosphate-buffered saline containing 0.05% (v/v) Tween-20 and 0.02% (w/v) sodium azide (PBS-AT)) and further incubated with 200 μl blocking buffer (PBS-AT containing 5% (w/v) milk powder) for 2 h at 37°C. The wells were washed five times with wash buffer as before and incubated with 100 μl diluted primary antibody or pre-immune serum overnight at 37°C. Dilutions of antibody or pre-immune serum were carried out with antibody buffer (PBS-AT containing 1% (w/v) milk powder). The wells were then washed five times with wash buffer as described above, and incubated with 100 μl goat anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad labs, Richmond, CA) diluted to 1:3000 in antibody buffer. The wells were again washed five times with wash buffer before being incubated with 100 μl substrate (1 mg/ml *p*-nitrophenyl phosphate (disodium salt) (Sigma) in 10 mM diethanolamine, 1 mM $MgCl_2$, pH 9.8) at room temperature. Absorbance was determined at 405 nm after 30 min using an Anthos microplate reader (Denley, U.K.).

2.6. Membrane preparation and solubilization

Ovarian tissue from pseudopregnant rats (about 100 mg) was homogenized in 1 ml PBS (10 mM sodium phosphate, 140 mM NaCl, pH 7.4) containing 5 mM EDTA (BDH) and 5 mM *N*-ethylmaleimide (Sigma, U.K.), and centrifuged at 120 × *g* for 5 min, whereafter the supernatant was further centrifuged at 27000 × *g* for 30 min. The crude membrane pellet was solubilized in 1 ml Triton X-100 (Sigma, U.K.) PBS containing 5 mM EDTA, 5 mM *N*-ethylmaleimide, and 20% (v/v) glycerol (BDH, U.K.), by stirring on ice for 30 min. The suspension was centrifuged at 100000 × *g* for 1 h at 4°C, and the resulting supernatant was concentrated using Amicon centriprep-30 concentrators (Amicon, Inc., Bevenly, MA 0195, USA) (Keinanen et al., 1987). The membranes from rat testes and liver were prepared by the same method. The solubilized membranes were then used for electrophoresis or stored at −70°C. The membranes of MA10 cells and the endothelial cell line were prepared as described previously by Hipkin et al. (1992).

2.7. Bio-Rad protein assay

The amount of protein was assayed by the Bio-Rad protein assay method. The stock solution of concentrated Bio-Rad dye (Bio-Rad Laboratories Ltd, Herts, U.K.) was diluted 1:5 in distilled water and filtered. Aliquots of solubilized membrane were diluted 1:10 with distilled water. A standard BSA curve was constructed in the range 0–1 mg/ml in distilled water. Twenty μl of standard or sample were added to 1 ml dye, mixed and incubated for 5–10 min, after which the absorbance was assessed at a wavelength of 595 nm.

2.8. Enzymatic deglycosylation

The deglycosylation of the receptors with endoglycosidase-F (*N*-glycosidase F-free; Boehringer Mannheim Biochemica, East Sussex, U.K.) was carried out according to the method described previously by Sojar and Bahl (1989).

2.9. Western blot analysis

Solubilized membrane proteins were separated by discontinuous sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) with 5% (w/v) stacking and 10% (w/v) separating gels. Aliquots of 50–100 μg protein were mixed with an equal volume of SDS sample buffer and

incubated for 15–30 min at room temperature before being applied to the gel.

Prestained low range markers of known molecular weight between 27.5 and 106 kDa (BioRad, U.K.) were used as standards. After SDS-PAGE, the resolved proteins from acrylamide gels were transblotted onto nitrocellulose Hybond-C transfer membranes (Amersham, Bucks, U.K.) using an LKB 2117 Multiphor II electrophoresis unit at a constant current of 150 mA for 90 min in transfer buffer (39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS and 5% (v/v) methanol). After transfer, each piece of nitrocellulose was placed in a 150 ml sterilin plastic bottle and washed for 10 min in wash buffer (Tris-buffered saline (TBS) 20 mM Tris, 500 mM NaCl, pH 7.5) on a roller mixer. Blocking solution (50 ml) (TBS-containing 0.2% (v/v) Tween 20 (TTBS) and 5% (w/v) milk powder) was added and incubated for 2 h rolling gently at room temperature. The membranes were then washed with wash buffer (TTBS) twice for 5 min. Primary antibody (2 μg/ml of affinity-purified antibody) or pre-immune IgG (at the same concentration) was added in antibody buffer (1% (w/v) milk powder in TTBS) and incubated overnight at room temperature. Membranes were then washed three times for 15 min with 100 ml TTBS with gentle agitation and then 30 ml antibody buffer containing 10 μl goat anti-rabbit IgG-alkaline phosphatase conjugate (1:3000) (Bio-Rad, U.K.) was incubated for 1–2 h at room temperature. The membranes were again washed 3 times with 100 ml TTBS and then further washed twice for 10 min with TBS to remove the Tween-20.

The binding of antibody to the receptor was detected using the chromagens 5-bromo-4-chloro-3-indolylphosphate (15 mg) (Sigma, U.K.) and nitroblue tetrazolium (30 mg) (Sigma, U.K.) mixed in 100 ml sodium carbonate buffer (0.1 M sodium bicarbonate, 1 mM $MgCl_2$, pH 9.8).

2.10. Dot and slot blots

Increasing amounts of proteins (100 ng to 100 μg) were immobilized on nitrocellulose membranes using a milliblot system. Proteins were reacted with antibodies and antibody binding was detected as described for the Western blots.

2.11. Iodination of hCG

Highly purified hCG (CR-127) (donated by NIADDK, NIH, Bethesda, MD, U.S.A.) iodinated with ^{125}I (Amersham, U.K.) to a specific activity of 100 Ci/g according to the lactoperoxide method of Thorell and Johanson (Thorell and Johansson, 1971) was purified by Sephadex G-25M (Pharmacia Biotech, Uppsala, Sweden) chromatography. ^{125}I-hCG was stored at −20°C and used within 4 weeks of preparation. Estimation of the specific activity of ^{125}I-hCG was carried out by competitive binding with known concentrations of purified hCG.

