MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 6 July 2004.....
....
Sec. Research Graduate School Committee

Pharmacological characterisation of relaxin and the relaxin receptor

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This thesis is presented to the
Faculty of Medicine, Monash University
for the degree of

Doctor of Philosophy

Department of Pharmacology
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February 2004

Abstract

The work presented in this thesis entitled "Pharmacological characterisation of relaxin and the relaxin receptor" investigated the pharmacological interaction of various relaxin analogues with the human relaxin receptor expressed (i) endogenously in THP-1 cells, (ii) recombinantly in CHO K1 and HEK293 cells, and (iii) physiologically in uterine tissue. Investigation of the structural interaction between HRLX and the relaxin receptor involved the development of a method for the recombinant production of relaxin peptides, which in conjunction with the use of bioassays utilising human receptors, would allow determination of features important for relaxin activity.

Chapter One provides a general overview of relaxin, including the history of relaxin discovery and isolation, the structure-activity relationships and physiological characterisation of relaxin in various species, and the identification of the relaxin receptor. Chapter Two details the methods used in this thesis to investigate the interaction between relaxin and the human relaxin receptor.

The interaction between the human relaxin receptor endogenously expressed in THP-1 cells and various relaxin and INSL3 analogues is described in Chapter Three. Relaxin-like affinity was determined using a high through-put binding assay and the selective relaxin radioligand, [^{33}P]-H2RLX. Affinity for the relaxin receptor was influenced by changes in the structural-integrity of the relaxin peptide and the presence of the relaxin binding motif along the alpha helix of the B chain. The results demonstrated distinct differences in the interaction between the relaxin analogues and the human versus the rat relaxin receptor. To investigate novel areas within human relaxin that influence the affinity for the relaxin receptor, a mammalian recombinant peptide expression system was developed, described in Chapter Four. Although it appeared that the recombinant relaxin peptide was successfully synthesised, isolation of the protein failed, halting further peptide production.

Chapter Five confirmed the identity of the putative relaxin receptor, LGR7. Binding and cAMP assays analysing the interaction between various relaxin analogues and

the human LGR7 receptor transiently expressed in CHO K1 cells, correlated with previous studies at the relaxin receptor expressed in THP-1 cells. Chapter Six investigated the interaction between relaxin analogues and the LGR7 and LGR8 receptors stably expressed in HEK293 cells. While all relaxin analogues could interact with LGR7, they selectively interacted with LGR8. The pharmacological interaction between relaxin and the two receptors also differed, with evidence suggesting binding to a single population of sites at LGR7 and binding to two sites on LGR8.

Chapter Seven provides evidence for a specific role for relaxin in human reproduction. Relaxin receptor binding studies and examination of gene expression in human uterine tissue showed a distinct cyclic expression of the receptor, peaking during the early-mid secretory phase. This information coupled with known circulating levels of relaxin peptide suggests a role for relaxin in implantation.

The contribution of the results presented in this thesis to the existing literature is discussed in Chapter Eight. Overall, this thesis has extended the knowledge of the interaction between relaxin and the human relaxin receptor, shown distinct differences in the ability of relaxin analogues to interact at LGR7 and LGR8 and provided further evidence supporting a function of relaxin in human reproduction.

Declaration

I declare that the contents of this thesis have not previously been accepted for the award of any other degree or diploma in any other university or institution. Furthermore, this thesis contains no material previously published by another person except where due reference is made.

I certify that the writing of this thesis with the results, interpretation, opinions and suggestions presented are entirely my own work.

This thesis is less than 100,000 words, excluding tables, figure legends and references.

Courtney Peta Judkins

February 2004

Acknowledgments

I would like to take this opportunity to thank those who gave their support, encouragement and most importantly, their friendship during the course of this thesis.

Firstly the boss, Roger, for never locking his door. Your time and patience was greatly appreciated. To Geoff, my home away from home supervisor, thank you for always smiling and your endless encouragement. To my past and present lab members, I have had a ball sharing the last four years with you. Thank you for your support, friendship and laughter. Thanks also to all the members of the Monash Pharmacology department, especially Emma for her support and friendship, and Dan for his endless assistance, even when it was inconvenient for him.

To my family and friends for always being there, and to my parents for the never-ending support and encouragement you have provided for me, I would not have accomplished as much without you.

And to my gorgeous husband Adam, you are my rock, this is yours as much as it is mine. Here's to us!

Publications

During the course of this research, the following publications have been published or submitted for publication. The publications which contain work not related to or not included in this thesis are denoted by (*).

Papers

*Mathieu, M.N., Wade, J.D., Catimel, B., Bond, C.P., Nice, E.C., Summers, R.J., Otvos Jr, L. & Tregear G.W. (2001). Synthesis, conformational studies and biological activity of N^{α}-*mono*-biotinylated rat relaxin. *Journal of Peptide Research*, **57**, 374-382.

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*Tan, Y.Y., Dawson, N.F., Kompa, A.R., Bond, C.P., Claasz, A.A, Wade, J.D., Tregear, G.W. & Summers, R.J. (2002). Structural requirements for the interaction of sheep insulin-like factor 3 with relaxin receptors in rat atria. *European Journal of Pharmacology*, **457**, 153-160.

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* Mathieu, M.N., Bond, C.P., Wade, J.D., Summers, R.J. & Tregear, G.W. (2001). N^{α}-*mono*-biotinylated rat relaxin: synthesis and activity. In: *Relaxin 2000: Proceedings of the Third International Conference on Relaxin & Related Peptides.* eds Tregear, G.W., Ivell, R., Bathgate, R.A. & Wade, J.D. p 239-240. Netherlands: Kluwer Academic Publishers.

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Abbreviations

ATP	adenosine 5'-triphosphate
BDM	2,3-butanedione monoxime
BHK cells	baby hamster kidney cells
bp(s)	base pair(s)
BSA	bovine serum albumin
cAMP	cyclic AMP
CHO-K1 cells	chinese hamster ovary cells
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FBS	foetal bovine serum
H1RLX	human gene 1 relaxin
H2RLX	human gene 2 relaxin
H3RLX	human gene 3 relaxin
HBS	hepes buffer saline
HEK293T cells	human embryonic kidney cells
IBMX	3-isobutyl-1-methylxanthine
INSL3	insulin 3
LGR7	leucine rich-repeat g-protein coupled receptor 7
LGR8	leucine rich-repeat g-protein coupled receptor 8
OVLT	organum vasculosum of the lamina terminalis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethylenimine
PMSF	phenylmethylsulphonyfluoride
PPS marker	precision protein standard marker
PRLX	porcine relaxin
RatRLX	rat gene 1 relaxin
Rat3RLX	rat gene 3 relaxin

RLX	relaxin
RMRLX	rhesus monkey relaxin
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TBS	tris buffered saline
TBS-T	TBS tween 20
TFA	trifluroacetic acid
THP-1 cells	human monocyte cell line
vmDTg	ventromedial dorsal tegmental nucleus

Chapter One

General Introduction

1.1 History of relaxin

In the early 1920's, F.L. Hisaw investigated the physiological changes that occur to the pubic symphysis during pregnancy in guinea pigs, including the relaxation of the pubis and the increase of connective tissue at the symphysis which caused separation of the bones. He reported that when virgin female guinea pigs, close to oestrus, were injected with serum taken from pregnant rabbits, there was relaxation of the pubic ligaments similar to that naturally occurring in pregnancy (Hisaw, 1926). The substance responsible for the relaxation response could be obtained from rabbit placenta and from the corpora lutea of sows, and was found to work in conjunction with oestrin (oestrogen). In 1930, a crude extract of this substance was extracted from the corpus lutea of sows, identified as a hormone, and given the name "Relaxin" (Fevold *et al.*, 1930).

Over the subsequent several decades, interest in relaxin lessened due to limited resources for the purification and testing of biological extracts. During this time however, there were several important findings made with impure porcine relaxin extracts. Relaxin was shown to inhibit spontaneous contractions of oestrogen-primed guinea pig myometrium (Krantz *et al.*, 1950) and to promote elongation of the interpubic ligament (Hall, 1947), identifying relaxin as a key hormone of pregnancy.

In the early 1970's, the successful purification of porcine relaxin (PRLX) from frozen sow ovaries was reported (Sherwood *et al.*, 1974), with the extract displaying strong relaxin-like biological activity in the mouse interpubic ligament bioassay (see Section 1.3.1.2). This led to the isolation and purification of relaxin from the ovaries, corpora lutea or placentae of various pregnant animals, including rats, sharks, whales, horses, dogs and rabbits (Sherwood, 1994), and the cloning of human relaxin in the 1980's (Hudson *et al.*, 1983).

1.2 Characterisation of Relaxin

1.2.1 Structure and molecular characterisation of relaxin

Early modelling of the relaxin structure, predicted from purified PRLX, indicated that the peptide was comprised of two polypeptide chains, an A chain and a B chain of 22 and 32 amino acids respectively (Schwabe *et al.*, 1976; Schwabe *et al.*, 1977a) with a molecular weight of around 6000 Daltons. The two chains were linked via two disulphide bridges, with an intra-chain disulphide bridge in the A-chain. When relaxin was first discovered, it shared very similar structural properties to insulin (Fevold *et al.*, 1930). Although PRLX contained only 11 residues that were identical to porcine insulin, the preservation of the hydrophobic residues and the position of the disulphide bridges was consistent with the structure of porcine insulin (Bedarkar *et al.*, 1977; Schwabe *et al.*, 1977b). This strengthened the suggestion that relaxin had structural homology with insulin and insulin-like growth factors (IGFs), and led to the successful modelling of relaxin into an insulin-like tertiary structure (Bedarkar *et al.*, 1977; Isaacs *et al.*, 1978) (Figure 1.1). The hypothetical structure was confirmed by an x-ray crystal structure of human relaxin (Eigenbrot *et al.*, 1991). While the x-ray crystal structure of relaxin highlighted that like insulin relaxin crystallises into a dimer, the orientation of the dimers and binding interfaces were completely different therefore suggesting that similarities in structure do not necessarily lead to similarities in receptor binding mechanisms.

With a similar structure to insulin, it was predicted that relaxin would have a precursor protein resembling pro-insulin (Snell *et al.*, 1975). Prepro-relaxin was shown by nucleotide sequence analysis of the cDNA to consist of a signal peptide, B-chain, connecting peptide and A-chain (Reddy *et al.*, 1992). The biosynthetic pathway of relaxin involves prepro-relaxin undergoing enzymatic cleavage by prohormone convertase (Layden *et al.*, 1996) of the signal peptide as it is translocated across the endoplasmic reticulum to produce pro-relaxin. Pro-relaxin is further cleaved at the A-chain/connecting peptide and B-chain/connecting peptide junctions to form relaxin (Reddy *et al.*, 1992).

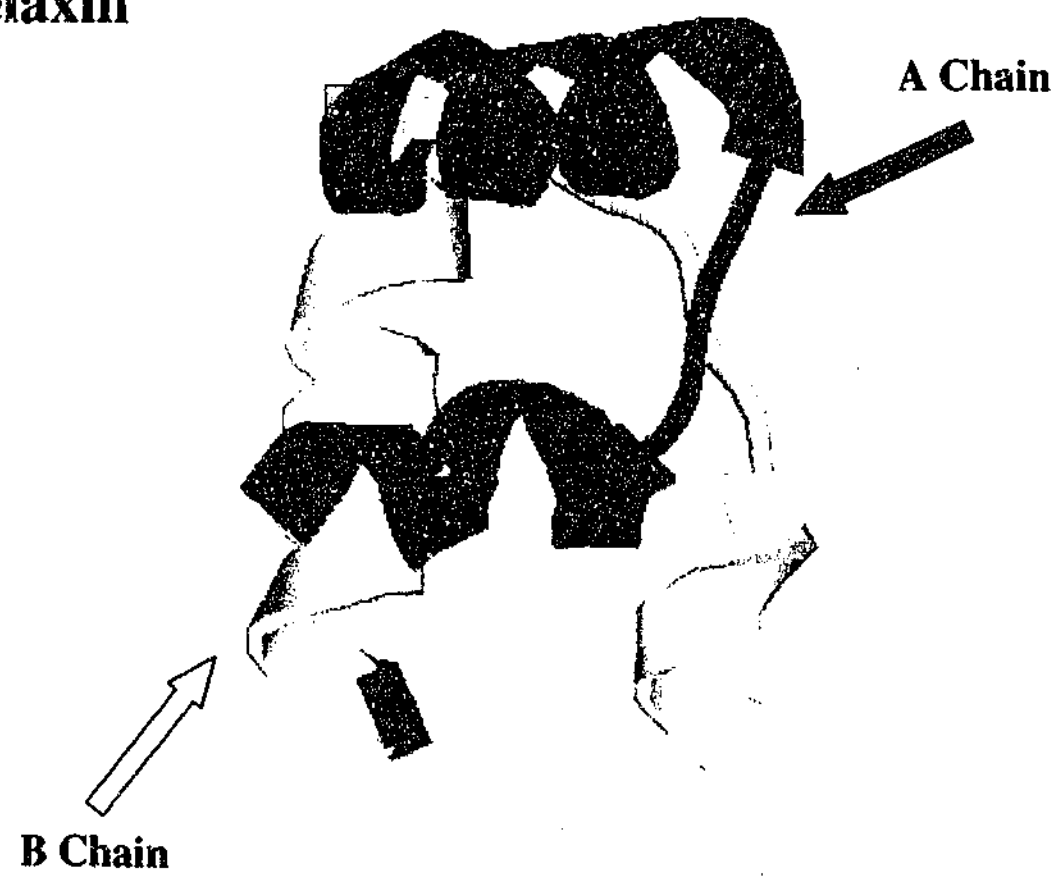
Figure 1.1

Schematic structure of human insulin and human relaxin peptides.

As with all members of the insulin gene superfamily, the insulin and relaxin peptides are comprised of two separate peptide chains, and A and a B chain.

Insulin

Relaxin



1.2.2 Relaxin and the insulin gene superfamily

Although the active form of relaxin in all species contains the A & B chain configuration, there is a large range of peptide sequence diversity between species homologues of relaxin. However the cysteine residues forming the disulphide bonds linking the two chain structure are highly conserved (Figure 1.2). Since the original cloning of the human relaxin gene in the 1980s (Hudson *et al.*, 1983; Hudson *et al.*, 1984), it was known that the human genome expresses two relaxin genes, human gene 1 (H1RLX) and human gene 2 (H2RLX) relaxin. The H2RLX peptide is predominantly expressed in the ovaries (Hudson *et al.*, 1984) but can also be locally expressed in a variety of other tissues, including mammary glands (Bongers-Binder *et al.*, 1991; Eddie *et al.*, 1989) and the prostate gland (Sokol *et al.*, 1989). The distribution of tissues expressing H2RLX mRNA however, is greater still than those that express the relaxin protein, and includes the cardiovascular and central nervous systems (Section 1.4). The expression and relevance of H1RLX is unclear although it is biologically active (Tan *et al.*, 1998; Wade *et al.*, 1996). However it was recently discovered that the human genome contained a third relaxin (Bathgate *et al.*, 2002), human gene 3 relaxin (H3RLX) and the expression and activity of this peptide is still under investigation. Until 2002, it was thought that most species only expressed one relaxin gene, equivalent to H2RLX, with only primates such as chimpanzee, gorilla, orang-utan and humans expressing two relaxin genes, equivalent to H1RLX and H2RLX. With the isolation of H3RLX came the discovery of an equivalent gene in mice and rats, termed M3RLX (Bathgate *et al.*, 2002) and Rat3RLX (Burazin *et al.*, 2002) respectively due to their homology with H3RLX.

Relaxin is a member of the insulin gene superfamily due to the structural similarities to insulin and insulin-like growth factors (Figure 1.3). The human insulin gene superfamily is made up of a growing number of hormones that regulate cell growth and metabolism, including insulin, insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), Leydig cell insulin-like protein (Ley-IL or INSL3), early placenta insulin-like peptide (EPIL or INSL4), insulin-like peptide 5 (INSL5), testis-

derived relaxin-like factor (INSL6) and the relaxin peptides (H1, H2 & H3RLX). The insulin-like peptides have structural features such as highly conserved cysteine residues that form disulphide bonds between the two peptide chains (Figure 1.3), and are essential for cell growth and differentiation, development and energy regulation.

INSL3, also known as relaxin-like factor (RLF), is the member of the insulin-gene superfamily most closely related to relaxin. The close relationship between INSL3 and relaxin is of interest since ruminants, such as sheep and cows, do not produce an active relaxin due to the numerous stop codons in the sequence corresponding to the C-peptide region (Roche *et al.*, 1993) yet respond to relaxin treatment (Porter *et al.*, 1981) indicating the presence of a receptor that can recognize relaxin. It has been suggested that ruminant INSL3 is a replacement for relaxin as INSL3 is produced in the ovary of ruminants, similar to relaxin in other mammalian species, although there has been no confirmation of this suggestion (Bathgate *et al.*, 2001).

Figure 1.2

The amino acid sequences of species homologues of relaxin.

A comparison of the amino acid sequence from a variety of species homologues of the relaxin peptide. The cysteine (C) residues that form the disulphide bonds (blue) are aligned and highlighted (pink). The arginine (R) and isoleucine (I) residues important for receptor binding (green) are situated in the B-chain.