2.12. Ligand blot

Proteins were transferred onto nitrocellulose membranes as described for the Western blot. The membrane protein samples were rinsed in PBS and incubated for 4 h at room temperature in PBS containing 0.1% (w/v) BSA (Sigma, U.K.), 0.25% (w/v) gelatin (Sigma, U.K.), 1% (w/v) haemoglobin (Sigma, U.K.) and 10% (v/v) glycerol. Thereafter the nitrocellulose strips were incubated overnight in 5 ml PBS containing 0.1% (w/v) BSA, 10% (v/v) glycerol and 1 × 10^6 counts/min of ^{125}I-hCG in the presence or absence of excess unlabelled crude hCG (10 IU/ml). The strips were then washed in 10 ml PBS containing 0.1% (v/v) Triton X-100 for 30 min, and finally rinsed in PBS, dried, and subjected to autoradiography on Kodak X-ray film (Amersham, U.K.) at −70°C for 2–3 days (Keinanen et al., 1987). The molecular size of the receptor protein was determined by running molecular weight standards in an adjacent lane.

2.13. ^{125}I-hCG competitive binding study

Antibody and pre-immune control IgG at concentrations of 10 and 1 μg/ml were incubated in 6-well plates containing 1 × 10^6 MA10 cells/well for 1 h at 37°C in Waymouth's medium + 0.1% (w/v) BSA. Cells were then incubated for 48 h at 4°C in the presence of ^{125}I-hCG (500 000 counts/min/well). The level of binding was determined by aspirating the medium, washing twice with ice cold PBS + 0.1% (w/v) BSA to remove unbound hormone, and dissolving the cells in 0.5M NaOH before counting in a γ-counter. Non-specific binding was determined in the presence of 300 IU/ml unlabelled crude hCG.

2.14. Effects of antibodies on LH actions

MA10 cells were grown in Waymouth's medium + 15% (v/v) horse serum and were sub-cultured at a density of 1 × 10^5 cells/ml. Cells were preincubated for 1 h with and without antibodies or pre-immune IgG in medium containing 0.1% BSA at antibody concentrations ranging from 0.01 to 100 μg/ml. The cells were then challenged for a further 2 h with 10 ng/ml LH (ovine LH batch oLH-26; potency = 2.3 U/mg) (NIADDK, NIH). In a second series of experiments, cells were incubated with a constant antibody concentration (10 μg/ml), before being challenged with a range of LH concentrations (0–1000 ng/ml). The reaction was then terminated by the addition of perchloric acid (final concentration 0.5 M) and neutralized with tripotassium orthophosphate (final con-

centration 0.23 M). Progesterone and cAMP were both measured in the medium by RIA.

2.15. Radioimmunoassays

cAMP was determined by the method of Steiner et al. (1972) modified by the acetylation procedure described by Harper and Brooker (1975).

A standard curve for progesterone (Sigma, U.K.) (0.25–31.79 pmol/ml) was aliquoted (in triplicate). One hundred μl of treated medium was aliquoted for totals, non-specific binding (NSB) and zero concentration of progesterone (Bo). Samples were diluted as appropriate in treated medium to a final volume of 100 μl. One hundred μl PAS-gelatin buffer (PGB) was added to totals and NSB tubes. Antibody diluted 1:4000 in PGB (100 μl) (characterized previously for use in an EIA (Hodges et al., 1988)) was added to all tubes except totals and NSB. One hundred μl [^{3}H]progesterone (Amersham, U.K.) in PGB containing 20 000 counts/min was added to all tubes. The mixture was vortex mixed and incubated overnight at 4°C. The following day, ice-cold PGB (500 μl) was added to the total tubes. Dextran (Pharmacia Biotech Ltd, St. Albans, Herts, U.K.) -coated charcoal (BDH laboratories supplies, Merck Ltd., Lutterworth, Leicestershire, U.K.) suspension (500 μl containing 2.5 mg charcoal/ml and 0.25 mg dextran/ml in 0.01 M phosphate buffer) was added to the remaining tubes at 4°C in order to separate the free [^{3}H]progesterone from the bound. The tubes were then vortex mixed and centrifuged at 1000 $\times$ g for 10 min at 4°C. The supernatant was decanted into scintillation vials and 2 ml of scintillation fluid (Ultima Gold, Canberra Packard Ltd, Pangbourne, Berks, U.K.) was added. The vials were then vortexed and counted for 5 min in a liquid scintillation counter to determine the radioactivity.

3. Results

3.1. Antibody specificity

The specificity of the antibodies was tested by cross-reacting them with the different peptides in an ELISA. It was found that peptide 1 was only recognized by antibody 1. Similarly peptides 2 and 3 were only recognized by antibodies 2 and 3, respectively. Each antibody versus its corresponding peptide gave a specific O.D. value of 1.50 ± 0.12, 1.51 ± 0.09 and 1.52 ± 0.02 for antibodies 1, 2 and 3 respectively. The O.D. values for the antibodies cross-reacted with the alternative peptides were negligible.

3.2. Dot and slot blotting

In dot blot analyses, again there was found to be no cross-reactivity between the antibodies and the different peptides. In addition, all three antibodies bound to solubilized proteins from superovulated rat ovaries but did not interact with liver proteins (negative control) nor blank wells (Fig. 1).

In the slot blots, the three antibodies (but not the pre-immune IgG) bound to the solubilized proteins from MA10 cells as well as proteins prepared from rat testes. Again there was no reaction with liver proteins nor with proteins prepared by the same method from an endothelial cell line (Fig. 2). The partial signal seen with pre-immune IgG in Fig. 2 is an artefact because it was only present in one of the triplicates of the testicular protein.