A Chain

	1					5					10					15					20				
H1RLX	R	P	Y	V	A	L	F	E	K			L	I	G		T	K	R	S	L	A	K	Y	
H2RLX	Q	L	Y	S	A	L	A	N	K			H	V	G		T	K	R	S	L	A	R	F	
H3RLX	D	V	L	A	G	L	S	S	S			K	W	G		S	K	S	E	I	S	S	L	
Porcine			R	M	T	L	S	E	K			Q	V	G		I	R	K	D	I	A	R	L	
Rat 1	Z	S	G	A	L	L	S	E	Q			H	I	G		T	R	R	S	I	A	K	L	
Rat 3	D	V	L	A	G	L	S	S	S			E	W	G		S	K	S	Q	I	S	S	L	
Mouse 1	E	S	G	G	L	M	S	Q	Q			H	V	G		S	R	R	S	I	A	K	L	Y
Mouse 3	D	V	L	A	G	L	S	S	S			E	W	G		S	K	S	Q	I	S	S	L	
Guinea pig	Z	L	D	M	T	V	S	E	K			Q	V	G		T	R	R	F	I	A	N	S	
Horse				Z	L	S	H	K				Y	W	G		T	R	K	E	L	A	R	Q	
Rabbit	Z	F	S	E	S	L	P	E	E			K	Y	G		P	R	Y	Y	L	L	M	Y	
Rhesus monkey	Z	L	Y	M	T	L	S	N	K			H	I	G		T	K	K	S	L	A	K	F	
Gorilla 1	R	P	Y	V	A	L	F	E	K			L	I	G		T	K	R	S	L	A	K	Y	
Gorilla 2	Z	L	Y	S	A	L	A	N	K			H	V	G		T	K	R	S	L	A	R	F	

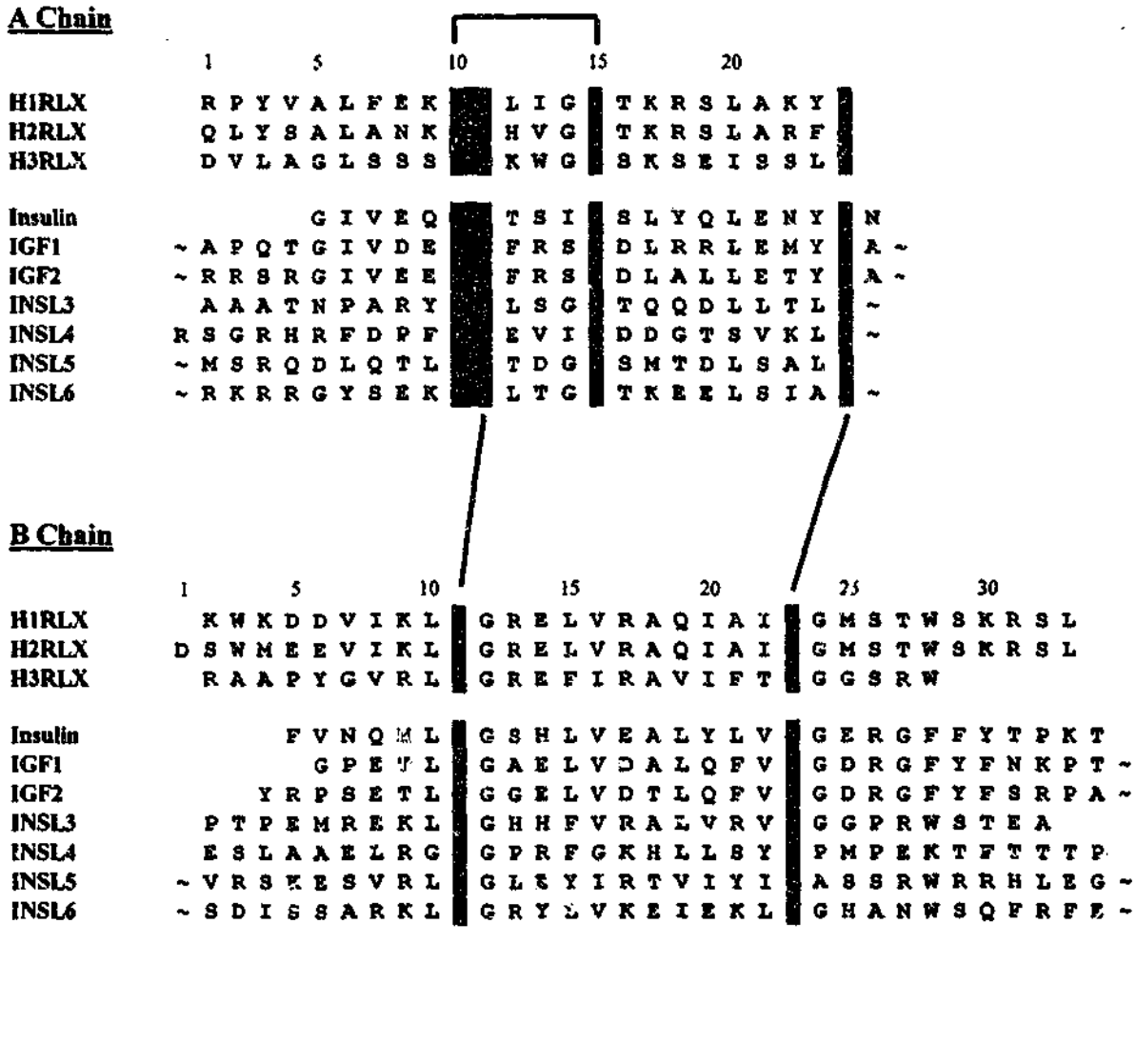
B Chain

	1					5					10					15					20					25					30				
H1RLX			K	W	K	D	D	V	I	K	L		G		E	L	V		A	Q		A	I		G	M	S	T	W	S	K	R	S	L
H2RLX	D	S	W	M	E	E	V	I	K	L			G		E	L	V		A	Q		A	I		G	M	S	T	W	S	K	R	S	L
H3RLX			R	A	A	P	Y	G	V	R	L		G		E	F	I		A	V		F	T		G	G	S	R	W					
Porcine						Q	S	T	N	D	F	I	K	A		G		E	L	V		L	W	V	E	I		G	S	V	S	W	G	R	T	A	L
Rat1	R	V	S	E	E	W	M	D	Q	V	I	Q	V		G		G	Y	A		A	W		E	V		G	A	S	V	G	R	L	A	L	
Rat3						R	P	A	P	Y	G	V	K	L		G		E	F	I		A	V		F	T		G	G	S	R	W				
Mouse1	R	V	S	E	E	W	M	D	G	F	I	R	M		G		R	Y	A		E	L		K	I		G	A	S	V	G	R	L	A	L	
Mouse3						R	P	A	P	Y	G	V	K	L		G		E	F	I		A	V		F	T		G	G	S	R	W				
Guinea pig						G	F	L	D	K	V	I	K	V		G		D	L	V		I	K		D	I		G	K	I	L	L	G	D	M	T	T	G
Horse						Q	K	P	D	D	V	I	K	A		G		E	L	A		L	R		E	I		G	S	L	S	W	K				
Rabbit	R	V	T	Y	E	W	M	M	E	N	V	K	I		R	N	D	F	V		T	A		E	V		G	H	V	H	L	E	R			
Rhesus Monkey				A	K	W	M	D	D	V	I	K	A		G		E	L	V		A	Q		A	I		G	K	S	T	L	G	K	R	S	L
Gorilla 1				A	K	W	K	D	D	V	I	K	L		G	C	E	L	V		A	Q		A	I		G	M	S	T	W	S	K	R	S	L
Gorilla 2				D	S	W	K	D	D	V	I	K	L		G		E	L	V		A	Q		A	I		G	M	S	T	W	S	K	R	S	L

Figure 1.3

The amino acid sequences of the human insulin-gene super family.

All members of the human insulin-gene superfamily contain an identical positioning of the cysteine (C) residues (pink) that form the connecting disulphide bond (blue). The ~ indicates the sequence is still continuing.


A Chain
1 5 10 15 20
H1RLX R P Y V A L F E K L I G T K R S L A K Y
H2RLX Q L Y S A L A N K H V G T K R S L A R F
H3RLX D V L A G L S S S K W G S K S E I S S L
Insulin G I V E Q T S I S L Y Q L E N Y N
IGF1 ~ A P Q T G I V D E F R S D L R R L E M Y A ~
IGF2 ~ R R S R G I V E E F R S D L A L L E T Y A ~
INSL3 A A A T N P A R Y L S G T Q Q D L L T L ~
INSL4 R S G R H R F D P F E V I D D G T S V K L ~
INSL5 ~ M S R Q D L Q T L T D G S M T D L S A L
INSL6 ~ R K R R G Y S E K L T G T K E E L S I A ~
B Chain
1 5 10 15 20 25 30
H1RLX K W K D D V I K L G R E L V R A Q I A I G M S T W S K R S L
H2RLX D S W M E E V I K L G R E L V R A Q I A I G M S T W S K R S L
H3RLX R A A P Y G V R L G R E F I R A V I F T G G S R W
Insulin F V N Q H L G S H L V E A L Y L V G E R G F F Y T P K T
IGF1 G P E T L G A E L V D A L Q F V G D R G F Y F N K P T ~
IGF2 Y R P S E T L G G E L V D T L Q F V G D R G F Y F S R P A ~
INSL3 P T P E M R E K L G H H F V R A L V R V G G P R W S T E A
INSL4 E S L A A E L R G G P R F G K H L L S Y P M P E K T F T T T P
INSL5 ~ V R S K E S V R L G L E Y I R T V I Y I A S S R W R R H L E G ~
INSL6 ~ S D I S S A R K L G R Y L V K E I E K L G H A N W S Q F R F E ~

1.2.3 Sources of relaxin

The primary source of relaxin synthesis varies slightly between species, being the corpus luteum in rodents, pigs, cows and humans, and the placenta in cats, dogs, horses and rabbits (Sherwood, 1994), with the highest levels detected during pregnancy. Local synthesis of relaxin has been identified in other reproductive tissues, including ovaries, uterus and mammary glands, as well as in a variety of non-reproductive tissues (see Section 1.4) suggesting a number of roles for relaxin.

1.2.3.1 Extraction of native relaxin

Early methods of extraction of relaxin from sow corpora lutea involved acid alcohol extraction, sodium chloride crystallisation and precipitation with picric acid (Fevold *et al.*, 1930), to yield approximately 30 mg of roughly purified hormone per kg corpora lutea. Forty years later developments in technology allowed highly purified PRLX to be prepared. This involved extraction and precipitation of protein from sow ovaries with acid-acetone, gel filtration on a Sephadex G-50 followed by further purification and concentration of the relaxin peptide with ion exchange chromatography on CM-cellulose (Sherwood *et al.*, 1974), yielding approximately 35 mg of a highly pure PRLX preparation per kg ovary. The purification of PRLX led to the determination of the physicochemical properties and the successful extraction of relaxin from other species including rat (Sherwood, 1979), sand tiger shark (Reinig *et al.*, 1981), rabbit (Eldridge *et al.*, 1985) and horse (Stewart *et al.*, 1986).

1.2.3.2 Synthetic production of relaxin peptides

As extraction of relaxin from tissue sources resulted in small yields and since human relaxin could not be easily prepared by this method, synthetic methods were developed. The synthetic production of relaxin peptides has also allowed investigation of the structure-activity relationships of relaxin. The first relaxin peptide to be synthesised was PRLX (Tregear *et al.*, 1982), followed by H1RLX

(Hudson *et al.*, 1983) and H2RLX (Hudson *et al.*, 1984). Further developments to the synthesis of relaxin have resulted in the current methodology which includes the solid-phase synthesis of the separate A and B chains, purification of the crude peptides, combination of the A and B chains and folding under oxidative conditions with the final product analysed for purity and correct folding of the peptide (Büllesbach *et al.*, 1994a; Büllesbach *et al.*, 1991; Rembiesa *et al.*, 1993; Tang *et al.*, 2003). However, synthetic production of relaxin peptides is not without its problems, with low yields of peptide due to substantial losses during the combination of A and B chains as a result of the poor solubility of the B chain (Büllesbach *et al.*, 1994a; Tregear *et al.*, 1995). The synthetic conditions for each relaxin peptide often differs due to the complexity and variability of the individual chain sequences, thus increasing the production time (Tregear *et al.*, 2001).

1.2.3.3 Production of relaxin by recombinant methods

To obtain amounts of relaxin peptide larger than that available by native extraction or synthetic means, recombinant methods were developed. Early methods included the combination of natural mRNA and synthetic DNA constructs to produce pro-PRLX in an *E.coli* culture (Stewart *et al.*, 1983). This was developed further with the incorporation of pro-PRLX DNA template into an *E.coli* expression vector (Reddy *et al.*, 1992), leading to the successful production of a pro-PRLX peptide. The pressure to develop a large scale, cost effective method for production of recombinant human relaxin was increasing due to the demand for use in clinical trials. Methods for recombinant production of human relaxin were developed by Genentech Inc. (CA, USA) and involved the separate production of the relaxin A and B chains in *E.coli* followed by the extraction and purification of large quantities of peptide (Canova-Davis *et al.*, 1991). The purified chains were combined and refolded with yields of approximately 60% relative to the amount of B chain incorporated into the reaction. A further advance was made with the development of a one chain process with the A and B chains connected by a "mini-C" peptide to produce a pro-relaxin form of the H2RLX peptide (Vandlen *et al.*, 1995), a method successfully employed in the production of recombinant mini-C pro-insulin (Wetzel *et al.*, 1981). The extracted mini-C pro-H2RLX peptide was then refolded and the mini-C peptide cleaved. The

synthesis with the mini-C peptide allowed correct folding of the recombinant peptide.

1.2.4 Structure-activity relationships of relaxin

The structural features of relaxin that defined the affinity and efficacy of the peptide and its receptor were examined by bioassays (see Section 1.3). The large range of relaxin sequence diversity between species has highlighted the importance of the highly conserved residues and researchers have exploited these structural features to investigate their role in relaxin activity.

1.2.4.1 A Chain

While the A chain appeared to have little direct interaction with the receptor due to its distal position to the B chain binding site (Eigenbrot *et al.*, 1991), its importance was established in maintaining structural integrity of the relaxin peptide complex. Shortening of the A chain by the removal of 3 or more amino acids from the N terminus caused a structural change to the relaxin peptide, leading to a subsequent loss of relaxin bioactivity in the mouse interpubic ligament assay (Büllesbach *et al.*, 1986b). Further investigation revealed that the identity of the N terminal amino acids is not as important for maintaining the structural integrity of the peptide as the presence of an N terminal helix (Büllesbach *et al.*, 1987). The importance of the A chain in the relaxin peptide was highlighted by the analysis of the activity of the separate A and B chains, revealing that the individual chains have no biological activity (Kakouris *et al.*, 1992; Tan *et al.*, 1998).

Substitution of highly conserved basic amino acids in the relaxin A chain with citrulline residues had no effect on the biological activity of the peptide (Rembiesa *et al.*, 1993), while substitution of the glycine (A14) residue with isoleucine dramatically reduced relaxin bioactivity by 100 fold (Büllesbach *et al.*, 1994a). Disruption of the internal A chain disulfide bond by substitution of the cysteine (A10 & A15) residues with alanine resulted in a significant decrease in biological activity (Büllesbach *et al.*, 1995a). While the above substitutions of A chain residues did

affect relaxin-like bioactivity, it was concluded that the decrease in bioactivity was indicative of the disruption of structural integrity of the relaxin peptide rather than a role of these amino acids in interactions with the receptor.

1.2.4.2 B Chain

Analysis of PRLX purified from pregnant sow ovaries revealed three forms of the hormone that differed in the length of the B chain at the carboxyl terminus (Sherwood *et al.*, 1974). The native form of PRLX was believed to have a B chain of 31 amino acids, while versions with a shortened B chain of either 29 or 28 amino acids were attributed to partial degradation during the isolation and purification process (Walsh *et al.*, 1980), although all three forms of PRLX were bioactive (Anderson, 1984; Sherwood *et al.*, 1974). This led to the examination of the role of the length of the B chain. Synthetic PRLX with three or seven amino acids removed from the N-terminus and C-terminus respectively showed no decrease in relaxin bioactivity, whereas the removal of two further amino acids from the C-terminus reduced relaxin bioactivity (Tregear *et al.*, 1983; Tregear *et al.*, 1982).

Based on the three-dimensional structure of insulin and with the completion of the x-ray crystallography of relaxin (Eigenbrot *et al.*, 1991), it was proposed that two highly conserved arginine (B13 & B17) residues in the B chain, which protrude into the surrounding water, would be crucial for interaction with the receptor (Büllesbach *et al.*, 1988). Substitution of these residues, separately and together, with either positively charged lysine or uncharged citrulline dramatically reduced receptor interaction and bioactivity of the relaxin peptide (Büllesbach *et al.*, 1991; Büllesbach *et al.*, 1992), indicating the importance of the arginine residues. However the specificity with which the altered relaxin peptides still interacted with the receptor suggested that there were other amino acids also involved in relaxin binding. Comparison of the known relaxin sequences from a variety of species yielded three highly conserved amino acids within the B chain binding site area; glycine B12, valine B16 and isoleucine B20 (Figure 1.2). Manipulation of these amino acids resulted in the exclusion of glycine B12 and valine B16 as active sites, and the unveiling of isoleucine (B20) as a critical component of relaxin binding (Büllesbach

et al., 2000). The relaxin binding "cassette", comprised of Arg-X-X-X-Arg-X-X-Ile, is positioned along the B chain alpha-helix allowing the arginine and isoleucine side chains to protrude and interact with the relaxin receptor in a unique "lock and key" mechanism.

1.3 Bioassays for relaxin

The original observations that relaxin caused relaxation of the pubic symphysis, elongation of the interpubic ligament and myometrial relaxation, led to the development of the three bioassays used to determine relaxin activity for more than 30 years. They include the guinea pig pubis symphysis palpation assay, mouse interpubic ligament assay and the uterine relaxation assay.

1.3.1 *In vivo* functional assays

1.3.1.1 Guinea pig pubis symphysis palpation assay

The first relaxin bioassay was based on the ability of relaxin to elongate the pubic symphysis of the guinea-pig (Fevold *et al.*, 1930). Virgin guinea-pigs in full oestrus were injected subcutaneously with a relaxin preparation. Ten to twelve hours later the pelvic ligaments were gently palpated vertically while holding the symphysis pubis stationary. The original guinea pig unit (GPU) was taken as the minimum amount of hormone needed to loosen the ligaments within ten to twelve hours (Fevold *et al.*, 1930). To ensure a quantitative assay, the definition of the GPU was redefined as the amount of hormone required to induce relaxation of the symphysis pubis within six hours in 70% of castrated female guinea pigs weighing between 350 – 800g, pre-treated daily with 0.85 µg oestradiol for 4 days (Abramowitz *et al.*, 1944). Although the assay now maintained a quantitative aspect, the overall validity of the assay had not improved due to the subjective nature of the analysis and the desensitization of tissues when relaxin was used repeatedly (Noall *et al.*, 1956; Steinetz *et al.*, 1982). To allow for a more objective assessment of relaxin activity, the palpation analysis was performed by two or more independent researchers under blind conditions and scored on a flexibility scale from 0 indicating no flexibility to 6

showing extreme flexibility and compared against a reference standard (Kroc *et al.*, 1959). Although useful for its ability to show the activity of relaxin from a variety of species (Büllesbach *et al.*, 1986a; O'Byrne *et al.*, 1978; Reinig *et al.*, 1981), the guinea pig pubis symphysis palpation assay still remains imprecise and expensive with 20 or more animals per dose needed to reduce error limits to less than 50% (Büllesbach *et al.*, 1994b; O'Byrne *et al.*, 1978; Reinig *et al.*, 1981; Steinetz *et al.*, 1969).

1.3.1.2 Mouse interpubic ligament elongation assay

The mouse interpubic ligament elongation assay was the first bioassay to be standardized and accepted as a relaxin bioassay that could be used for comparative studies without the large discrepancies that occurred in the guinea pig pubis symphysis palpation assay. Oestrogen-primed mice were subcutaneously injected with relaxin or vehicle, killed 18-24 hours later, the interpubic ligament dissected and measured against reference standards (Steinetz *et al.*, 1960). This bioassay allowed the reproducible assessment of relaxin activity to be determined in a dose-dependent manner and has been extensively used to characterise the activity of relaxin and synthetic analogues (Büllesbach *et al.*, 1996; Büllesbach *et al.*, 1990; Büllesbach *et al.*, 1993; Sherwood, 1994). Its simplicity and specificity make this bioassay more appealing, however it still has the same drawbacks as the guinea pig pubis symphysis palpation with regard to variation among animals, needing 20 mice per dose to reduce the error limits to less than 50%.

1.3.2 *In vitro* functional assays

1.3.2.1 Uterine contractility/relaxation assay

The *in vitro* uterine contractility or relaxation assay are essentially two complementary versions of the same bioassay, modified from an original *in vivo* bioassay based on the observation that relaxin extracts could decrease spontaneous uterine contractions in guinea pigs (Krantz *et al.*, 1950). The contractility bioassay utilises the spontaneous contractions produced in isolated uterine tissue from guinea pigs or rodents, which diminish in the presence of relaxin (Anderson, 1984; MacLennan *et al.*, 1991; Oliver *et al.*, 1978; Petersen *et al.*, 1991). The advantage of this bioassay over the *in vivo* bioassay is that it is sensitive and the relaxin response is immediate rather than over several hours. However the ability to quantitate relaxin activity is not easy since both the frequency and amplitude of the contractions are affected by relaxin and the consistency of the spontaneous contractions is not reliable, making a stable baseline hard to establish. Endogenous peptides, such as oxytocin, have also been shown to interfere with this assay (Fields *et al.*, 1983).

The uterine relaxation bioassay removes the dependence on the spontaneous contractions by contracting the tissue artificially using a low dose of prostaglandin F_2 alpha ($PGF_{2\alpha}$) (Nishikori *et al.*, 1983), electrical stimulation (Brenner *et al.*, 1984b) or a depolarizing potassium solution (St-Louis, 1982) and then relaxing the tissue with increasing concentrations of relaxin. Taking out the spontaneous component of the bioassay has allowed a more accurate and reliable quantitation of relaxin activity to be achieved.

1.3.2.2 Rat isolated atrial bioassay

More recentiy the rat isolated atrial bioassay was developed based on the observation that relaxin binding sites were described in the atria (Osheroff *et al.*, 1992; Tan *et al.*, 1999). Both lobes of the heart atria were isolated from the ventricles, the right

atria allowed to beat spontaneously to allow the determination of drug induced chronotropic effects, while the left atria was stimulated and the force of contraction measured to allow positive inotropic effects to be studied (Kakouris *et al.*, 1992; Tan *et al.*, 2002; Tan *et al.*, 1998; Ward *et al.*, 1992). Relaxin caused a slow developing, dose-dependent positive chronotropic and inotropic response which stabilised within 5 minutes. The response to relaxin was the result of a direct interaction between relaxin and its receptor, without the need of steroid treatment pre-priming, and there was no difference in results obtained in atria isolated from male or female rats (Kakouris *et al.*, 1992). The isolated atrial bioassay has proven to be a sensitive, reproducible and efficient bioassay, as an entire concentration-response curve to relaxin can be achieved using a single animal, therefore decreasing animal usage.

1.3.2.3 Cellular functional assays

More sensitive and precise bioassays were needed to accommodate the low levels of relaxin available from tissue extracts and the rapid advances in peptides synthesised for relaxin structure-activity relationships. Relaxin caused a dose-dependent increase in cAMP in newborn rhesus monkey uterus cells (Kramer *et al.*, 1990), rat myometrial cells (Hsu *et al.*, 1985) and human endometrial cells (Fei *et al.*, 1990; Osheroff *et al.*, 1990). The bioassays were sensitive, specific and the cAMP response to relaxin could be inhibited by the addition of anti-relaxin antibodies (Fei *et al.*, 1990; Kramer *et al.*, 1990). Limitations of these primary cell cultures include a relatively short life span, a scarcity of starting tissues and a degree of variability between cultures. These problems were solved by the use of a human monocyte cell line, THP-1, that naturally expresses relaxin receptors. Relaxin stimulates a cAMP response in THP-1 cells by binding specifically to receptors on the cell surface (Parsell *et al.*, 1996).

1.4 The physiological actions of relaxin

The actions of relaxin on the pregnant female reproductive tract were responsible for its discovery and isolation in 1926 (Hisaw, 1926), and for many years functional research focused on the role of relaxin in female reproductive organs. However, relaxin is becoming more than just a hormone of pregnancy, with research identifying a variety of functions in both reproductive and non-reproductive organs (Table 1.1).

1.4.1 Female reproductive tissues

The discovery of relaxin as a reproductive hormone has led to extensive study of its role in the rat and porcine reproductive systems. The primary source of circulating relaxin in most species is the corpus luteum and radioimmunoassays have determined serum levels to be highest during pregnancy (Sherwood *et al.*, 1980). The serum levels of relaxin vary slightly between species. The general pattern is that levels remain low during the first and second trimesters of pregnancy but dramatically increase in the third trimester before falling rapidly following birth (Sherwood *et al.*, 1975a; Sherwood *et al.*, 1980; Stewart *et al.*, 1992), correlating with the influence of relaxin on parturition.

The development of an anti-relaxin antibody allowed researchers to investigate the actions of relaxin in the reproductive process. The elimination of circulating relaxin caused disruption of birth and a decrease in the delivery of live rat pups (Hwang *et al.*, 1989), hindered development of mammary glands and nipples (Hwang *et al.*, 1991), and reduced growth and softening of the cervix and vagina (Zhao *et al.*, 1996). Similar results were found in pigs (Cho *et al.*, 1998; Min *et al.*, 1997; Winn *et al.*, 1994). The development of a relaxin gene knock out mouse that cannot express a functional relaxin (Zhao *et al.*, 1999) has allowed a detailed investigation of the physiological role of relaxin and is described in Section 1.4.6.

Direct administration of relaxin to isolated myometrial tissue from rats caused a dose-dependant inhibition of contractile activity (Petersen *et al.*, 1991), as well as reducing oxytocin induced spasm by preventing the release of Ca^{2+} from intracellular stores and the influx of extracellular Ca^{2+} (Hughes *et al.*, 1995). Similar results were found with isolated pig myometrial tissue, where exposure to relaxin caused a dose-dependent decrease of spontaneous contractility (MacLennan *et al.*, 1991; MacLennan *et al.*, 1995; MacLennan *et al.*, 1986a). It has been demonstrated in myometrial tissue obtained from a non-pregnant rat that relaxin receptors are regulated by oestrogen (Mercado-Simmen *et al.*, 1982). However, during pregnancy, the concentration of relaxin receptors determined in rat myometrial membrane-enriched fractions using [^{125}I]iodorelaxin do not correlate with oestrogen levels. Administration of exogenous relaxin significantly decreased relaxin receptor expression in the myometrium during late pregnancy (Mercado-Simmen *et al.*, 1982), which could account for the lack of relaxin activity seen in myometrial tissue obtained from late pregnancy in some studies (Longo *et al.*, 2003).

In humans however, the role of relaxin is uncertain with relaxin exhibiting less influence on remodelling for parturition but appearing to be more important in the early stages of pregnancy. Relaxin binding sites have been shown in both the endometrial and myometrial layers of the human uterus, although functional studies indicate that relaxin fails to inhibit the spontaneous contractility of human myometrial strips (MacLennan *et al.*, 1991; MacLennan *et al.*, 1995; MacLennan *et al.*, 1986a; Petersen *et al.*, 1991). Plasma levels of relaxin rise dramatically upon implantation, remain high for the first trimester and then steadily decrease until parturition (Eddie *et al.*, 1986). Relaxin appears to have little influence on the cervix as clinical trials reported that topically applied relaxin failed to affect any aspect of cervical ripening compared to controls (Brennand *et al.*, 1997), even though the cervix has been shown to contain relaxin binding sites (Kohsaka *et al.*, 1998). One study reported a positive correlation between relaxin serum levels and cervical growth during the early stages of the second trimester of pregnancy (Eppel *et al.*, 1999), with little correlation found at term.

The function of relaxin in a non-pregnant menstrual cycle appears to be influenced by the phase of the menstrual cycle. Relaxin in the uterus is thought to be influenced by progesterone and regulation of relaxin secretion is cycle dependent (Yki-Jarvinen *et al.*, 1985). Relaxin differentially regulates the secretion of vascular endothelial growth factor (VEGF) in isolated uterine cells with a negative influence in the proliferative phase, while in the secretory phase, relaxin treatment stimulated VEGF secretion (Palejwala *et al.*, 2002). The positive effect of relaxin on secretory phase VEGF secretion is thought to be responsible for an increased incidence of menometrorrhagia in relaxin-treated clinical trial patients (Unemori *et al.*, 1999)

1.4.2 Male reproductive tissues

Relaxin was first identified in the male when a crude extract taken from rooster testis demonstrated specific relaxin-like activity in the mouse pubis symphysis bioassay that could be inhibited with rabbit anti-PRLX antiserum (Steinetz *et al.*, 1964). The presence of an immunoreactive relaxin-like protein was readily identified in human male seminal plasma (Loumaye *et al.*, 1980), although the amount of this protein varied between the samples. The peptide was confirmed as relaxin and identified as a likely product of the prostate gland (Essig *et al.*, 1982). Expression of both H1RLX and H2RLX mRNA transcripts were identified in the prostate (Hansell *et al.*, 1991) however only H2RLX peptide was identified (Winslow *et al.*, 1992), again questioning the physiological relevance of H1RLX. Human chorionic gonadotropin (hCG) has been shown to influence relaxin secretion in females (Quagliarello *et al.*, 1980), as injection of hCG into a non-pregnant female produces detectable levels of relaxin in the serum a couple days later. In males, hCG has no effect on relaxin secretion as ejaculate from male subjects with no hCG contains detectable amounts of relaxin (Colon *et al.*, 1994).

Early physiological studies showed that PRLX improved motility of washed human sperm (Lessing *et al.*, 1986). and improved the ability of the sperm to penetrate bovine cervical mucus (Brenner *et al.*, 1984a). Studies with human relaxin have shown that treatment of washed human sperm with 1ng/ml of human relaxin increased penetration significantly better than when treated with 100ng/ml of PRLX

(Colon *et al.*, 1989). However, treatment of washed human sperm with relaxin did not have a greater or lesser effect on penetration when compared to human sperm in semen, indicating that fresh, normal semen has an optimum amount of relaxin, and that the addition of more relaxin has no further effect. Another possible role for relaxin in the male may be connected with the development of prostatic hyperplasia, as the expression of relaxin gene transcripts and the presence of relaxin binding sites increases with age in the rat prostate (Hornsby *et al.*, 2001).

1.4.3 Cardiovascular system

An increase in the heart rate of pregnant women occurs within the same time frame as changes in the plasma relaxin concentrations (Stewart *et al.*, 1990). It was therefore hypothesized that relaxin may have a direct effect on the heart. It was also observed that anaesthetised female rats injected with PRLX, intravenously into a cannulated femoral vein, responded with a subsequent increase in blood pressure and heart rate (Parry *et al.*, 1990). Relaxin binding sites were identified in the atrium of both male and female rats (Osheroff *et al.*, 1992), and further characterisation demonstrated no difference in affinity for relaxin but a greater density of receptors in the female than the male (Tan *et al.*, 1999). Oestrogen treatment did not alter relaxin binding in atria from female rats as in the uterus, but did reduce the density of relaxin binding sites in atria from male rats. The rat isolated atrial bioassay (Section 1.3.2.2) provided evidence of a direct effect of relaxin to cause positive inotropic and chronotropic responses, with relaxin more potent than angiotensin II, isoprenaline and adrenaline (Kakouris *et al.*, 1992). Gene transcripts of both H1RLX and H2RLX were identified in human atrial and ventricle tissue (Dschietzig *et al.*, 2001) however local synthesis of the relaxin peptides has yet to be confirmed (Samuel *et al.*, 2003a).

The influence of relaxin on the cardiovascular system extends beyond direct stimulation of the heart. Relaxin may regulate vascular tone as administration of relaxin increased coronary flow in guinea pig isolated hearts (Bani-Sacchi *et al.*, 1995), caused a delayed increase in renal plasma flow comparable to that seen in pregnancy (Danielson *et al.*, 1999) and induced potent dilation of small systemic

resistance arteries (Fisher *et al.*, 2002). The vasodilator effects of relaxin have been suggested to be: a direct consequence of relaxin stimulation (Fisher *et al.*, 2002); mediated through nitric oxide production (Bani *et al.*, 1988; Danielson *et al.*, 1999), endothelin production (Danielson *et al.*, 2000) and atrial natriuretic peptide secretion (Toth *et al.*, 1996); endothelium dependent (Reid *et al.*, 2001); and vary between vascular beds (Samuel *et al.*, 2003a).

While the mechanism of action of relaxin in the cardiovascular system remains unclear, there is increasing evidence that relaxin may play a protective role in heart failure and in chronic heart disease. In guinea pigs with induced myocardial damage, relaxin significantly increased coronary flow, improved heart contractility and decreased the generation of markers associated with myocardial damage (Masini *et al.*, 1997). In patients with chronic heart failure, there was a significant increase in the synthesis of relaxin gene transcripts and in relaxin plasma levels, which correlated directly with the severity of the disease (Dschietzig *et al.*, 2001). In a rat model of myocardial infarction the inotropic response to relaxin was diminished while the chronotropic response was unaffected (Kompa *et al.*, 2002). Gene transcription of Rat3RLX was also shown to be significantly increased in this model of myocardial infarction, further suggesting a protective role for relaxins in the development of heart disease.

1.4.4 Brain

The first indication of a central role for relaxin came with the observation that central administration of relaxin inhibited the release of oxytocin, and affected milk ejection during suckling in the anaesthetized lactating rat (Summerlee *et al.*, 1984). Further investigation indicated that the subfornical organ, situated in the ventricular system, was important in mediating the inhibitory action of relaxin, since lesions to the subfornical organ prevented the suppression of oxytocin release (Summerlee *et al.*, 1987). Injection of relaxin directly into the lateral and dorsal portions of the third ventricle induced a sustained rise in blood pressure while injection into the ventral portion of the third ventricle caused a short rise in blood pressure (Mumford *et al.*, 1989). Lesions to the subfornical organ prevented the sustained pressor action

of relaxin but did not prevent the acute rise in blood pressure resulting from relaxin administration into the ventral portion, indicating a biphasic action of centrally administered relaxin. The angiotensin II system was thought to be involved in the central actions of relaxin (Parry *et al.*, 1990). Blockade of the central angiotensin system prevented the pressor response to icv relaxin, but only partially blocked the pressor response to an iv administration of relaxin (Parry *et al.*, 1991), with the remaining pressor response removed by a peripheral vasopressin antagonist. Blockade of the central angiotensin system reduced the relaxin-stimulated release of vasopressin and oxytocin (Geddes *et al.*, 1994) and water consumption of rats (Sinnayah *et al.*, 1999).

Binding studies have revealed high concentrations of relaxin binding sites in several circumventricular organs, including the subfornical organ and the organum vasculosum of the lamina terminalis (OVLT), areas known to be associated with control of blood pressure and fluid balance, and in the magnocellular regions of the paraventricular and supraoptic nuclei, regions responsible for the release of oxytocin and vasopressin (Osheroff *et al.*, 1991). Relaxin binding sites have been identified in layer V of the cerebral cortex of rat brain (Osheroff *et al.*, 1991; Tan *et al.*, 1999) although the physiological function of these relaxin binding sites is still unclear. Fos immunoreactivity studies confirmed results from the binding studies since icv injection of relaxin caused fos-positive neurons to appear in the outer layer of the subfornical organ, dorsal part of the OVLT, throughout the median preoptic nucleus, supraoptic nucleus and the hypothalamic paraventricular nucleus (McKinley *et al.*, 1997). Neurons in the outer layer of the subfornical organ and the dorsal part of the OVLT have efferent connections to the supraoptic nucleus and the hypothalamic paraventricular nucleus (McKinley *et al.*, 1998), implying that it is possible that relaxin releases oxytocin and vasopressin from the supraoptic and paraventricular nuclei indirectly via the subfornical organ and the OVLT. The administration of anti-relaxin antibodies into the circumventricular organs of the rat brain caused a specific central effect to disrupt the timing of birth (Summerlee *et al.*, 1998), whereas peripheral administration of anti-relaxin antibodies had no influence on delivery or survival of pups (Hwang *et al.*, 1989).

Since relaxin has a specific central role and cannot cross the blood-brain barrier, it seems likely that relaxin is produced locally in the brain. Low levels of relaxin mRNA have been identified in the anterior olfactory nucleus, dentate gyrus and neocortex (Burazin *et al.*, 2001; Osheroff *et al.*, 1993), suggesting that the relaxin peptide is localised to few areas while the relaxin receptor is widely distributed within the brain. This has led to the hypothesis that the relaxin peptide is synthesised in the cell bodies and transported to nerve terminals with relaxin binding sites possibly indicating the location of peptide release (Burazin *et al.*, 2001). With the identification of the third human relaxin gene, an equivalent rat relaxin gene 3 (Rat3RLX) was isolated (Burazin *et al.*, 2002) and found to be abundantly expressed in the ventromedial dorsal tegmental nucleus (vmDTg) while low levels of rat relaxin gene 1 (Rat1RLX) were found in areas previously reported to express relaxin mRNA (Osheroff *et al.*, 1993). Relaxin receptors are also expressed in the vmDTg (Burazin *et al.*, 2002), an area shown to connect afferents and efferents with forebrain areas (Goto *et al.*, 2001) leading to the hypothesis that the vmDTg was integrated into the brainstem network that regulates behaviour. This information, combined with the localisation of relaxin binding sites in the cerebral cortex (Tan *et al.*, 1999), suggests that relaxin may influence behaviour (Burazin *et al.*, 2002). The ratio of Rat1RLX to Rat3RLX expression in rat brain has led to the hypothesis that Rat3RLX may be the predominant centrally acting relaxin while Rat1RLX may be the predominant peripherally acting relaxin.