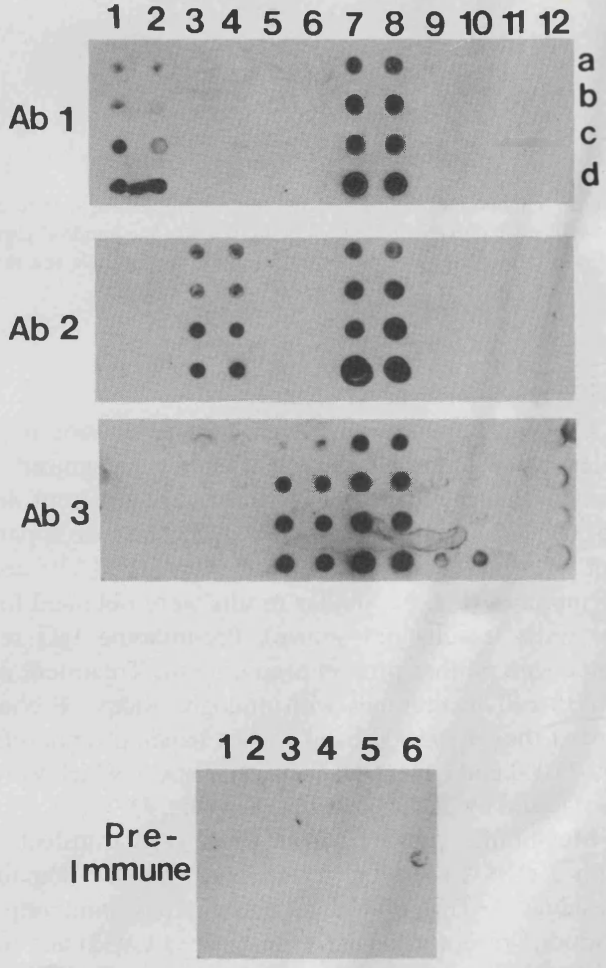

Fig. 1. Demonstration of antibody specificity by dot blotting. Dot blots containing (a) 100 ng, (b) 1000 ng, (c) 10 μg and (d) 100 μg of peptides 1 (lanes 1 and 2), 2 (lanes 3 and 4) or 3 (lanes 5 and 6), of solubilised rat ovarian membranes (lanes 7 and 8), of solubilised rat liver membranes (lanes 9 and 10) or no protein (lanes 11 and 12) were stained with the antibodies indicated (10 μg/ml) or with pre-immune IgG (10 μg/ml) and binding of the antibodies to the membranes was detected as described in Materials and methods. For the pre-immune panel, lanes 1, 2 and 3 refer to peptides 1, 2 and 3, respectively. Lanes 4 and 5 refer to ovarian and liver membranes and lane 6 contains no protein. Experiments were repeated three times with similar results for the peptides, ovarian and liver membranes.

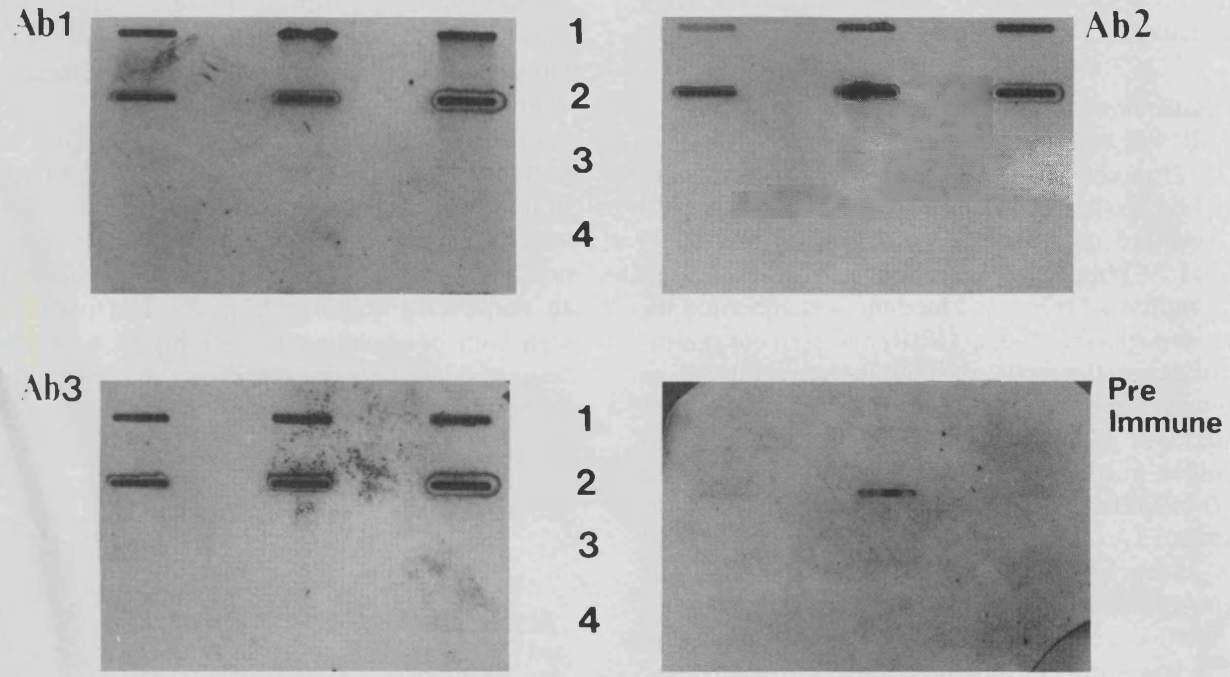

Fig. 2. Immunoslot blotting with the three antibodies against testicular membranes (row 1), MA10 cell membranes (row 2), liver membranes (row 3) and endothelial cell membranes (row 4). One hundred μg of proteins in triplicate lanes were used and the amount of primary antibody (10 μg/ml) bound was detected as described in Materials and methods. Experiments were repeated three times with similar results.

3.3. Western and Ligand blotting

On Western blots of membranes from superovulated rat ovaries, all three antibodies recognized a protein that migrated as a broad band of apparent M_r 95 000–100 000. A somewhat sharper band of apparent M_r 95 000 was labelled on blots of MA10 cell membranes (Fig. 3). Similar results were obtained for rat testis (results not shown). Pre-immune IgG reacted with neither protein preparations. Treatment of MA10 cell membranes with endoglycosidase F converted the M_r 95 000 band to two bands of apparent M_r 75 000 and 64 000, respectively, both of which were recognised by all three antibodies (Fig. 4).

Membranes prepared from CHO cells transfected with a cDNA encoding for the extracellular domain (residues 1–415) of the human thyroid stimulating hormone receptor (hTSHr) (designated ExG2) and an antibody raised to this protein (antiserum R14) were used to establish cross-reactivity with the LH receptor antibodies. Fig. 5 shows that the ExG2 protein (M_r 60 000) was recognized by the TSH R14 antibody only and not by the LH receptor antibodies 1, 2 and 3. Antibodies 1, 2 and 3, but not pre-immune IgG, only recognized the M_r 95 000 protein found in MA10 membranes, whereas the hTSHr antibody R14 failed to bind to the M_r 95 000 protein. The M_r 68 000 band obtained using the R14 antibody in both the ExG2 and MA10 membranes is a non-specific band discussed previously by Harfst et al. (1994).

A protein in MA10 cell preparations of M_r 95 000, identical to that of the band recognised by the antibodies, was also labelled by ^{125}I-hCG in ligand blotting experiments (Fig. 6). Binding of ^{125}I-hCG to this band was completely displaced by excess unlabelled hCG. Binding of hCG was also displaced by antibodies 1, 2 and 3 (10 μg/ml); the two N-terminal antibodies were more effective than the C-terminal antibody (Fig. 6). When liver membranes were used in ligand blotting there was no reaction with any of the three antibodies (data not shown).

3.4. ^{125}I-hCG competitive binding studies

In addition to their inhibition of hCG binding to the protein of M_r 95 000 on ligand blots, the antibodies also affected the binding of the hormone to intact MA10 cells (Fig. 7). At a concentration of 1 μg/ml antibody 1 inhibited specific ^{125}I-hCG binding to the cells by 76% compared to the binding measured in the presence of an equivalent concentration of pre-immune IgG. At the same concentration, neither antibody 2 or 3 had any greater effect than the pre-immune IgG. The latter itself inhibited binding to a small but significant extent relative to the IgG-free control. At the higher concentration of 10 μg/ml both antibodies 1 and 2 showed significant inhibition of binding relative to the pre-immune IgG controls, with antibody 1 showing the greater inhibition (92 and 67% inhibition, respectively.) In contrast, the effect of antibody 3 at this concentration did not differ from that of the pre-immune control (Fig. 7).

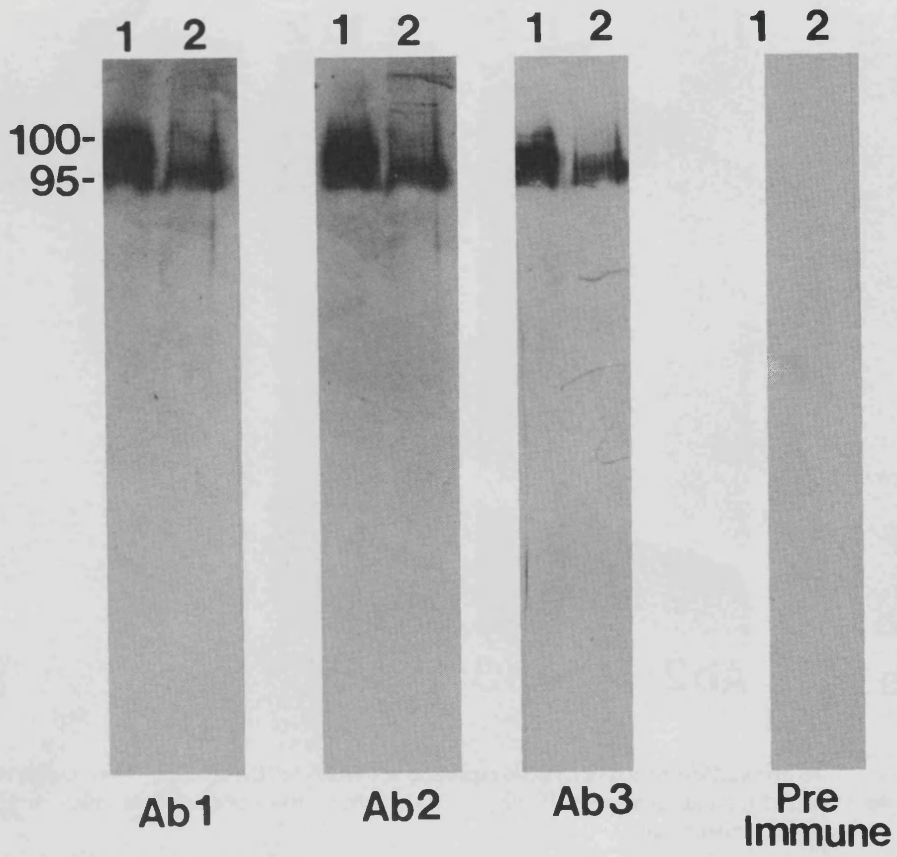

Fig. 3. Western blotting of membrane proteins obtained from superovulated rat ovarian cells (lane 1) and MA10 cells (lane 2) using the above antibodies and pre-immune IgG. Ten percent SDS-gels were used to resolve 50–100 μg membrane proteins and then transblotted onto nitrocellulose membranes. Twenty μg affinity purified primary antibody was added and binding was detected as described in Materials and methods. Experiments were repeated at least four times with similar results for the ovarian membranes and the MA10 cells.