1.4.5 Connective tissue and cell growth

Relaxin remodels connective tissue to cause the elongation of the pubic symphysis and facilitate parturition, but little was known about the effects of relaxin on connective tissue outside the reproductive system. Relaxin administration to a cell culture of human dermal fibroblasts produced an increase in the secretion of metalloproteinase, procollagenase and a decrease in the expression of the tissue inhibitor of metalloproteinase expression (Unemori *et al.*, 1990). A decrease in expression of interstitial collagens was also noted, resulting in an overall increase in collagen matrix turnover and a reduction in overall tissue collagen content. Addition of transforming growth factor beta (TGF-β) and interleukin 1-beta (IL-1β) combined

with indomethacin, which is known to increase collagen secretion in these fibroblast cells, failed to inhibit relaxin induced collagen turnover, indicating that relaxin could modulate collagen synthesis in presence of collagen over-expression (Unemori *et al.*, 1990).

Similar results were obtained in scleroderma fibroblast (Unemori *et al.*, 1992) and lung fibroblast cell cultures (Unemori *et al.*, 1996), as collagen over-expression was reduced by relaxin treatment, while relaxin did not markedly alter the basal collagen levels. Relaxin treatment of an *in vivo* murine model of bleomycin-induced lung fibrosis showed that relaxin prevented and reversed the effects of fibrosis (Unemori *et al.*, 1996), without any effect on non-fibrotic lung tissue. A similar result was obtained in a cell culture model of rat liver fibrosis, with relaxin treatment decreasing collagen synthesis and deposition by decreasing the secretion of two collagenase inhibitors that prevent collagen breakdown, TIMP-1 and TIMP-2 (Williams *et al.*, 2001). An *in vivo* model of renal interstitial fibrosis treated with relaxin showed renal protection with a subsequent decrease in renal disease symptoms, including serum creatinine and proteinuria (Garber *et al.*, 2001; McDonald *et al.*, 2003).

Based on its specific anti-fibrotic actions, relaxin has been trialled for the treatment of Scleroderma (Seibold *et al.*, 1998). Phase II clinical trials suggested that relaxin reduced scleroderma-related skin thickening, leading to an improvement of forced vital capacity and hand function (Seibold *et al.*, 2000). Although the results from Phase III clinical trials showed a similar trend, the relaxin treated patients were not significantly different from placebo (Erikson *et al.*, 2001).

1.4.6 Relaxin gene knockout mouse

Recently, inactivation of the relaxin gene by homologous recombination in embryonic stem cells was used to develop a relaxin knockout mouse (relaxin -/- mouse) (Zhao *et al.*, 1999). The phenotype of the relaxin -/- mice displayed no gross anatomical or behavioural differences compared to the normal wild type mouse. Both the male and female relaxin -/- mice were fertile and produced normal litters in

direct contrast to anti-relaxin antibody studies performed in rats (see Section 1.4.1), although male -/- mice displayed stunted growth of the testis, epididymis and prostate compared to age matched wildtype mice (Samuel *et al.*, 2003b). In agreement with the anti-relaxin antibody studies, the mammary gland and nipple development in relaxin -/- female mice was impaired, partly due to a lack of connective tissue development, which resulted in pups being unable to suckle and survive unless fostered to wild type mothers (Zhao *et al.*, 1999). Closer inspection of the pubic symphysis showed that while relaxin -/- mice had an elongated interpubic ligament compared to virgin mice, it was significantly smaller than wild type mice, reiterating the importance of relaxin in this function.

The lack of mammary gland and nipple development led to a further investigation of the influence of relaxin on collagen regulation (see Section 1.4.5). It was found that relaxin -/- mice failed to regulate collagen degradation in the nipple and vagina while the collagen fibres of cervix and mammary glands appeared more densely packed compared to wild-type mice (Zhao *et al.*, 2000). Relaxin -/- mice showed an increase in age-related development of fibrosis in non-reproductive tissues, including the lung and kidneys, when compared to age-matched wild-type mice (Samuel *et al.*, 2003c; Samuel *et al.*, 2004). The influence of relaxin on fibrosis was further highlighted when the fibrosis in relaxin -/- mice was shown to be reversed to wild type mice levels following relaxin treatment. The relaxin -/- mouse has provided an important scientific tool for elucidating the role of relaxin in the regulation of collagen and in the development of fibrosis.

Table 1.1

A brief summary of the rat and human physiological systems that express the relaxin receptor, the relaxin peptide and the function of relaxin in these systems. N/A denotes information not available

Physiological System	Species	Relaxin receptors expressed	Source of relaxin peptide	Functional relevance
Reproductive system	Rat - female	Myometrium, cervix, vagina, nipples, mammary glands, pubic symphysis ligament (Weiss *et al.*, 1982; Osheroff *et al.*, 1990; Kuenzi *et al.*, 1995; Yang *et al.*, 1992)	Corpus luteum (Sherwood, 1994)	Maintenance of pregnancy, parturition, mammary gland and nipple development.
	Rat - male	Prostate (Hornsby *et al.*, 2001)	Prostate (Gunnersen *et al.*, 1995; Hornsby *et al.*, 2001)	Involved in the development of testis, epididymis and prostate.
	Human - female	Fallopian tube, uterus, cervix, mammary glands and nipples (Tang *et al.*, 1995; Kohsaka *et al.*, 1998)	Corpus luteum (Sakbun *et al.*, 1990; Johnson *et al.*, 1991)	Decidualisation, possibly involved in implantation.
	Human - male	LGR7 mRNA transcripts identified in prostate and testis (Hsu *et al.*, 2002)	Prostate (Essig *et al.*, 1982; Winslow *et al.*, 1992)	Possibly involved in sperm motility.
Cardiovascular system	Rat	Atria (Osheroff *et al.*, 1992; Tan *et al.*, 1999)	Rat3RLX mRNA detected in in atria and ventricles (Samuel *et al.*, 2003c)	Influences blood pressure and heart rate, protection against myocardial infarction.
	Human	No binding sites identified in heart, receptors identified through functional studies in systemic resistance arteries (Tan 1999; Fisher *et al* 2002)	H1RLX and H2RLX mRNA identified in the atrium and ventricle (Dschietzig *et al.*, 2001)	Cardiovascular regulation through dilator actions, possible protection against chronic heart failure.
Central nervous system	Rat	OVLT, hypothalamic paraventricular and supraoptic nuclei, cerebral cortex layer V, vmDTg (Osheroff *et al.*, 1991; McKinley *et al.*, 1997; Tan *et al.*, 1999)	Anterior olfactory nucleus, dentate gyrus, neocortex, vmDTg (Osheroff *et al*, 1993; Burazin *et al*, 2002)	Stimulates oxytocin and vasopressin release, influences behaviour and reproductive system.
	Human	Deep layer of the parietal, entorhinal and frontal cortex (Tan 1999)	N/A	N/A

1.5 Relaxin receptor binding assays

Relaxin radioligands were developed to examine the characteristics and localisation of relaxin receptors in various tissues.

1.5.1 Specific relaxin radioligands

1.5.1.1 Radioiodinated relaxin

[^{125}I] labelled PRLX was originally developed to assess serum levels of relaxin using radioimmunoassay (Sherwood *et al.*, 1975b). Synthesis of [^{125}I]-PRLX involved the incorporation of a tyrosine residue into the porcine relaxin sequence to allow radio-iodination to occur, with the resulting [^{125}I]-PRLX peptide appearing to be biologically active in an immunological assay. Simultaneously another method was developed that utilised the iodination of an acylating agent, known as the Bolton & Hunter iodination method (Bolton *et al.*, 1973), which was then reacted with PRLX to produce a [^{125}I]-PRLX. This method does not involve exposing the peptide to the harsh treatment inherent in the first method and does not rely on the presence of a tyrosine. [^{125}I]-PRLX was then purified on reverse phase HPLC (Yang *et al.*, 1992), although the stability of the ligand was decreased (Osheroff *et al.*, 1990).

Although [^{125}I]-PRLX has been used extensively to localise relaxin receptors, the properties of the ligand are altered slightly by the addition of the ^{125}I. The iodine is thought to cause conformational changes to the relaxin peptide and the labelled peptide has a lower binding affinity (Yang *et al.*, 1992) and reduced bioactivity (Cheah *et al.*, 1980). Iodination of H2RLX has advantages over PRLX as it naturally contains a tyrosine residue in the A chain (A3; Figure 1.2) and incorporation of the iodine does not affect the binding affinity (Palejwala *et al.*, 1998) although the bioactivity of this peptide has not been determined. One common problem of iodinated relaxin ligands is that the specific activity can vary considerably between preparations, ranging from 80 – 185 μCi/μg (Gates *et al.*,

1981; Palejwala *et al.*, 1998; Sherwood *et al.*, 1975b) as the unlabelled relaxin peptide can not be easily separated from the labelled relaxin peptide.

1.5.1.2 Phosphorylated relaxin

High specific activity labelled relaxin was produced using cAMP-dependent protein kinase to phosphorylate H2RLX at the serine (B32) with [γ-^{32}P]-ATP, resulting in [^{32}P]-H2RLX (Osheroff *et al.*, 1990). Phosphorylation was specific to the serine (B32) and attempts to phosphorylate H2RLX with B chain lengths of 29 and 31 residues failed. [^{32}P]-H2RLX has a specific activity of >5000 Ci/mmol (Osheroff *et al.*, 1990) and unlike [^{125}I]-RLX, the unlabeled relaxin peptide can be separated from the labelled relaxin peptide using cation exchange HPLC (Section 2.3.1), maintaining consistent specific activity between preparations. H2RLX but not other insulin-like factors competed with [^{32}P]-H2RLX binding to relaxin receptors and biological activity was unaffected by phosphorylation. Although the half life of this ligand is shorter than that of iodinated relaxin, the stability and specificity of [^{32}P]-H2RLX made it a favourable option.

To increase the resolution of autoradiography performed with [^{32}P]-H2RLX, a modification was made by substituting the [γ-^{32}P]-ATP with [γ-^{33}P]-ATP to produce [^{33}P]-H2RLX (Tan *et al.*, 1999). The sharpness of the autoradiography images were improved due to the lower energy of [^{33}P] β particle emission (Hudson, 1993).

1.5.2 *In vivo* localisation of relaxin receptors

Early relaxin receptor localisation studies were performed *in vivo* by injecting whole animals with [^{125}I]-RLX and examining individual tissues for ligand localisation that can be displaced by administration of unlabeled hormone. Injection of [^{125}I] PRLX into female guinea pigs identified ligand localised to the uterine cervix, pubic symphysis and to a lesser degree to the uterine horns that could be blocked with administration of unlabeled PRLX (Gates *et al.*, 1981). There was no localisation of the ligand into other tissues, such as mammary gland, ovary, liver, spleen and connective tissue. Similar results were obtained in rats where localisation in tissues

other than the uterus was not displaced by unlabeled relaxin indicating non-specific uptake of the ligand (Cheah *et al.*, 1980). Injection of [^{125}I]-RLX into mice caused uptake of the ligand into the kidneys, with radioactivity also in heart and other major vessels but not in the uterus or pubic joint (O'Byrne *et al.*, 1982). This assay was acknowledged to be unreliable due to metabolism of the radioligand (Cheah *et al.*, 1980) and the difficulty of transporting a large ligand across the blood vessel walls into the target tissues (O'Byrne *et al.*, 1982).

1.5.3 *In vitro* localisation of relaxin receptors

Receptor autoradiography performed on isolated tissue sections allows fine-detail localisation of relaxin receptors within tissues and has proven to be a reliable assay that can be optimised to ensure specific binding of the radioligand. Exposure of tissue sections to ligands has identified relaxin binding sites throughout the reproductive system of rodents, including the myometrial and endometrial layers of the uterus (Osheroff *et al.*, 1990; Tan *et al.*, 1999; Weiss *et al.*, 1982), in the cervix and vaginal tract (Kuenzi *et al.*, 1995; Osheroff *et al.*, 1990; Weiss *et al.*, 1982), within the epithelial cells of the mammary gland as well as the epithelial and smooth muscle cells of nipples (Kuenzi *et al.*, 1995). High resolution autoradiography demonstrated relaxin binding sites in the peripheral layers of the ovary and within the pubic symphysis ligament (Yang *et al.*, 1992). Sites were also identified in the heart, localised to the atrium (Osheroff *et al.*, 1992; Tan *et al.*, 1999) and in specific areas in the brain, including the fifth layer of the cortex and the subfornical organ within the OVLT (Tan *et al.*, 1999). These studies provide evidence that supports the wide range of physiological functions of relaxin (Section 1.4) that could not be confirmed by the *in vivo* receptor localisation studies.

Autoradiographic localisation of relaxin receptors in the human is limited to a few studies, possibly due to the difficulty in obtaining human tissues. Relaxin binding sites were identified using biotinylated PRLX throughout the reproductive organs, including the fallopian tube (Tang *et al.*, 1995), both the endometrial and myometrial layers of the uterus, cervix, vagina and mammary glands and nipples (Kohsaka *et al.*, 1998). However functional responses to relaxin in human tissues do not support all

areas identified with relaxin binding, with for example minimal physiological function found in isolated myometrial tissue where PRLX and HRLX failed to inhibit the spontaneous contractions produced by isolated human myometrial strips (MacLennan *et al.*, 1991; MacLennan *et al.*, 1995; MacLennan *et al.*, 1986a; Petersen *et al.*, 1991). Autoradiographic studies of human heart sections failed to demonstrate specific relaxin binding sites, while receptors were identified in brain sections, specific to the parietal and entorhinal cortexs and within a deep cerebral cortical layer (Tan, 1999). A recent study has identified specific relaxin binding within the synovial lining cells, stromal fibroblasts and blood vessels lining in the female anterior cruciate ligament while no relaxin receptors were identified in the male ligament (Dragoo *et al.*, 2003), demonstrating a sex-specific localisation of relaxin receptors in a non-sex dependant tissue that has not been reported in animals.

1.5.4 Pharmacokinetic binding assays

Membrane preparations have been used to characterise relaxin receptors in a variety of tissue and cell types. Cell culture has allowed the identification of relaxin receptor in human foetal membranes (Garibay-Tupas *et al.*, 1995; Koay *et al.*, 1986) thus identifying a novel target for relaxin in the development of the foetus. Binding of [^{32}P]-H2RLX to human isolated uterine cells provided the first indication of the size of the relaxin receptor, calculated to be ~220kDa (Osheroff *et al.*, 1995). Relaxin bound to a single site from which it could not be displaced by insulin, IGF's or INSL3, indicating that relaxin modulates cell function through a distinct relaxin receptor (Parsell *et al.*, 1996).

The combination of cellular binding assays and functional bioassays has provided valuable insights into the role of relaxin (Section 1.3). Human isolated endometrial stromal and glandular epithelial cells, and lower uterine fibroblast cells have been shown to express a functional relaxin receptor (Palejwala *et al.*, 1998; Palejwala *et al.*, 2002) that appears to stimulate different signalling pathways depending on the cell preparation. This indicates a variable role for relaxin within the reproductive system that can be further investigated using the cellular bioassays described in Section 1.3.2.3.

1.6 Identification of the relaxin receptor

Although relaxin was one of the first reproductive hormones to be identified, the isolation and characterisation of the relaxin receptor eluded researchers for decades. Since relaxin is related to insulin and other members of the insulin-gene superfamily which signal through tyrosine kinases, it was initially proposed that the relaxin receptor would also be a tyrosine kinase, related to but distinctly different from the insulin receptor. The relaxin receptor was reported to be comprised of two components, of 220 and 36 kDa, similar to the insulin receptor (Osheroff *et al.*, 1995). The same study reported that binding of [^{32}P]-relaxin to human uterine cells was unaffected by GTP, indicating that the relaxin-receptor was unlikely to be a G-protein coupled receptor.

However, and surprisingly given the indications noted above, the relaxin receptor was recently identified as an orphan leucine-rich repeat-containing G-protein coupled receptor (LGR). Two LGR orphan receptors, previously cloned as LGR7 & LGR8, were identified as receptors that could interact and be activated by relaxin (Hsu *et al.*, 2002). The original relaxin receptor appeared to be LGR7 as relaxin displayed similar affinity and potency to that previously described in functional and binding studies. The LGR8 receptor, while displaying a lower affinity and potency for relaxin, had high affinity and potency for INSL3, and appears to be the INSL3 receptor.

1.6.1 Leucine-rich repeat-containing G-protein coupled receptor family

LGR receptors are large G-protein coupled receptors containing the typical seven transmembrane domains with a unique large ectodomain that includes the leucine-rich repeat (LRR) regions (Figure 1.4). The ectodomain is important for interaction with the large proteins that target these receptors. The LGR family consists of three subfamilies of receptors; Type A LGRs include the luteinizing hormone receptor (LHR), follicle-stimulating hormone receptor (FSHR) and the thyroid-stimulating hormone receptor (TSHR); Type B LGRs include the orphan LGR4, LGR5 & LGR6

receptors; Type C LGRs consisting of the relaxin receptor (LGR7) and the INSL3 receptor (LGR8) (Hsu, 2003). Each group of LGRs is classified according to their ectodomain structure. Type A LGRs ectodomains contain 9 LRR, Type B contain 17 LRR in their ectodomains, while Type C LGRs contain 9 LRRs with a unique N-terminal low-density lipoprotein (LDL) receptor-like cysteine-rich domain (Figure 1.5).

1.6.2 LGR7, the putative relaxin receptor

Comparison of recent research on the LGR7 receptor with previously reported data on the relaxin receptor suggests that they are one and the same receptor. LGR7 mRNA transcripts have been identified in a variety of tissues that are known to display relaxin binding or functional responses, including ovary, uterus, brain and heart (Hsu *et al.*, 2000). The LGR7 receptor transiently expressed in HEK293T cells produces cAMP when stimulated by PRLX (Hsu *et al.*, 2002) with similar potency to that reported in the THP-1 cell line which endogenously expresses the relaxin receptor (Bartsch *et al.*, 2001).

1.7 Aims of the present study

The aim of this study is to pharmacologically characterise the interaction of relaxin with the human LGR7 receptor, and thereby confirm that LGR7 is indeed the relaxin receptor. In order to achieve this aim, a high throughput binding assay was developed, utilising THP-1 cells which endogenously express the human relaxin receptor, CHO cells transiently transfected with the LGR7 receptor and HEK293 cells stably transfected with the LGR7 receptor. To further investigate the structural interaction between HRLX and the relaxin receptor, methods were developed to recombinantly express HRLX peptide and employ molecular cloning techniques to modify the HRLX sequence in order to deduce novel areas within the relaxin peptide that are important for its activity.

To investigate the characteristics of an endogenously expressed human relaxin receptor within a tissue known to display relaxin function, human uterine tissue was

utilised. The expression of relaxin and the relaxin receptor was investigated to gain further insights into the role of relaxin in the human reproductive system.

Figure 1.4

Schematic diagram of a leucine-rich repeat-containing G-protein coupled receptor, LGR7.