3.5. Effects on LH stimulated cAMP and progesterone production

Antibody 1 significantly inhibited LH-stimulated cAMP and progesterone production in a dose-dependent manner (Fig. 8). At concentrations of 1 and 10 μg/ml, antibody 1 inhibited both of these responses completely. Antibody 2 only significantly inhibited the two responses at a concentration of 10 μg/ml. Anti-

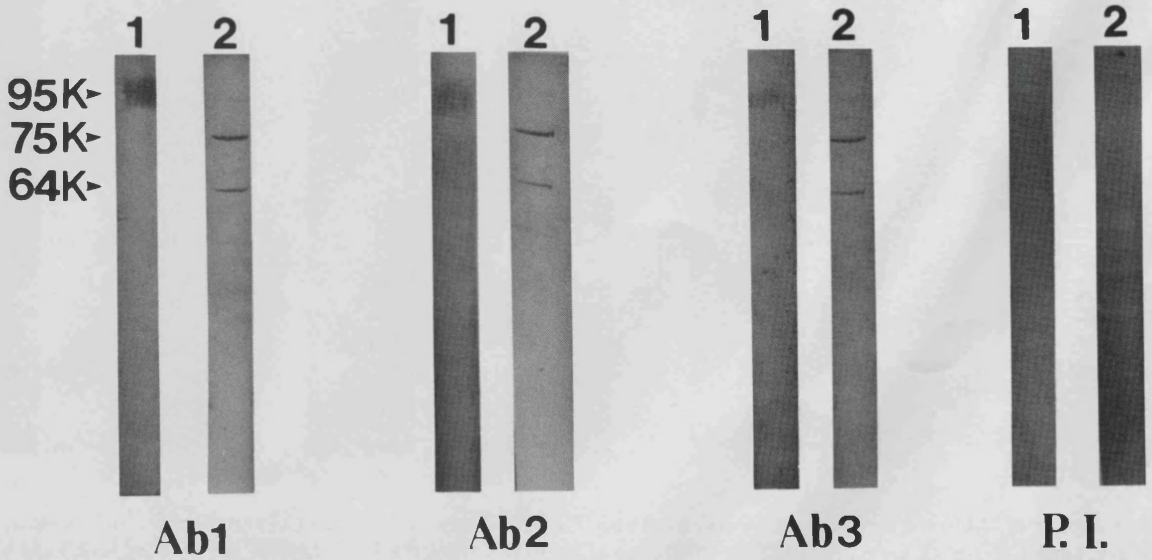

Fig. 4. Western blotting of MA10 membrane proteins (lane 1) before and after treatment with endoglycosidase F (lane 2), using the three antibodies and pre-immune IgG. Experiments were repeated three times with similar results.

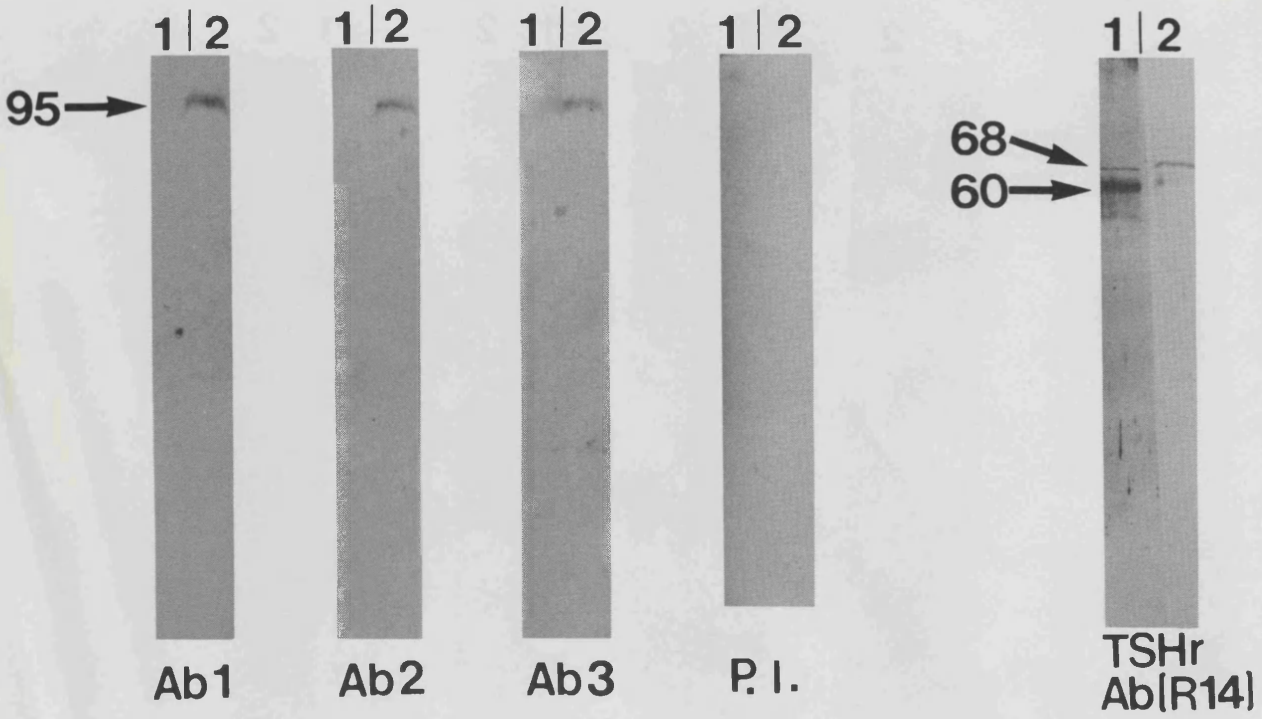

Fig. 5. Western blot analyses of the extracellular region of hTSHR expressed in CHO cells (ExG2) (lane 1) and the MA10 membranes (lane 2) with antiserum R14 and the three LH receptor antibodies. Binding of the antibodies was detected as described in Materials and methods. Experiments were repeated twice with similar results.