LGR7 has the typical seven transmembrane spanning regions positioned within the cell membrane. The large extracellular domain contains the 9 leucine-rich repeat regions and the unique N-terminal low-density lipoprotein receptor-like cysteine-rich (LDLR) domain.

Schematic diagram kindly provided by Daniel Scott, Howard Florey Institute of Experimental Physiology and Medicine, Vic, Aust.

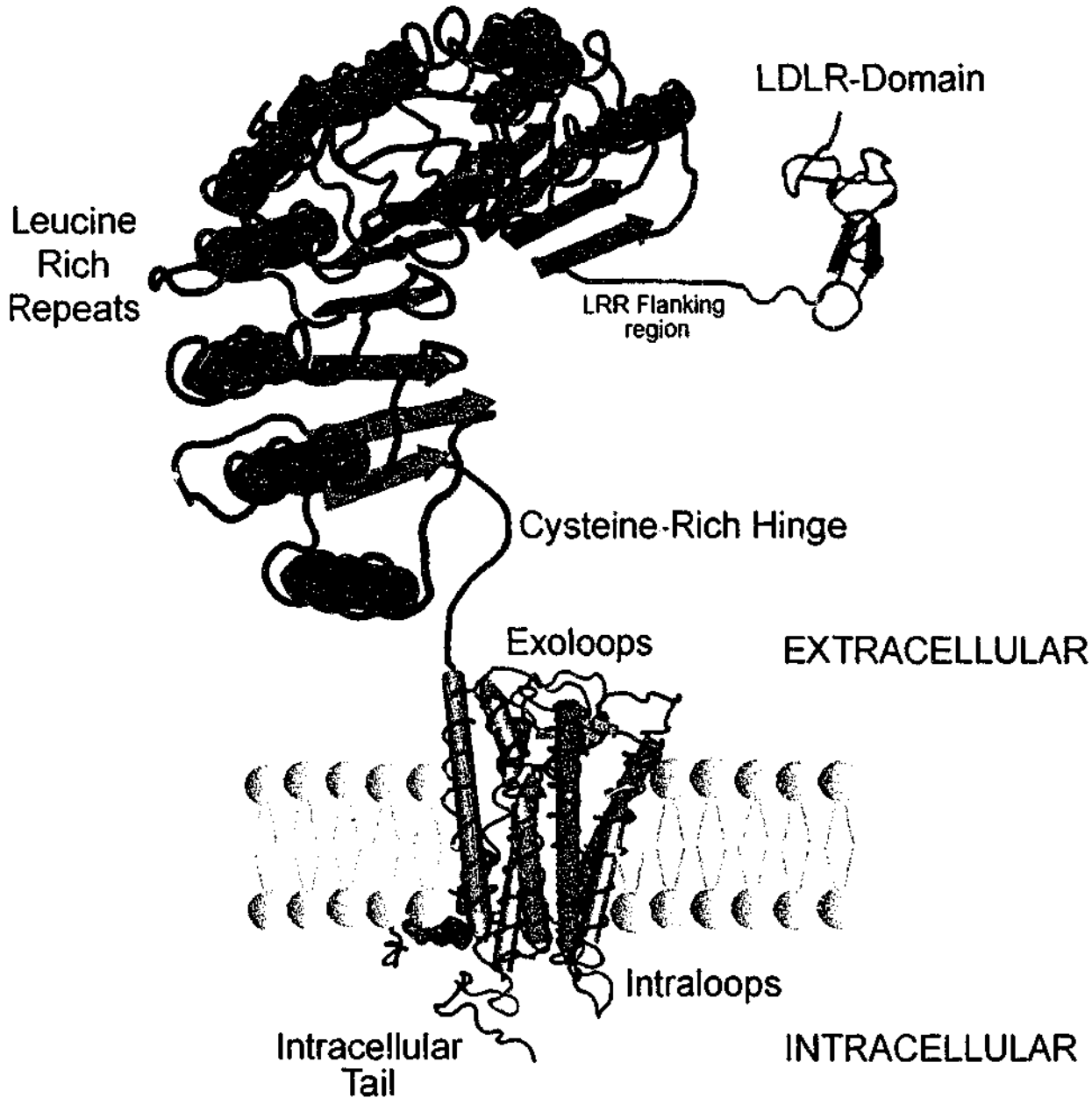
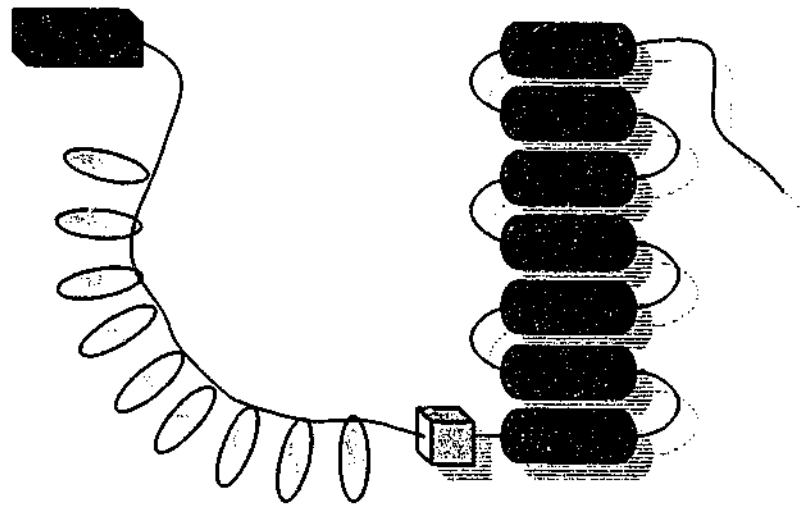

LDLR-Domain
Leucine
Rich
Repeats
LRR Flanking
region
Cysteine-Rich Hinge
Exoloops
EXTRACELLULAR
Intraloops
Intracellular
Tail
INTRACELLULAR

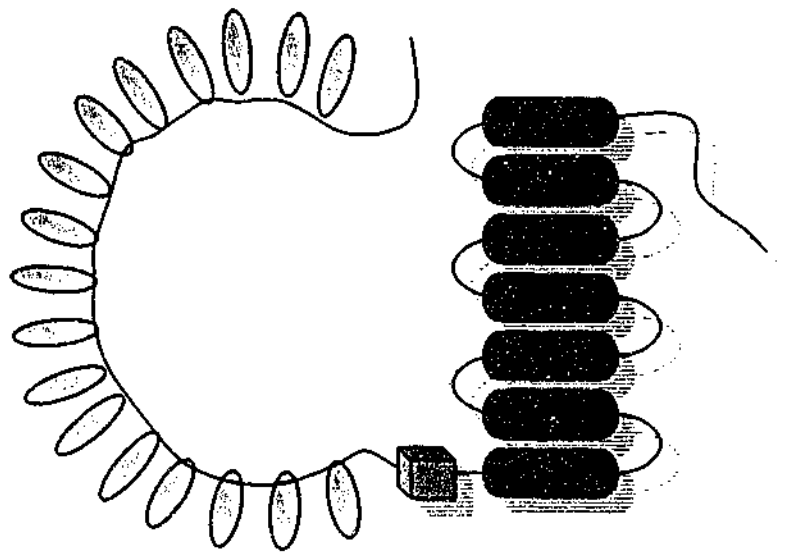
Figure 1.5

Schematic diagram of the three distinct ectodomains that characterise the LGR receptor family.

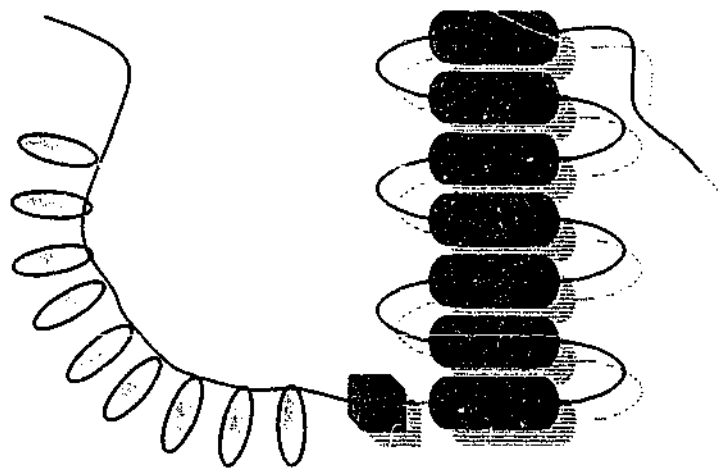
The three subfamilies of LGR receptors are characterised by the structure of the ectodomain; Subfamily A receptors contains 9 leucine-rich repeat regions (LRR,), Subfamily B receptors contain 17 LRR, while Subfamily C receptors contain 9 LRRs with a unique N-terminal low-density lipoprotein (LDL,) receptor-like cysteine-rich domain. Each subfamily contains a distinct hinge region ( ) as denoted by the different colours.



Subfamily C LGR



Subfamily B LGR



Subfamily A LGR

Chapter Two

General Methods

2.1 Cell culture

2.1.1 Maintenance of cell lines

All cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA), except BHK cells which were obtained from Invitrogen (Mount Waverley, Vic, Aust). All cell culture was performed in the cell culture laboratory in PS2 laminar flow hoods under sterile conditions using sterilised equipment. Cells were maintained at 37°C, 5% CO_2, 85% humidity in a CO_2 water-jacketed incubator (Forma Scientific, Marietta, OH, USA). The basic cell culture techniques were as described in Freshney (1994). The cell environment was optimised with the appropriate growth media and nutrients to ensure the continuation of viable cell lines (Freshney, 1994).

2.1.1.1 Non-adherent cell lines

To split cells grown in suspension and renew media, the growth media was transferred from the tissue culture flask to a sterile centrifuge tube, centrifuged for 5 minutes at 1000g to pellet cells and the supernatant removed. Cells were gently washed with Hepes-buffered saline (HBS; 10mM Hepes, 150mM NaCl, pH 7.4) and resuspended in new media. Cells were counted and the appropriate number of cells was transferred into new flasks with media.

THP-1 cells

THP-1 (a human monocyte cell line) cells were maintained in RPMI 1640 medium containing glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 µg/mL) and heat inactivated foetal bovine serum (FBS; 10% v/v). Optimal growth and cell survival was ensured by maintaining the density of cells within $2x10^5$ and $1x10^6$ cells/mL.

2.1.1.2 Adherent cell lines

As the extent of cell growth varied with the adherent cell lines and composition of the medium, splitting of cells and media changes depended on the cell type. Media changes were conducted by removal of the old media from the tissue culture flask and replacement with new media. Once the cells had reached 80-90% confluency, they were split as follows: medium was removed from the culture flask, the cells gently washed 1-2 times with sterilised phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and trypsin/ethylenediaminetetraacetic acid (EDTA) (0.1% trypsin w/v, 0.02% EDTA w/v) solution added. Cells were incubated at 37°C for 5-10 minutes. Cells detached from the flask surface and clumps were disaggregated by pipetting to form a cell suspension. Cells were removed and placed in a new flask with new media, or centrifuged for counting and replating.

BHK cells

BHK (baby hamster kidney) cells were maintained in alpha Modified Eagle's Medium (αMEM) media containing glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 µg/mL) and FBS (5% v/v).

CHO-K1 cells

CHO-K1 (chinese hamster ovary) cells were maintained in 50:50 Dulbecco's Modified Eagle's Medium (DMEM): Ham's F-12 medium containing glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 µg/mL) and heat inactivated FBS (10% v/v).

HEK293T cells

HEK293T (human embryonic kidney) cells were maintained in RPMI 1640 media containing glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 µg/mL) and heat inactivated FBS (10% v/v). All tissue culture plates and flasks were coated with poly-L-lysine (0.1 mg/mL) prior to cell culturing to ensure HEK293T cell adhesion.

2.1.2 Freezing and thawing of cells

2.1.2.1 Freezing of cells

Cells were split according to the cell type, transferred to a sterile centrifuge tube and centrifuged for 5 minutes at 1000g to pellet cells. The supernatant was discarded and the cells resuspended in FBS. FBS containing 20% dimethyl sulfoxide (DMSO) was added to 1 mL resuspended cells/FBS to give a final concentration of DMSO of 10%. Cells were aliquotted (1 mL) into cryotubes, slowly frozen in a -70°C freezer for 24 hours and transferred to liquid nitrogen storage to ensure long term viability of cells.

2.1.2.2 Thawing of cells

Media was warmed to 37°C in a tissue culture flask for 30 minutes. Cells were taken from liquid nitrogen storage and immediately placed in water at 37°C. Thawed cells were added to the pre-warmed tissue culture flask and allowed to settle for 2 hours. Media was replaced with fresh media to remove the DMSO used in the freezing process.

2.1.3 Cell counting

A haemocytometer and cover slip were cleaned with ethanol and the cover slip placed over channels in the haemocytometer. Cells were lifted if necessary and approximately 100 µL of resuspended cells added drop wise to the haemocytometer. Cells were counted under a microscope in the 16 squares and the average number of cells per square calculated. The average number of cells per square was multiplied by 2.5×10^5 to give the number of cells/mL.

2.1.4 Transient transfection of adherent cells

Approximately 18 hours before transfection, cells were split, counted and plated at $8x10^5$ cells/cm^2 in a tissue culture flask with normal media. The transfection solution was prepared under tissue culture conditions and contained the DNA construct (0.136 μg/cm^2), the transfection reagent LIPOFECTAMINE™ (1.088 μL/cm^2) and the transfection media OPTI-MEM® (22.6 μL/cm^2). The transfection mix was incubated for 30-45 minutes at room temperature. Cells were washed 1-2 times with OPTI-MEM® and OPTI-MEM® (90 μL/cm^2) was added to the transfection mix. Cells were then incubated with the transfection mix at 37°C for 4 hours. Serum enriched media (113.4 μL/cm^2) was added to the cells according to cell type and incubated at 37°C for a further 20 hours. Transfection mix media was replaced with normal media and incubated at 37°C for 24 hours. Transfected cells were used 48 hours after transfection.

2.1.5 Production of a cell line stably expressing a foreign protein

Production of a cell line stably expressing the LGR7 receptor involved transiently transfecting cells in 2 wells of a 12-well plate with LGR7 DNA ligated into the pcDNA3.1-Zeo expression vector (Invitrogen, Vic, Aust) containing the zeocin resistance gene. Cells were maintained for 72 hours following transfection, then the contents of both wells were transferred to a 175 cm^2 tissue culture flask with normal media containing zeocin (800 μM) for specific selection. Cells not containing the LGR7 receptor DNA were selectively destroyed by zeocin, while cells containing the desired DNA continued to grow and replicate. Cells were maintained for 1-2 weeks. At 80% confluency, cells were split and resuspended in media containing zeocin at 8 cells/mL. Cells were plated out into 6 x 96-well plates (100 μl/well) and incubated at 37°C for 2-3 weeks. Colonies produced from single cells were selected, screened for expression of relaxin receptor and maintained as individual cell lines.

2.2 Production of recombinant protein.

The Sindbis Expression System (Invitrogen, Vic Aust) was used to produce recombinant relaxin protein in a mammalian expression system. The pSinHis plasmid (Invitrogen, Vic Aust) and the relaxin peptide sequences were analysed for relevant restriction sites. A Pml I restriction site was designed for insertion into the multiple cloning site of the pSinHis plasmid and into the 5' end of the relaxin peptide segment. Oligonucleotides were designed (Table 2.1) to incorporate the Pml I restriction site and an appropriate sequence for the correct cloning of the relaxin peptide sequence into the pSinHis plasmid (Figure 2.1).

2.2.1 Incorporation of Pml I restriction site into the pSinHis plasmid

The pSinHis plasmid sequence was altered to include the Pml I restriction enzyme cleavage site (Figure 2.2) using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, CA, USA) according to the manufacturer's instructions. Briefly, the mutagenesis reaction (20 µL) contained the Pml I restriction site, forward and reverse primers (Table 2.1; 80 ng each), 1x reaction buffer, dNTPs (1 mM), pSinHis A plasmid DNA (100 ng) and Pfu enzyme (2.5 U). The reaction parameters included heating to 92°C for 2 minutes, 18 cycles of [95°C for 30 seconds, 55°C for 1 minute, 68°C for 25 minutes], followed by 25°C for 5 minutes and ending on 4°C. Although this was a set of single amino acid changes, 18 cycles was chosen due to the large size of the plasmid (10 kB). Following the mutagenesis reaction, the DNA was incubated for 2 hours at 37°C with Dpn1 to remove any non-methylated DNA, precipitated and resuspended in sterilised deionised water (10 µL).

Table 2.1

Nucleotide sequences of primers used in the production of recombinant relaxin peptides.

Primer Name	Sequence (5' ⇒ 3')
Primers for pSinHis Pml I site mutagensis	
pSinHis Pml for	CTG GTG GAC AGC ACG TGG GTC GGG ATC TG
pSinHis Pml rev	CAG ATC CCG ACC CAC GTG CTG TCC ACC AG
Primers for RLX insert amplification	
RLX for	GGT GGA CAG CAC GTG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GAC TCA TGG ATG GAG GAA G
RLX rev	CGG CCA GTG GCA TGC TAA GCT TCA GCA AAA TCT AGC
Primers to sequence PCR-Script/RLX insert ligation	
T3	AAT TAA CCC TCA CTA AAG GG
T7	TAA TAC GAC TCA CTA TAG GG
Primers to sequence pSinHis plasmid/RLX insert ligation	
pSinHis Seq for	CTA CAA CAC CAC CAC CTC TAG ACA CCA TGG G
pSinHis Seq rev	CCT CGG AAG TAC ATC GAG TTT TGC TGG TCG G
pRLX Seq for	CAC CTA GAC CAG TGG CAG AAA TTG TGC CAT CC
pRLX Seq rev	CTG AAG GAC TGC TGT CTG CGG CTT CAC

Figure 2.1

Schematic diagram of the insertion of relaxin peptide DNA into the pSinHis expression vector.

The relaxin insert generated for ligation into the pSinHis multiple cloning site incorporated the EK recognition and cleavage sequence to allow use of the Pml I restriction site and to maintain the EK enzyme restriction site for removal of the polyhistidine tag from the relaxin peptide following purification.

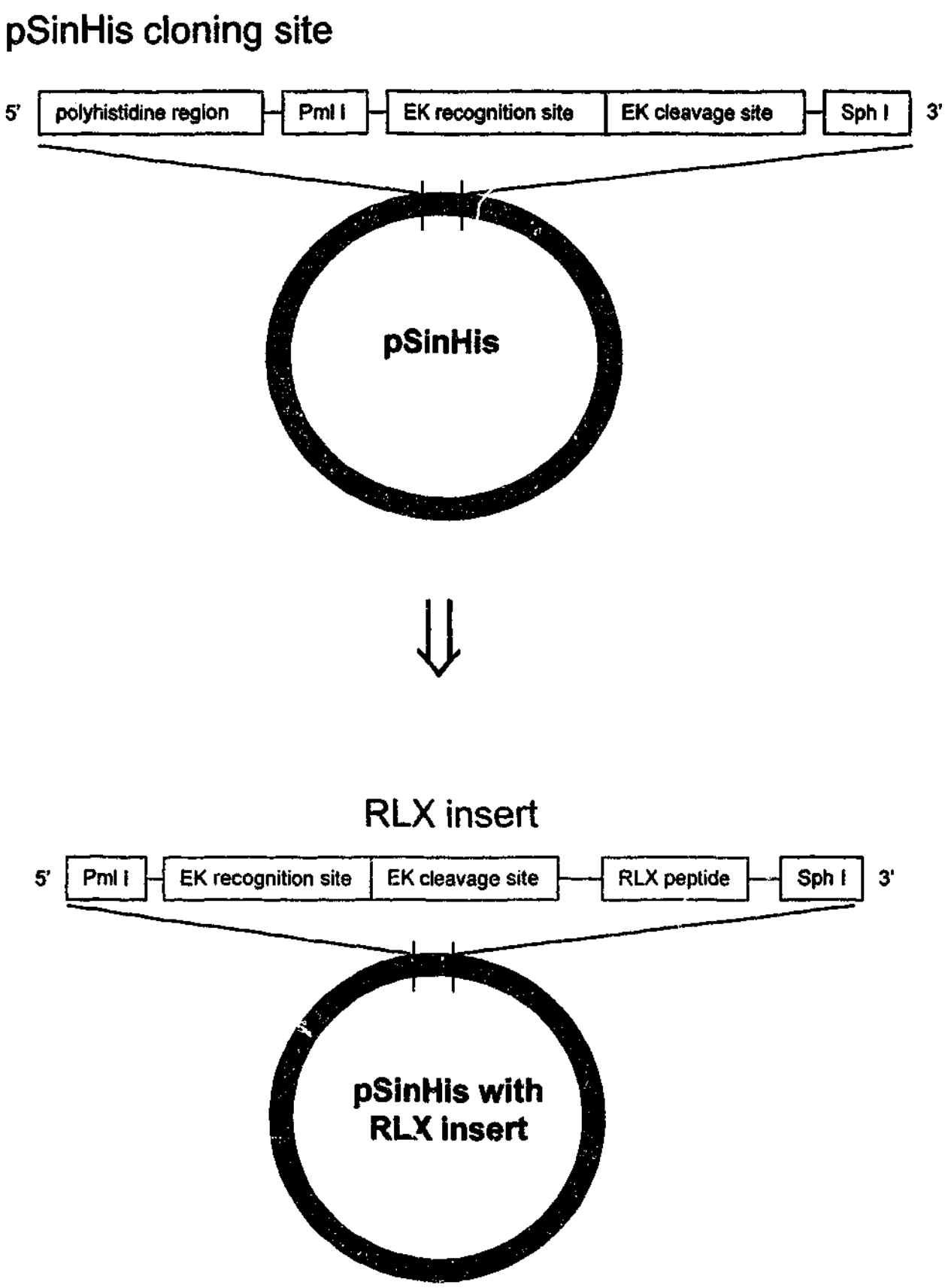
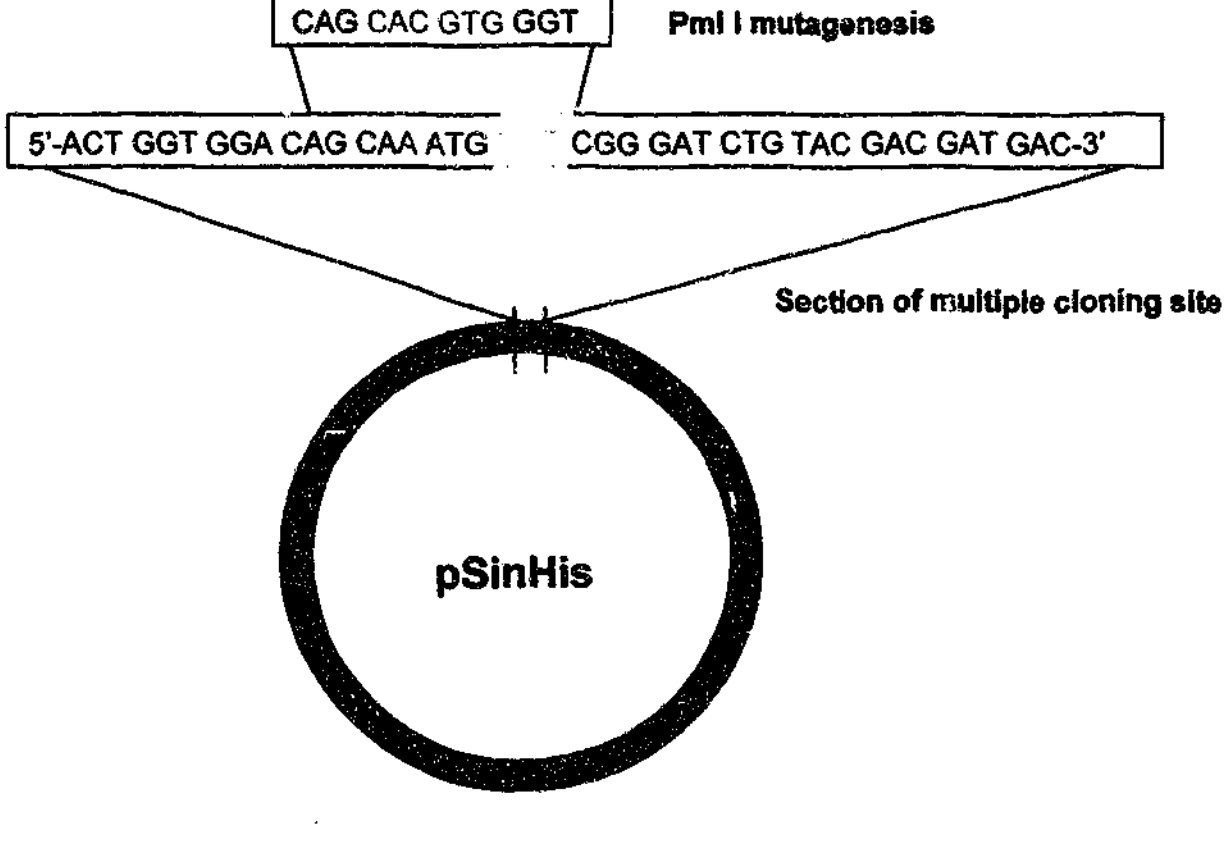

pSinHis cloning site
5'
polyhistidine region
Pml I
EK recognition site
EK cleavage site
Sph I
3'
pSinHis
RLX insert
5'
Pml I
EK recognition site
EK cleavage site
RLX peptide
Sph I
3'
pSinHis with
RLX insert

Figure 2.2

Schematic diagram of the Pml I restriction site mutagenesis.

The insertion of the Pml I restriction site involved the mutation of two nucleotides within the pSinHis multiple cloning site sequence.


Pml I cleavage site
CAG CAC GTG GGT
Pml I mutagenesis
5'-ACT GGT GGA CAG CAA ATG
CGG GAT CTG TAC GAC GAT GAC-3'
Section of multiple cloning site
pSinHis

2.2.1.1 Transformation of pSinHis plasmid into $DH_{5\alpha}$ cells

The altered pSinHis plasmid was transformed into competent $DH_{5\alpha}$ cells (Sharma *et al.*, 1996). The pSinHis DNA (3 µL) was combined with 50 µL of competent $DH_{5\alpha}$ cells, incubated on ice for 5 minutes and transferred to a BioRad Gene Pulser/*E. coli* Pulser cuvette (0.2 cm electrode gap). Electroporation was carried out at 2500 V, 25 µF capacitance and 200 Ohms using a Gene Pulser II electroporation system (BioRad Laboratories, CA, USA). Immediately following transformation, 1 mL of pre-warmed SOC media (20 g/L casein hydrolysate, 5 g/L yeast extract, 0.5 g/L NaCl, 2.5 mM KCl, pH 7, 10 mM $MgCl_2$ and 20 mM glucose) was added to the cuvette. Cuvette contents were gently mixed, transferred to a microcentrifuge tube and incubated in an orbital shaker (37°C for 1 hour). The transformed cells were plated onto agar LB plates (LB broth: 1% tryptone, 0.5% yeast, 1% NaCl, 75 mg/L ampicillin; agar LB plates: LB broth, 1% agar) and incubated at 37°C for 16 hours.

2.2.1.2 Colony selection

Single colonies of cells grown on the agar LB plates were selected, picked with sterile toothpicks under sterile conditions and set in miniprep cultures according to the Wizard Plus Miniprep DNA Purification System (Promega, WI, USA; including cell suspension, lysis, neutralization, wash and elution buffers). Briefly, selected colonies were placed in 5 mL LB broth and incubated for 16 hours at 37°C in an orbital shaker. Cells were pelleted (2 minutes, 10,000xg), the supernatant discarded and cells resuspended in cell suspension solution (250 µL). Lysis buffer (250 µL) was added and the mixture gently inverted. Cell lysis was halted by addition of chilled neutralisation solution (350 µL), gently mixed by inversion and the cell debris pelleted (10 minutes, 10,000xg at 4°C). The supernatant containing the DNA was transferred to a miniprep filter tube contained in a collection tube and centrifuged (1 minute, 14,000xg) to harvest the DNA onto the filter. The filtrate was discarded, the filter tube reconnected to the collection tube, and wash buffer (700 µL) was added and centrifuged (1 minute, 14,000xg). The filtrate was discarded and

the filter tube centrifuged (1 minute, 14,000xg) to ensure a dry and clean filter. Any additional filtrate was discarded and the filter tube was reconnected to a sterilized Eppendorf tube. DNA was eluted from the filter with additional elution buffer (100 µL) and centrifugation (30 seconds, 14,000xg). DNA was stored at -20°C. For long term storage, glycerol stocks of the miniprep cell culture were made (cells in 50% glycerol) and stored at -70°C.

The selected colonies of pSinHis plasmid DNA were checked for insertion of the Pml I restriction site. The purified plasmid concentrations were calculated as described in Section 2.5. Due to the large size of the plasmid (10 kB), 1 µg of purified plasmid DNA was digested in the reaction mixture of 1x One-Phor-All buffer, 0.01% BSA, 20 U Xho I enzyme and 40 U Pml I enzyme in a total volume of 100 µL (37°C for 2 hours). A sample of the digestion (10 µL + 2 µL loading dye) was run on a 1.3% agarose gel and appropriate band sizes were visualized for pSinHis containing the Pml I restriction site (450 bp dropout).

2.2.1.3 Amplification of altered pSinHis plasmid DNA

The DNA samples containing the correct digestion profile were further amplified with the Wizard Plus Midipreps DNA Purification System (Promega, WI, USA; including cell suspension, lysis, neutralization and wash buffers, purification resin and midicolumns). Briefly, 100 µL of glycerol stocks of the selected DNA samples were placed in 100 mL LB broth and incubated for 16 hours at 37°C in an orbital shaker. Cells were pelleted (10 minutes, 4000xg at 4°C), supernatant discarded and cells resuspended in cell suspension solution (3 mL). Lysis buffer (3 mL) was added, gently mixed by inversion of the tube and cell lysis terminated by addition of Neutralization buffer (3 mL). Cell debris was pelleted (15 minutes, 14,000xg at 4°C) and the supernatant containing the DNA transferred to a separate tube. Purification resin (10 mL) was added and swirled to mix. A Midiprep purification column was attached to a vacuum manifold. The resin and DNA solution was transferred and passed through the column allowing the plasmid DNA to adhere to the filter/resin. After washing with buffer (30 mL) the column was removed from the vacuum manifold. Filter/resin containing plasmid DNA was cut from column,

placed into a sterile Eppendorf tube and centrifuged (2 minutes, 10,000xg) to remove excess wash buffer. The resin filter was transferred to a new tube, preheated sterilised deionised water (70°C, 300 µL) added and the DNA removed from the exhausted resin by centrifugation (20 seconds, 10,000xg). The purified plasmid concentrations were calculated (Section 2.5) and DNA was stored at -20°C.

2.2.2 Production of the relaxin DNA fragment

The relaxin peptide DNA sequence was obtained from Dr Ross Bathgate (Howard Florey Institute of Experimental Physiology and Medicine, Vic, Aust) with the relaxin peptide DNA in the pMAL plasmid. Primers were designed to incorporate a Pml I restriction site, the pre-relaxin sequence of the pSinHis plasmid and the 4' end of the relaxin sequence (Table 2.1) to amplify by PCR a relaxin DNA segment for ligation. The relaxin DNA segment amplification PCR reaction (20 µL) contained forward and reverse primers (50 ng each), pMAL vector with relaxin DNA (25 ng), 1x reaction buffer, $MgSO_4$ (1.5 mM), dNTPs (1 mM). with Pfx polymerase (2.5 Units). The PCR conditions included 35 cycles with an annealing temperature of 62°C to form a blunt end DNA fragment.

The PCR product was run on a 1.3% low melting temperature agarose gel to visualize the DNA fragment. The DNA fragment size of ~500 bp was the expected size of the relaxin DNA fragment.

2.2.2.1 Purification of relaxin PCR DNA fragment

The relaxin DNA band was carefully cut from gel and purified using the Wizard PCR Preps DNA Purification System (Promega, WI, USA; including purification resin, buffers and columns). Briefly, the agarose slice of DNA was placed in a sterile Eppendorf tube and melted at 70°C. Resin (1 mL) was added to the melted agarose and gently mixed. A minicolumn was attached to a 3 mL syringe barrel, resin mix was added and pushed through the syringe with DNA adhering to the filter/resin in the column. Isopropanol (80%, 2 mL) was washed though the column, the minicolumn detached, placed in a sterile Eppendorf tube and centrifuged (2

minutes, 10,000xg) to remove excess isopropanol. The Minicolumn was transferred to a clean tube and DNA eluted with 50 µl of nuclease-free water (20 seconds, 10,000xg). The DNA concentration was calculated (Section 2.5) and stored at -20°C.

2.2.2.2 Ligation of relaxin DNA fragments into PCR-script.

To ensure correct digestion of relaxin DNA fragment to allow for correct ligation into pSinHis, the relaxin PCR fragment was ligated into PCR-script using a PCR-Script™ Amp Electroporation-Competent Cell Cloning Kit (Stratagene, CA, USA; including cloning vectors, reaction buffers, enzymes and selection tools). Briefly, the ligation reaction included 10 ng pPCR-script Amp SK(+) cloning vector, 1x reaction buffer, 0.5 mM rATP, 170 ng relaxin PCR fragment, 5 U Srf I restriction enzyme and 4 U T4 DNA ligase in a 10 µL reaction (2 hours at room temperature). The reaction was inactivated by incubation at 65°C for 10 minutes, precipitated and resuspended in 10 µL of deionised water.

The ligated DNA was transformed into XLI-Blue MRF competent cells as previously described with $DH_{5\alpha}$ competent cells (Section 2.2.1.1). X-gal (20 mg/mL) was spread over the agar LB plates 30 minutes prior to plating out transformed cells. The XLI-Blue MRF competent cells on the X-gal will show cells containing the ligated relaxin PCR fragment as white, while cells containing the vector without the relaxin PCR fragment will appear blue in colour, allowing for an accurate selection of colonies containing the ligated DNA. The transformed cells were plated onto the agar LB plates and were placed in an orbital shaker (37°C for 16 hours). Single white colonies of cells grown on the agar LB plates were selected, picked in a sterile environment and set in miniprep cultures according to the Wizard Plus Miniprep DNA Purification System (Promega, WI, USA) as previously described (Section 2.2.1.2). The DNA was purified from the miniprep cultures, the concentration calculated and screened by digestion to ensure that the correct relaxin PCR fragment was present. The digestion reaction mix included 1x NE 1 buffer (New England Biolabs; 10 mM Bis Tris Propane-HCl, 10 mM MgCl, 1 mM DTT, pH 7), 0.01% BSA, 5 U Sph I enzyme, 20 U Pml I enzyme and 1 µg purified DNA

in a total volume of 20 µL and was incubated at 37°C for 2 hours. A sample of the digest (10 µL + 2 µL loading dye) was run on a 1.3% agarose gel and an appropriate band size for the relaxin DNA fragment (~500 bp) was seen in various samples.

2.2.3 Ligation of relaxin DNA fragment into the pSinHis plasmid

The altered pSinHis plasmid (5 µg) and PCR-script vector containing the relaxin fragment (2 µg) were digested in a reaction mixture including 1x NE 1 buffer (New England Biolabs), 0.01% BSA, 25 U Sph I enzyme and 100 U Pml I enzyme in a total volume of 100 µL at 37°C for 2 hours. The digested DNA was loaded onto a 1.3% low melting temperature agarose gel where the relaxin DNA fragment bands (~500 bp) and linearised pSinHis DNA bands (~10 kB) were carefully cut from the gel. The DNA was purified using a Freeze and Squeeze phenol chloroform extraction method. The pieces of agarose gel were weighed, cut into small pieces and placed in sterile Eppendorf tubes with an equal volume of phenol (0.1g agarose to 100 µL phenol). The DNA was frozen at -70°C for 10 minutes, centrifuged for 15 minutes (14,000xg at 4°C) and the aqueous phase carefully transferred to a clean tube and the volume determined. Phenol (50% of aqueous phase volume) was added to the aqueous phase, mixed and centrifuged for 5 minutes (14,000xg at 4°C). An equal volume of chloroform was then added (50% of original aqueous phase volume), DNA solution was mixed and centrifuged for 10 minutes (14,000xg at 4°C). The upper phase containing the DNA was transferred to a clean tube. DNA was precipitated by the addition of 1/10 volume of sodium acetate (NaOAc; 3 M, pH5.2) and 2.5 x volume of absolute ethanol, mixed and incubated at -20°C for 20 minutes. DNA was centrifuged for 25 minutes (14,000xg at 4°C), supernatant discarded and the DNA pellet was carefully washed twice with 70% ethanol. The DNA pellet was air dried, resuspended in sterilized deionised water (20 µL), and the concentration calculated (Section 2.5) before storage at -20°C.