body 3 and pre-immune IgG had no effect. On the basis of these results, an IgG concentration of 10 μg/ml was chosen as the most appropriate for use in further investigation of the effects of the antibodies on LH action. The antibodies alone had no effect on basal progesterone production and cAMP accumulation. However, at LH concentrations of 10 ng/ml or greater, antibody 1 significantly inhibited the concen-

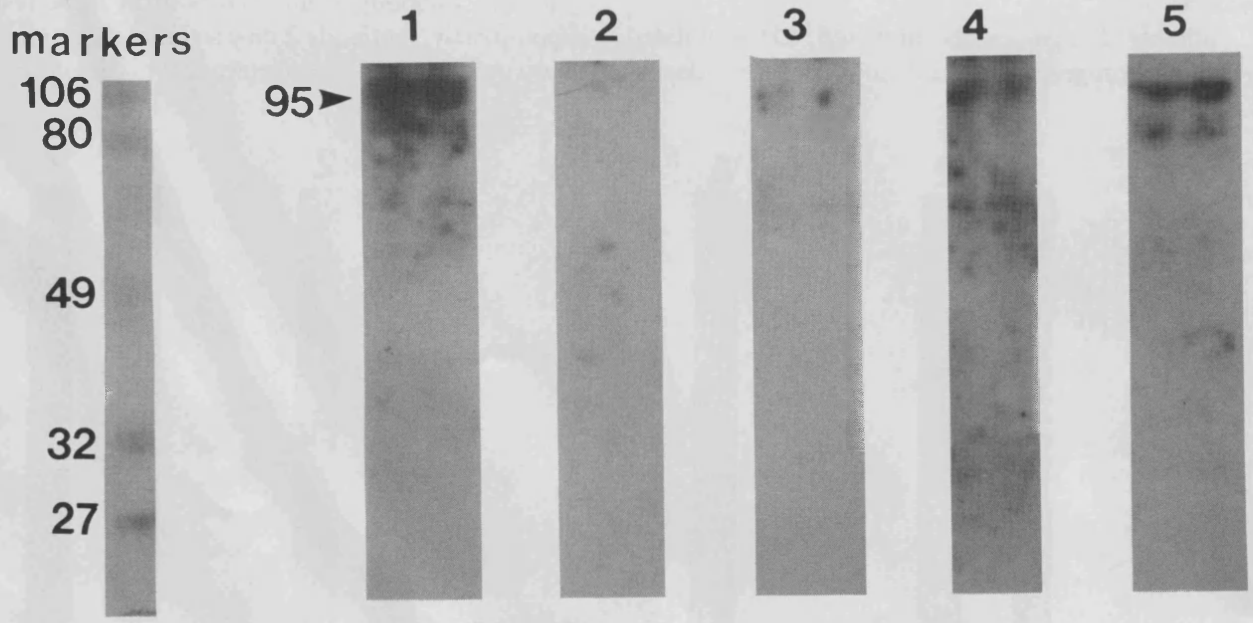

Fig. 6. Ligand blotting of MA10 cell membranes (100 μg/lane) with ^{125}I-hCG. Excess unlabelled hCG and the three antibodies were used to displace binding. Lane 1, ^{125}I-hCG. Lane 2, ^{125}I-hCG + unlabelled hCG (10 IU/ml). Lane 3, ^{125}I-hCG + Ab 1 (10 μg/ml). Lane 4, ^{125}I-hCG + Ab 2 (10 μg/ml). Lane 5, ^{125}I-hCG + Ab 3 (10 μg/ml). Experiments were repeated three times with similar results.

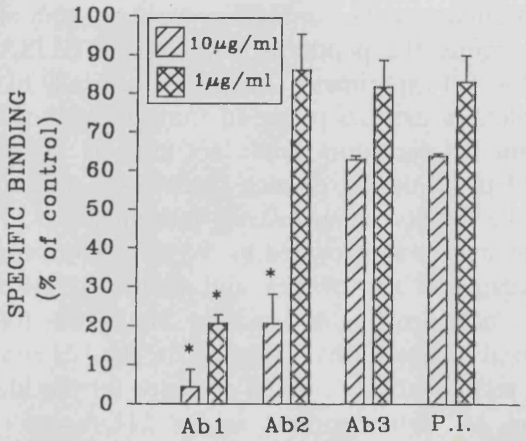

Fig. 7. The effect of antibodies on the specific binding of ^{125}I-hCG to MA10 cells. The effects of the three anti-peptide antibodies and pre-immune IgG at the concentrations indicated were measured on the binding of ^{125}I-hCG., relative to an IgG-free control. MA10 cells were plated at a density of 1×10^6 cells/well in 6-well plates and preincubated with antibody or pre-immune IgG (at 1 and 10 μg/ml) for 1 h at 37°C. ^{125}I-hCG binding was determined as described in Materials and methods. Values given are the mean ± S.E.M. of three independent experiments (triplicate determinations for each treatment in each experiment). * Indicates a significant difference ($p < 0.05$) between the effect of an antibody and that of its pre-immune control (unpaired t-test).

tration-dependent stimulation of both cAMP and progesterone production by LH (Fig. 9). The extents of inhibition were relatively constant at about 81.7 ± 4.8% (mean ± S.E.M.) and 74.7 ± 6.3%, respectively, for LH concentrations of 10–1000 ng/ml. In contrast, neither antibodies 2 or 3, nor pre-immune IgG, had a significant effect on progesterone or cAMP production at any LH concentration tested. When dbcAMP, cholera toxin and forskolin were used instead of LH, there was no inhibition of cAMP or progesterone production with any of the antibodies (data not shown).