The ligation of the relaxin fragment into the pSinHis plasmid was carried out in a reaction mixture of 200 ng pSinHis plasmid DNA, 40 ng relaxin fragment DNA, 1x One-Phor-All buffer, 1 mM ATP and 4 U T4 DNA ligase in a total volume of 20 µL

which was incubated overnight at room temperature. Ligation mix was precipitated and resuspended in sterilized deionised water (10 µL). Ligation DNA was transformed into $DH_{5\alpha}$ competent cells as previously described (Section 2.2.1.1), plated onto agar LB plates, colonies grown overnight, single colonies selected, set in miniprep cultures and DNA purified according to the Wizard Plus Miniprep DNA Purification System (Promega, WI, USA) as previously described (Section 2.2.1.2). The purified ligated DNA was digested to screen the colonies for correct ligation of relaxin fragment into pSinHis. The digestion reaction included 1x Buffer H (Amersham Pharmacia Biotech, England, UK), 0.01% BSA, 0.01% Triton X-100 (Amersham Pharmacia Biotech, England, UK), 5 U Not I enzyme and 0.5 µg purified DNA in a total volume of 20 µL incubated at 37°C for 2 hours. Digestion with Not I enzyme will only produce a dropout band of ~850 bp if the relaxin fragment is ligated into pSinHis plasmid in the correct orientation. Successful ligation was confirmed in several of the selected colonies when the digested DNA was visualised on a 1.3% agarose gel.

2.2.3.1 Sequencing of pSinHis plasmid containing the relaxin construct

Before sequencing, DNA was further purified by polyethylene glycol (PEG) precipitation. Briefly, 0.16 x volume of NaCl (5 M) and 1x total volume of 13% PEG was added to the DNA. The mixture was incubated on ice for 20 minutes, centrifuged for 25 minutes (14,000xg at 4°C), the supernatant discarded and the DNA pellet gently washed twice with 70% ethanol. The pellet was air dried, dissolved in 2/3 x original volume of sterilized deionised water and the concentration of DNA calculated (Section 2.5).

The sequencing amplification reaction mix (20 µL) contained PEG purified pSinHis plasmid DNA containing the relaxin insert (800 ng), sequencing primer (4 pmol), BigDye Terminator Mix (v3.1, 4 µL; Applied Biosystems, CA, USA) and DMSO (5%). The PCR sequencing parameters were heating at 96°C for 1 minute, 25 cycles of [96°C for 10 sec, 50°C for 5 sec, 60°C for 4 minutes], ending on 4°C. At completion of the PCR reaction, DNA was precipitated with 95% ethanol and NaOAc (0.3 M, pH 5.2), washed with 70% ethanol and air dried at room temperature

before being processed by electrophoresis through a denaturing polyacrylamide gel (Micromon Sequencing Facility, Monash University, Vic, Aust; Sambrook *et al.*, 2001).

2.2.4 RNA transcription of DNA templates

The pSinHis plasmid, with and without the relaxin construct (~20 µg), was digested with Xho I (2 hours, 37°C) to linearise the DNA. The digestion was terminated with 1/20 volume of 0.5M EDTA and the DNA extracted by phenol/chloroform extraction. The DNA was precipitated with 1/15 x volume 7.5 M ammonium acetate (RNase free, Sigma) and 2x volume 100% ethanol (-20°C for 20 minutes, centrifugation for 25 minutes at 4°C), washed twice with 70% ethanol (400 µL, centrifugation for 5 minutes at 4°C) to ensure a clean DNA template for RNA transcription. The pellet was dried at room temperature and resuspended in 15 µL RNase-free Tris-EDTA buffer (TE buffer; Promega). DNA concentration was measured (Section 2.5) and adjusted accordingly to obtain a final DNA concentration of 0.6 µg/µL.

The RNA transcription method was based on a combination of the 'SFV gene expression system' manual and products (Invitrogen, page 23) and 'Sindbis expression system' manual (Invitrogen, page 17-18). The 50 µL reaction was set up in a 1.5 mL tube (autoclaved) containing NTP mix (20 µL), 5x SP6 RNA polymerase buffer (10 µL), m7G5'ppp5'G RNA cap analogue structure (5 µL), 10 mM DTT (5 µL), RNasin (40 U/µL; 1.3 µL), RNase free TE (3.7 µL), linearised plasmid (1.5 µg; 2.5 µL) and SP6 RNA polymerase (38 U; 2.5 µL). The reaction was incubated for 2 hours at 37°C.

A sample of RNA was examined on a 1% agarose gel to check the quality of the RNA. RNA was precipitated with 1/15x volume 7.5 M ammonium acetate (RNase free, Sigma) and 2x volume 100% ethanol (-20°C for 20 minutes, centrifugation for 25 minutes at 4°C), washed twice with 70% ethanol (400 µL, centrifugation for 5

minute at 4°C) and the pellet resuspended in 20 µL RNase-free (DEPC treated) dH_2O. RNA concentration was measured (Section 2.5) and stored at -70°C.

2.2.5 Transfection of pSinHis RNA transcripts into BHK cells

Approximately 18 hours before transfection, BHK cells were split, counted and plated at $8x10^5$ cells/cm^2 in a tissue culture flask with αMEM media. The transfection solution included the RNA transcript of pSinHis plasmid containing the relaxin construct (0.17 µg/cm^2), the transfection reagent DMRIE™ (1.37 µL/cm^2) and the transfection media OPTI-MEM® (120 µL/cm^2). Cells were washed 1-2 times with OPTI-MEM® and incubated with the transfection mix at 37°C for 4 hours. Following the 4 hours, transfection solution was removed from cells and replaced with serum enriched αMEM media (120 µL/cm^2) incubated at 37°C for a further 20 hours. Some transfected BHK cells were harvested after 24 hours following transfection, while the remaining BHK cells were given a media change and were harvested 48 hours after transfection.

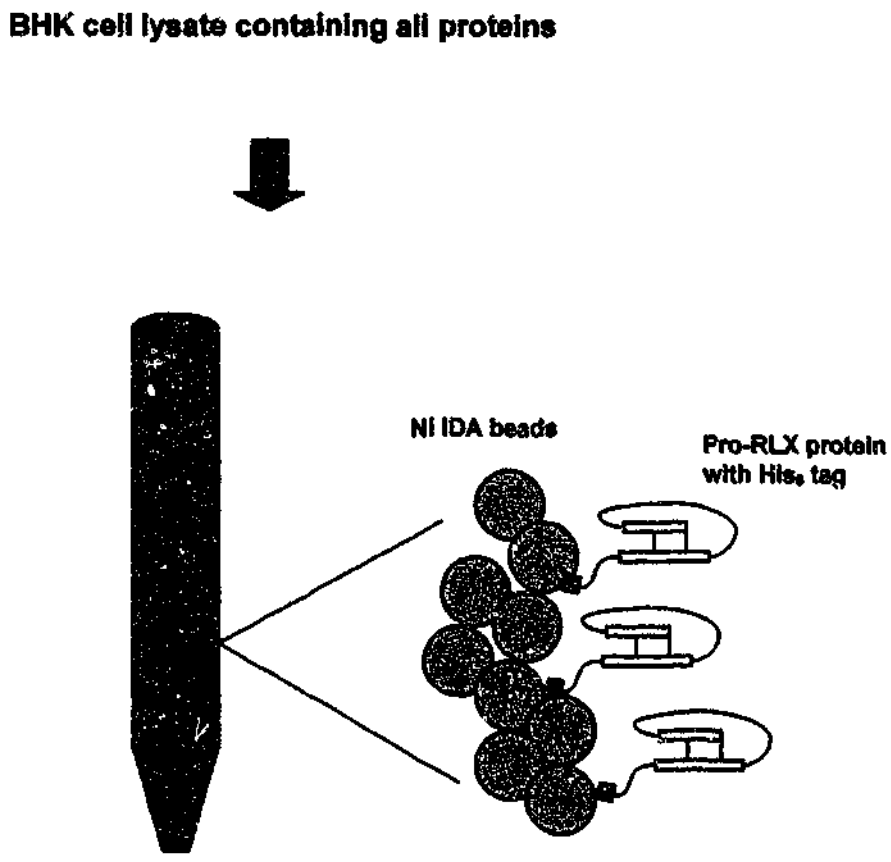
2.2.6 Protein extraction

The ProBond™ Purification System (Invitrogen, Vic, Aust) was used to extract and purify the polyhistidine-RLX protein. The polyhistidine tail displays high affinity for the pre-equilibrated nickel IDA bead column (Scientifix, Vic, Aust), binding the relaxin peptide and removing all excess lysate. The relaxin peptide was cleaved from its His_6 tag and purified from the nickel column (Figure 2.3).

Figure 2.3

Purification of polyhistidine tagged-RLX protein.

The expressed pro-relaxin peptide was purified from the BHK cell lysate through a nickel IDA column. The polyhistidine tag attached to the pro-relaxin peptide displayed high affinity for the activated column, binding to the beads and allowing the cell debris to be washed through. The pro-relaxin peptide was then cleaved from the polyhistidine tag with the EKMax™ enzyme and eluted from the column.


BHK cell lysate containing all proteins
Ni IDA beads
Pro-RLX protein with His6 tag
Incubation with EKMax™ enzyme
pro-RLX peptide

2.2.6.1 Prepare column for purification

The nickel beads in the column were gently resuspended in the storage mixture using a Pasteur pipette and storage solution eluted without allowing the column to dry out. The resin was washed twice with 7 mL sterilized deionised water, equilibrated with 7 mL binding buffer and excess buffer eluted. The equilibration process was repeated three times before protein extraction.

2.2.6.2 Protein purification

Transfected BHK cells were washed with PBS, lifted and transferred into a 15 mL centrifuge tube. Cells were centrifuged, supernatant removed and the cells resuspended in 4 mL binding buffer (20 mM $NaPO_4$, 500 mM NaCl; pH 7.6). The cells were lysed by freezing in a dry ice/ethanol bath and thawing in a 42°C water bath. The freeze/thaw method was repeated twice. The cell lysis suspension was passed through an 18-gauge needle four times to shear unwanted DNA. The cell debris was pelleted by centrifugation and the supernatant transferred to a new tube (containing the protein) and kept on ice. The pellet was kept for analysis.

The cell lysate preparation was added to the pre-equilibrated resin, column was sealed and incubated with gentle rocking at room temp for 20 minutes. The supernatant was eluted and kept for analysis. The resin was washed twice with 2x bed volume of binding buffer (pH 7.8), twice with 2x bed volume of wash buffer (20mM $NaPO_4$, 500 mM NaCl; pH 6.0) and twice with 2x bed volumes of binding buffer (without NaCl; pH 7.8). After each wash the supernatant was eluted and kept for analysis.

After the final wash, the resin was resuspended in 500 µL binding buffer (without NaCl; pH 7.8) and 50 µL 10x EKMax buffer (Invitrogen). EKMax enzyme (10 U) was added to the suspension, column was sealed and rocked overnight at room temperature. The supernatant containing the protein was eluted and collected. The

resin was resuspended three times in 2x resin volume of wash buffer and the supernatant containing the fusion protein (His_6 tag) was eluted into a new tube.

The resin was stripped with 3 x column volume of buffer containing 40 mM EDTA (pH 9.0). The resin was recharged by washing with 3-5 volumes dH_2O, followed by 50 mM $NiSO_4$ (1 mL $NiSO_4$ per 1 mL resin). The resin was stored in 50 mM phosphate buffer containing 20% ethanol at 4°C.

2.2.7 Analysis of recombinant protein

Separated protein samples were visualised on a 16% polyacrylamide gel (Section 2.7). Protein samples were mixed with an equal volume of 2 x SDS buffer (without dithiothreitol) and boiled for 5 minutes. Samples were loaded (15 µL) together with a Prestained Precision Protein Standard marker (PPS, 10µL; BioRad, CA, USA) and H2RLX peptide as a positive control. For the Western blotting and immunostaining protocol, please refer to Section 2.7. Relaxin was visualised on the membrane using a relaxin specific polyclonal primary antibody (1:2000 dilution) and an anti-rabbit secondary antibody (1:1000 dilution).

2.3 Radioligand binding studies

2.3.1 Preparation of [^{33}P]-H2RLX

2.3.1.1 Labelling of recombinant H2RLX with [γ-^{33}P]-ATP.

H2RLX was labelled with [γ-^{33}P]-adenosine triphosphate (ATP) using the procedure of Osheroff *et al* (1990) with modifications (Figure 2.4). The reaction was carried out in siliconised tubes which contained 4 µg recombinant H2RLX (2 mg/mL stock), 5 µL of reaction buffer (20 mM Tris-HCl, 100 mM NaCl, 20 mM $MgCl_2$, pH7.5), 5 µCi [γ-^{33}P]-ATP (specific activity 2000 Ci/mmol) and 400 U of cAMP-dependent protein kinase (cAMP-dPK) isolated from bovine heart. The reaction was carried out in a 37°C water bath for 1 hour and terminated by placing on ice.

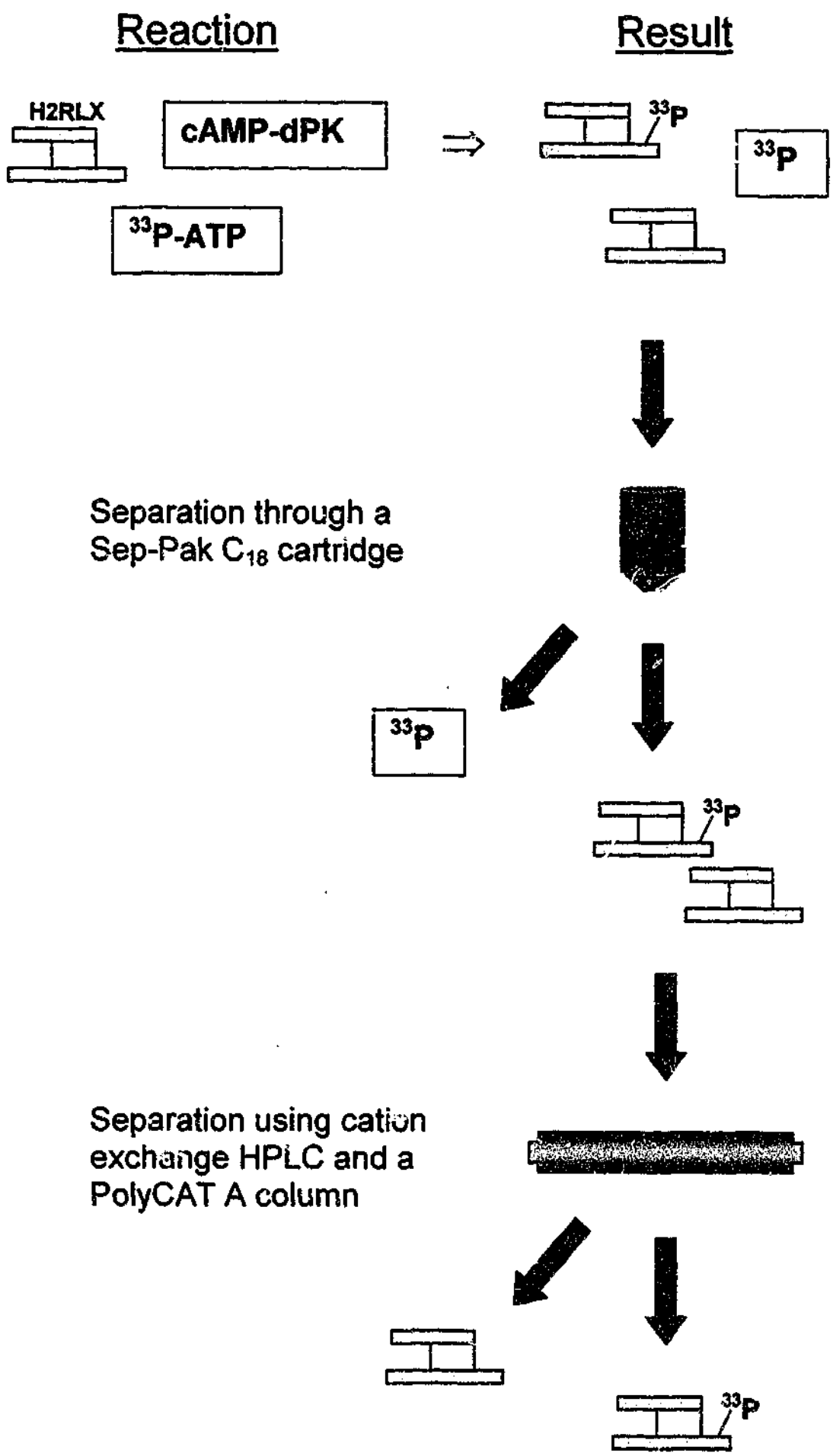
2.3.1.2 Separation of unreacted [γ-^{33}P]-ATP from labelled relaxin.

Separation of unreacted [γ-^{33}P]-ATP from labelled and unlabelled relaxin was accomplished using a Sep-Pak C_{18} cartridge (Waters) (Figure 2.4). A 6 mL syringe barrel was attached to the cartridge. The cartridge resin was activated with acetonitrile, making sure that no air bubbles were introduced which hinder the flow through the cartridge. The resin was washed with deionised water, followed by BSA solution (1 mg/mL BSA in 0.1% TFA), and finally allowed to equilibrate with ATP (1 mM ATP in 0.1% TFA solution). The reaction mixture was added to the syringe with a total of 3 mL ATP (1 mM solution), followed by 4 mL 10% acetonitrile in 0.1% TFA and fractions collected (1 mL) in glass test tubes. The labelled and unlabelled relaxin was eluted using 3 mL 80% acetonitrile in 0.1% TFA solution. A sample of each fraction (10 µL) was analysed by liquid scintillation spectrometry (TRI-CARB model 1900 TR, Perkin-Elmer, Vic, Aust).

Figure 2.4

Schematic diagram of the [^{33}P] labelling of H2RLX

H2RLX was labelled with [^{33}P]-ATP in a reaction catalysed by cAMP dependent protein kinase (cAMP-dPK) resulting in labelled H2RLX, unlabelled H2RLX and free radiolabel [^{33}P]. The free [^{33}P] was removed via purification through a Sep-Pak C_{18} cartridge. A pure sample of [^{33}P]-H2RLX was obtained by separating the unlabelled H2RLX from the labelled H2RLX through a PolyCAT A column on a cation exchange HPLC.


Reaction
Result
H2RLX
cAMP-dPK
33P-ATP
33P
33P
Separation through a
Sep-Pak C18 cartridge
33P
33P
Separation using cation
exchange HPLC and a
PolyCAT A column
33P

2.3.1.3 Separation of labelled and unlabelled relaxin

The labelled relaxin was separated from the unlabelled relaxin using cation exchange HPLC (Waters) incorporating a PolyCAT A column (Poly LC, USA). The PolyCAT A column is negatively charged, allowing the adhesion of positively charged peptides. Peptides are eluted from the column according to the strength of their positive charge by an increasing salt gradient applying positively charged counter-ions. The relaxin peptide containing the [γ-^{33}P] has an extra negatively charged phosphorous ion, forcing it to be eluted earlier than the unlabelled relaxin peptide (Figure 2.4).