4. Discussion

The first report on the production of antibodies against the LH/CG receptor was published over 15 years ago (Luborsky and Behrman, 1979). In this study, these antibodies bound the hCG-receptor complex solubilized from rat luteal tissue, as well as rat testes, but did not recognize the hCG-receptor complex solubilized from ovine or human ovaries. In addition these antibodies did not inhibit the binding of hCG to rat luteal membranes or intact luteal cells, although they inhibited hCG-stimulated progesterone production in these cells. The titre of these antibodies was also reported to be very low.

Metsikko and Rajaniemi (1981) reported raising antibodies against a partially purified LH receptor preparation. These antibodies did not bind the hCG-receptor complex, but were able to inhibit the binding

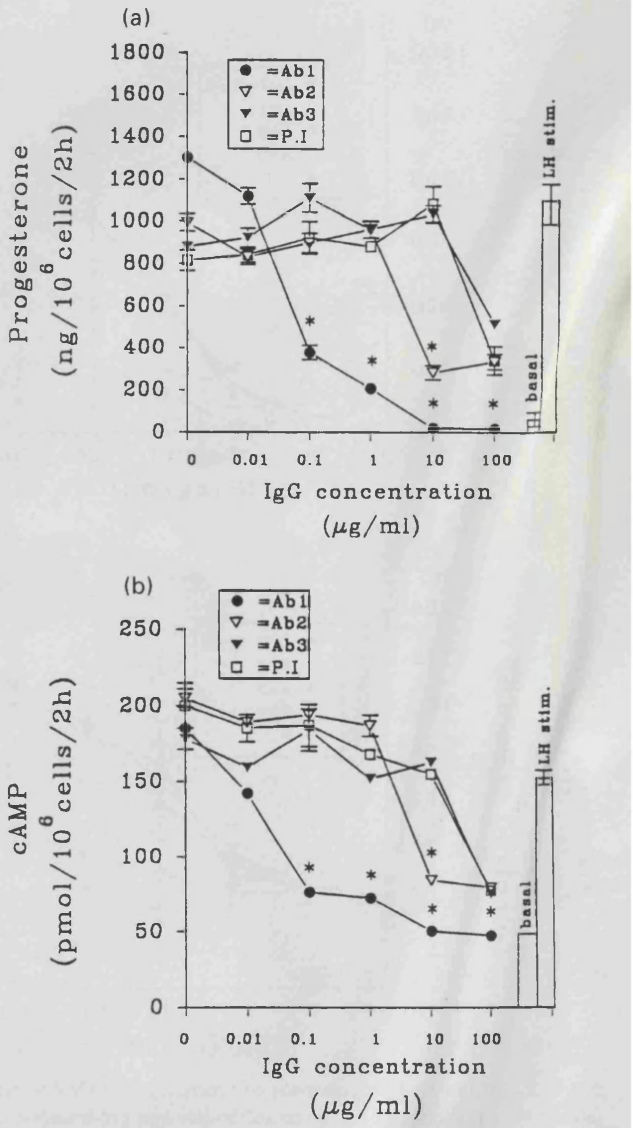

Fig. 8. Dose-dependant effects of the antibodies on LH-stimulated progesterone production and cAMP accumulation by MA10 cells. MA10 cells were plated at a density of 1×10^5 cells/ml in 96-well plates and preincubated for 1 h with and without antibodies or pre-immune IgG at concentrations ranging from 0.01 to 100 μg/ml. The cells were then challenged for 2 h with 10 ng/ml LH. Reactions were stopped by the addition of perchloric acid and assayed for cAMP and progesterone by RIA as stated in Materials and methods. The open bars show basal and maximal LH-stimulated values obtained in the absence of IgG. Values given are the mean ± S.E.M. of four independent experiments (triplicate determinations for each treatment in each experiment). * Indicates a significant difference ($p < 0.05$) between the effect of an antibody and that of its pre-immune control (unpaired t-test).

of hCG to homogenates of ovarian tissue. The same authors have also reported the production of antibodies raised against an affinity-purified preparation of the rat luteal LH/CG receptor (Metsikko and Rajaniemi, 1984). These were shown to bind the hCG-receptor complex, and to inhibit the binding of hCG to a homogenate of rat luteal tissue. These antibodies also recognized an M_r 90000 band in

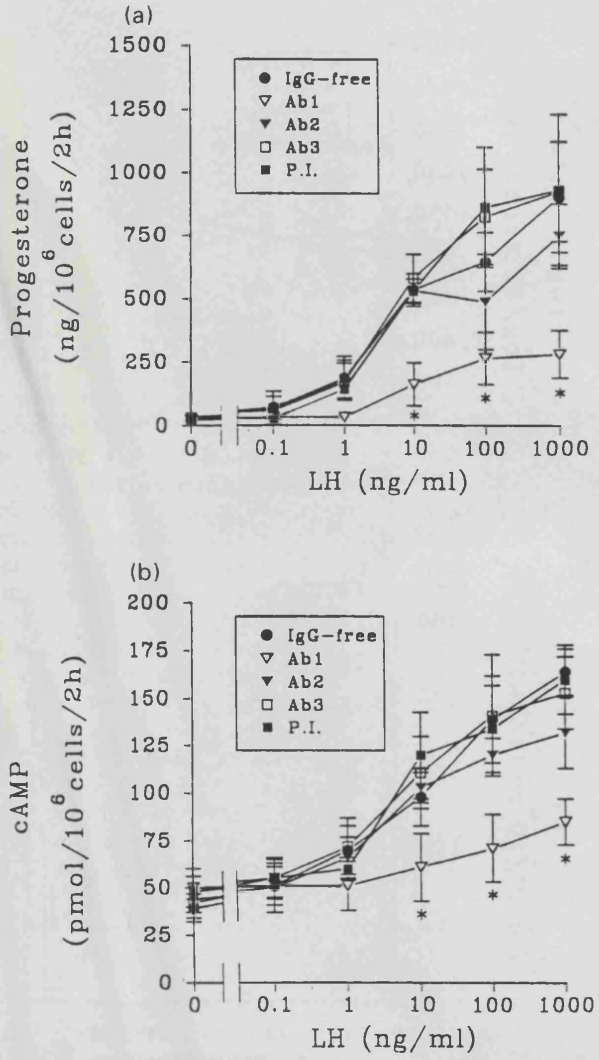

Fig. 9. LH-stimulated progesterone production and cAMP accumulation in the presence of the three antibodies and pre-immune IgG. MA10 cells were plated at a density of 1×10^5 cells/ml in 96-well plates and preincubated for 1 h with and without antibodies or pre-immune IgG at a concentration of 10 μg/ml and then stimulated for 2 h with a range of LH concentrations (0–1000 ng/ml). Reactions were stopped by the addition of perchloric acid and assayed for cAMP and progesterone by RIA as stated in Materials and methods. Values given are the mean ± S.E.M. of four independent experiments (triplicate determinations for each treatment in each experiment). *Indicates a significant difference ($P < 0.05$) between the effect of an antibody and that of its pre-immune control (unpaired t-test).

Western blots of the affinity purified rat ovarian LH/CG receptor. Rosemblit (1988) also produced polyclonal antibodies against the rat luteal LH/CG receptor. These antibodies recognized an M_r 93 000 single band on Western blots of partially purified rat luteal receptor, but failed to inhibit the binding of hCG to intact rat luteal cells or to detergent extracts thereof.

In this study we report the production and characterization of antibodies that bind to the LH receptor and affect both its binding of LH/CG and its signalling function. The antibodies were not only shown to recognise the peptides themselves in ELISA and the dot-blot experiments, but also specifically to stain slot blots of extracts prepared from tissues known to contain LH receptors, while not staining tissues assumed to be devoid of such receptors. Confirmation that the antibodies specifically recognise the LH receptor itself was provided by Western blots of both superovulated rat ovaries and mouse MA10 cells, where only proteins of apparent M_r 95 000–100 000, identical in size to that reported for the LH receptor were recognised. Additional evidence for the identity of the M_r 95 000 protein as the LH receptor was provided by its ability to bind ^{125}I-hCG in ligand blot experiments. The differences in mobility seen for the bands recognised by the antibodies on the Western blots of the rat and mouse tissues are probably attributable to differences in the N-linked glycosylation pattern in ovary and testis, as discussed previously by Minegishi et al. (1989). The two bands observed after treatment with endoglycosidase-F (M_r 75 000 and M_r 64 000) are in agreement with the results obtained previously by Sojar and Bahl (Sojar and Bahl, 1989). This also suggests that the antibodies recognize the receptor regardless of the sugar residues, i.e., the carbohydrate residues do not interfere with the binding of the antibodies, and the fact that the C-terminal antibody binds to the M_r 64 000 fragment confirms that the latter is not the extracellular domain of the receptor. The specificity of the antibodies was also confirmed in the cross-reaction Western blot experiment using the hTSH receptor protein (ExG2) and antibody R14. The three LH receptor antibodies 1, 2 and 3 failed to identify the M_r 60 000 hTSH receptor extracellular protein, but only reacted with the M_r 95 000 protein in the adjacent lane containing the MA10 membranes. Similarly antibody R14 bound specifically only to the M_r 60 000 protein from the ExG2 protein but not with the LH receptor found in the MA10 membranes.

The ability of antibodies 1 and 2 to inhibit ^{125}I-hCG binding is consistent with the fact that the N-terminus of the LH receptor includes the ligand binding domain. Similarly, the lack of effect of antibody 3 is consistent with the C-terminal region of the receptor being intracellular. The observation that the C-terminal antibody and pre-immune IgG have a small effect on ^{125}I-hCG binding indicates non-specific protein interference between the ligand and the receptor. Antibody 1 inhibited both LH stimulated cAMP accumulation and steroidogenesis illustrating that this antibody binds to a region of the N-terminus that affects both ligand binding and signal transduction, and also shows that this antibody is more potent than antibody 2 which is also raised to recognize a se-

quence in the N-terminus of the receptor. There was no inhibition when dbcAMP, cholera toxin and forskolin were used to stimulate replacing LH, suggesting that the inhibition of LH action is occurring at the ligand-receptor step. According to the study by Roche et al. (1992) which used a series of overlapping synthetic peptides to the extracellular domain of the LH/CG receptor to identify the hormone-binding regions, neither antibodies 1 nor 2 would be predicted to interact with those regions of the N-terminus implicated in ligand binding. However antibody 1 has been raised to a peptide sequence (residues 48–65) which is closer to a putative region involved in ligand binding (residues 21–41) than the peptide sequence used to raise antibody 2 (residues 187–206). Therefore, it is probable that the binding of antibody 1 decreases ligand binding and action via steric hinderance and/or induction of conformational changes in the hormone binding domain.

In conclusion, the antibodies developed in this study recognize the LH receptor from both the rat testis, the rat ovary, and mouse tumour MA10 cells, and are sensitive enough to detect normal levels of LH receptor expression (i.e., they do not require overexpression of the receptor in a heterologous expression system nor stimulation of overexpression in gonadal cells). In addition to this, the failure of the three antibodies to identify the G2 (hTSHr extracellular domain) indicates that these antibodies are specific for the LH receptor. They will therefore be invaluable for the study of trafficking of the LH receptor and the cellular mechanisms via which LH regulates both testicular and ovarian steroidogenesis.

Acknowledgements

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