The PolyCAT A column was equilibrated with Buffer A (25 mM KH_2PO_4, 25% acetonitrile, pH 7.4). The radioactive sample from the first elution step (Section 2.3.1.2) was injected onto the column. The column was eluted with a linear gradient of 0 – 0.5 M KCl in Buffer A at 1 mL/minute for a 50 minute period through a spectrophotometer measuring the absorbance at 214 nm. Elution fractions were collected at one-minute intervals in glass test tubes until the excess unlabelled relaxin peptide was detected by its large absorbance peak. A sample of each fraction (10 µL) was analysed by liquid scintillation spectrometry, and the fraction containing the peak radioactivity corresponding to [^{33}P]-H2RLX was transferred into a siliconised tube containing 1 mg of BSA, stored at 4°C and used within seven days.

2.3.2 Quantitative Autoradiography

2.3.2.1 Tissue preparation

Animal tissue preparation

Male Sprague-Dawley rats (200-250g) were anaesthetized with 80% CO_2/20% O_2 gas mixture and decapitated. The heart was removed and washed in chilled 1:1 0.32M sucrose: Krebs phosphate (119 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 10 mM $NaH_2PO_4.2H_2O$) solution. The atria were dissected from the ventricles and frozen in isopentane chilled by liquid nitrogen. The brain was dissected, the brain stem and occipital lobe removed and the brain frozen on a cork platform floating in liquid nitrogen. Tissues were stored at –70°C for up to four weeks. Frozen rat atria and brain were mounted in OCT in the cryostat, and 10 µm sections cut at -18°C, and mounted on precleaned microscope slides subbed with poly-l-lysine (0.01% solution). Slide mounted sections were stored at -20°C.

Human uterine tissue preparation

Full thickness endometrial tissue was collected by qualified nurses at 27 hysterectomy operations performed on ovulating women suffering from menorrhagia (age range 34-46). Subjects had not received exogenous hormones in the three months prior to hysterectomy, and endometrial tissue appeared normal by routine histological examination. Ethical approval for this study was obtained from the Southern Health Human Research and Ethics Committee B and informed consent was received from each subject. Tissue blocks containing the intact endometrial layer and several mm of myometrium were frozen in liquid nitrogen and stored at –80°C. Frozen blocks of uterine tissue were mounted in OCT in the cryostat, cut into 10 µm sections at -18°C and collected on precleaned microscope slides subbed with poly-l-lysine (0.01% solution) identified only by their catalogue number. Slides were stored at -20°C. Following the completion of the binding assay, the uterine sections were analysed on ImageQuaNT™ (Version 4.1; Molecular Dynamics, U.S.A.) and the data grouped according to the menstrual phase in GraphPad PRISM (GraphPad Inc., San Diego, U.S.A.).

2.3.2.2 [^{33}P]-H2RLX quantitative autoradiography binding protocol

Slides were removed from storage at -20°C and equilibrated to room temperature in sealed containers with silica gel to prevent development of condensation. A moist chamber was pre-heated to 25°C, and slides were pre-incubated for 30 minutes with the section covered with 100 µL of binding buffer (25 mM Hepes, 300 mM KCl) containing 1 mM phenylmethylsulphonyfluoride (PMSF). Excess buffer was removed and the slides were incubated for 90 minutes in 100 µL of altered binding buffer (25 mM Hepes, 300 mM KCl, 1 mg/mL BSA) containing [^{33}P]-H2RLX ± cold relaxin and relaxin-like analogues. Slides were briefly rinsed in deionised water, washed in binding buffer (2x10 minutes), followed by another brief rinse with deionised water and blown dry. Slides were apposed to a phosphorimaging plate for 2-3 days, developed on a Phosphorimager (Molecular Devices, USA), the image displayed and the density of binding quantified using ImageQuaNT™ (Figure 2.5). Data was analysed using GraphPad PRISM (Section 2.8).

Saturation binding protocol

Saturation binding was determined using [^{33}P]-H2RLX, in a concentration range of 50 - 1500 pM. Specific binding was calculated by the total binding – non-specific binding (defined by 10 µM H2RLX) for each concentration. Data was analysed using GraphPad PRISM (Section 2.8) and the pK_D and B_{max} values calculated.

Competition binding protocol

Competition binding studies were carried out using [^{33}P]-H2RLX at a concentration of 100 pM. The ability of relaxin-like analogues to compete with the radioligand for the relaxin receptor was tested over a concentration range of 10 nM – 10 µM, with non-specific binding determined using 10 µM H2RLX. Data was analysed using GraphPad PRISM (Section 2.8) and pK_i values calculated.

Figure 2.5

Quantitative autoradiography

Tissue sections, cut on a cryostat at 10μm and collected on poly-L-lysine coated slides, were placed in a moist chamber for the binding experiments. [^{33}P]-H2RLX, in the presence or absence of cold relaxin analogues, was incubated on the tissue sections for 90 minutes before slides were washed and exposed to a phosphorimager plate for 3 days. The phosphorimager plates were developed and analysed using ImageQuant™.

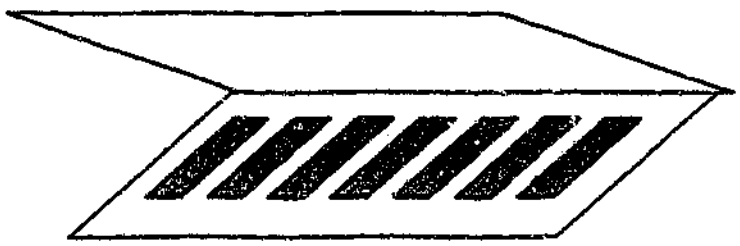
Tissue sections
10μm, poly-L-lysine
coated slides

Placed in
moist chamber

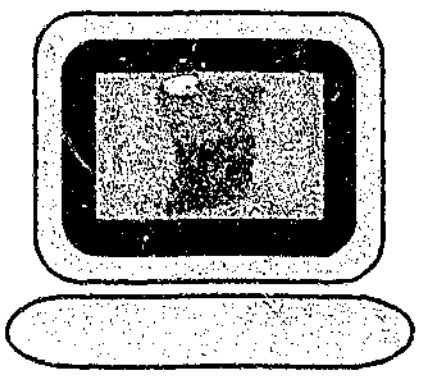
[^{33}P]-H2RLX
± H2RLX or PRLX
25°C, 1.5 hours

Wash 2 x 10 minutes

Expose to
phosphorimager
plate for 3 days



Analysed using
ImageQuant™



2.3.2.3 Phosphorimaging

Receptor expression and saturation binding images were expressed as arbitrary phosphorimager units that were converted into dpm/mm^2 using [γ-^{33}P]-ATP standards.

2.3.2.4 Preparation of [γ-^{33}P]-ATP reference standards

Rat skeletal muscle tissue was ground in a pestle at 0°C to produce a smooth thick paste which was weighed out into eleven lots. Serial dilutions of [γ-^{33}P]-ATP were mixed thoroughly with each separate paste lot and then carefully inserted into prepared moulds of aluminium foil. Moulds were snap frozen in liquid nitrogen and stored at -70°C. Frozen tissue standards were mounted in OCT in the cryostat, cut into 10 µm sections at -18°C and either mounted into poly-L-lysine coated microscopes slides to be apposed together with slide-mounted uterus sections or 30 sections of each mould were placed in liquid scintillation vials for direct determination of radioactivity.

2.3.2.5 High resolution autoradiography using photographic emulsion

Tissue sections were processed using the [^{33}P]-H2RLX quantitative autoradiography binding protocol (Section 2.3.2.2), with slides being incubated with 100 pM [^{33}P]-H2RLX for 90 minutes to determine total binding with non-specific binding defined by incubation with unlabelled H2RLX (1 µM). Labelled sections were washed and dried with a stream of air. Slides were stored overnight in a vacuum-sealed container containing silica gel to ensure that they were completely dry before commencing the emulsion protocol.

LM-1 hypercoat emulsion (Amersham Pharmacia Biotech, U.S.A.) was melted at 42°C (under darkroom conditions) for 1 hour and then transferred to a dipping chamber. Each slide was dipped into the emulsion twice, drained and allowed to dry in a light-proof box overnight. Slides were then transferred into a slide box,

wrapped in foil to prevent exposure to light and stored at 4°C for 3 weeks. Prior to development, slides were equilibrated at room temperature for 1 hour. Emulsion was developed in D-19 developer (5 minutes; Schreiber Photographics, Vic, Aust), stop solution (1 minute; Schreiber Photographics), fixative (10 minutes; Schreiber Photographics) and gently rinsed in running water (15 minutes). Slides were air-dried and examined under a dark-field microscope. For histological analysis, slides were stained with Gill's haematoxylin (distilled water 730 mL, ethylene glycol 250 mL, haematoxylin 2g, sodium iodate 17.6g and glacial acetic acid 20 mL) and Eosin (1% solution).

2.3.3 High through-put cellular binding assay

2.3.3.1 Cell preparation

THP-1 cells

From a large flask of cells, a sample was removed with a glass pipette and counted (Section 2.1.3) with Trypan Blue dye (0.5%; Trace Biosciences, NSW, Aust) to identify the proportion of live cells. The remaining cells were pelleted (5 minutes, 1000xg), media removed and the pellet was washed with sterile HBS to remove the dead cells sitting loosely on the cell pellet. The cell pellet was resuspended in binding buffer (20 mM HEPES, 1.5 mM $CaCl_2$, 50 mM NaCl, 1% BSA, 0.1 mg/mL Lysine, 0.01% NaN_3, pH 7.5) and recounted to confirm that the concentration of cells was approximately $4x10^6$ cells/100 µL.

HEK293T cells

Cells were washed and lifted from the tissue culture flask using 0.45 mM EDTA solution, pelleted (5 minutes, 1000xg), the supernatant removed and the cells resuspended in binding buffer (20 mM HEPES, 1.5 mM $CaCl_2$, 50 mM NaCl, 1% BSA, 0.1 mg/mL Lysine, 0.01% NaN_3, pH 7.5).

2.3.3.2 [^{33}P]-H2RLX cell binding assay

A high through-put cell binding assay was set up in a 96 well plate format. The 96 well plates were coated with Sigmacote® to prevent peptide binding. Final assay volume was 100 µL/well, consisting of [^{33}P]-H2RLX (20 µL) ± cold relaxin and relaxin-like analogues (10 µL) with resuspended cells (50 µL), which was incubated for 90 minutes at 25°C. Cells were harvested onto GF/C filters (Skudtek Scientific, Vic, Aust) that had been pre-soaked in 0.5% polyethylenimine (PEI, 4°C overnight). Filters were dried (42°C), liquid scintillant added (30 µL/well) and the filters incubated overnight at room temperature. Filters were counted on a Topcount scintillation counter (Perkin-Elmer, Vic, Aust) and data was analysed using GraphPad PRISM (Section 2.8).

Saturation binding protocol

Saturation binding was determined using [^{33}P]-H2RLX, in a concentration range of 25 pM – 3 nM. Specific binding was calculated by the total binding – non-specific binding (10 µM H2RLX) for each concentration. Protein concentration was determined as described in Section 2.6 and data was expressed as fmol/mg protein. Data was analysed and the pK_D and B_{max} values were calculated using GraphPad PRISM (Section 2.8).

Competition binding protocol

Competition binding was determined using [^{33}P]-H2RLX at a concentration of 100-200 pM. The ability of relaxin-like analogues to compete with the radioligand for the relaxin receptor was tested over a concentration range of 10 nM – 3 µM, with non-specific binding determined using 10 µM H2RLX. Data was analysed using GraphPad PRISM (Section 2.8) and pK_i values calculated.

2.4 Cellular functional assays

2.4.1 cAMP assay

CHO-K1 cells were transiently transfected with LGR7 receptor cDNA (see Section 2.1.4). 16 hours prior to experiment, cells were plated at $1x10^5$ cells/well into 96-well tissue culture plates in normal growth media (see Section 2.1.1.2). Incubation medium was prepared (phenol-red free media, 0.1% FBS, 2 mM IBMX, 1 μM forskolin) and drug dilutions prepared in PBS. Growth media was aspirated from cells and incubation media added (90 μL/well). Cells were exposed to relaxin or relaxin analogues (10 μL; 10 pM – 30 nM) in duplicate wells, and incubated at 37°C for 30 minutes. Stimulation was halted by the addition 0.5M HCl (25 μL/well). Plates were stored at -20°C until analysis.

cAMP was determined using the NEN FlashPlate RIA (Perkin-Elmer, Vic, Aust) according to the manufacturers instructions. Briefly, cAMP standards were diluted in water (0.1 – 15 pmol/well) and the experimental samples defrosted. Assay detection buffer was prepared (50 mM sodium acetate trihydrate, 0.8mM EDTA, pH 6.2) and added to the FlashPlate, 90 μL/well for unknown samples and 80 μL/well for cAMP standards. Unknowns (10 μL/well) and cAMP standards (20 μL/well) were added to the FlashPlate. [^{125}I]-cAMP was diluted 1:100 in tracer diluent (50 mM sodium acetate trihydrate, 0.1% NaN_3, 12 mM calcium chloride, pH 6.2) and aliquotted into FlashPlates (100 μL/well). The FlashPlates were sealed, incubated at room temperature overnight and read on a Topcount scintillation counter (Perkin-Elmer, Vic, Aust). Data was analysed using GraphPad PRISM (Section 2.8).

2.4.2 AlphaScreen™ cAMP assay

HEK293T cells stably expressing the LGR7 or LGR8 receptors were plated into a 96-well tissue culture plate at $1x10^4$ cells/well in normal culture media and incubated for 24 hours under normal cell culture conditions. Media was aspirated and assay stimulation buffer (20μL; Hank's balanced salt solution (HBSS), 1 mg/mL BSA, 0.5

mM IBMX, 0.5 M HEPES, pH 7.4) added to each well. Cells were exposed to relaxin and relaxin analogues (20µL; 1 pM – 1 µM) for 30 minutes (37°C), and stimulation was performed in duplicate wells. Stimulation solution was aspirated, cells were lysed with 100 µL lysis buffer (0.5 M HEPES, 1 mg/mL BSA, 0.5 mM IBMX, 0.3% Tween-20, pH 7.4) and snap frozen at -70°C. Plate was stored at -20°C.

cAMP accumulation was analysed using the cAMP Alphascreen Kit (Perkin-Elmer, Vic, Aust) according to the manufacturers instruction with modifications. Experimental samples were defrosted and cAMP standards (1 µM – 10 pM) diluted in detection buffer (0.4% HBSS, 3mM HEPES, 0.2% Tween 20, 0.1% BSA, pH 7.4). 5 µL of unknown samples and cAMP standards were transferred into a white 384-well plate. Acceptor bead (anti-cAMP acceptor bead diluted in detection buffer) and Donor bead (streptavidin donor beads diluted in detection buffer, 1M biotinylated cAMP) solutions were prepared under light limiting conditions in light sealed containers and keep on ice. In minimal light, 5 µL of Acceptor bead solution was added to each well and incubated in the dark for 30 minutes. Also in light limited conditions, the Donor bead solution was added (15 µL/well), the plate sealed and incubated in the dark overnight. The plate was read by the Fusion™ α microplate reader (Perkin-Elmer, Vic, Aust). Data was analysed using GraphPad PRISM (Section 2.8).

2.5 Determination of RNA and DNA concentration

The absorbance of DNA and RNA samples were measured by UV spectrophotometer (Ultrospec 2000, Pharmacia Biotech). A reference standard of sterilised water was set before measuring absorbance of the samples at 260 and 280 nm. Concentration was calculated using a constant of 50 ng/µL for double strand DNA, 20 ng/µL for single strand DNA and 40 ng/µL for RNA. Purity of the samples was determined using a ratio of the absorbance readings, 260/280 nm, with a ratio of 1.8 depicting a pure sample.

2.6 Protein determination

Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951). Cell samples were diluted 10-20 fold, and a 50 µL sample made up to 150 µL with distilled water. BSA standards (0 – 150 µg) were made up in 150 µL water. All unknowns and standards were measured in duplicate. Samples were digested with 2 M NaOH (50 µL) and incubated at room temperature for 30 minutes. Alkaline copper solution (0.01% $CuSO_4$, 0.02% NaK Tartrate, 2% anhydrous Na_2CO_3; 1 mL) was added and the incubation continued for 10 minutes. Folin's reagent (30%, 100 µL) was added, the samples vortexed and covered with foil. The colour change was allowed to develop in the dark for 30 minutes. Absorbances were measured at a wavelength of 750 nm on a Novaspec II spectrophotometer (Pharmacia LKB Biochrom, England). BSA standards were used to produce a standard curve (GraphPad PRISM) and unknown protein values interpolated from this curve.

2.7 Protein analysis

Protein samples were visualized for Western blotting and immunostaining (Harlow *et al.*, 1988). Polyacrylamide gels (10%-16% acrylamide) were produced in the Mini Protean 3 electrophoresis module (BioRad, Hercules, CA, USA). The separation gel (12% acrylamide gel) comprised 30% acrylamide (4.8 mL), Tris/SDS buffer (1.5 M Tris, pH 8.8, 0.4% SDS; 6 mL), 5% glycerol (0.8 mL), 6 mg ammonium peroxide sulphate and TEMED (5 µL). The separation gel was allowed to set before assembly of the stacking gel, comprised of 30% acrylamide (0.5 mL), Tris/SDS buffer (0.5 M Tris, pH 6.8, 0.4% SDS; 2.5 mL), distilled water (2 mL), bromophenol blue solution (4 mg in 0.5M Tris, pH 6.8; 20 µL) 2.5 mg ammonium peroxide sulphate and TEMED (5 µL). Once set, gels were assembled into the electrophoresis module with running buffer (25 mM Tris, 0.2 M glycine, 0.1% SDS), samples loaded with a PPS marker and separated at a constant 150 V (2 hours).

Following electrophoresis, the separation gel was soaked in transfer buffer 2 (25 mM Tris, 20% methanol, pH 10.4) for 5-10 minutes with gentle shaking. The separation

gel was electrotransferred onto a Hybond-C Extra nitrocellulose membrane (0.45 μm pore size) with a semi-dry blotter (CBS Scientific, Del Mar, CA, USA) for 30 minutes at a constant current in the range 2-2.5 mA/cm^2 of membrane. Electrotransfer consisted of separation gel, nitrocellulose membrane and Whatman 3MM paper in the order of: positive electrode, 6 sheets of 3MM paper pre-soaked in transfer buffer 1 (0.3 M Tris, 20% methanol, pH 10.4), 3 sheets of 3MM paper pre-soaked in transfer buffer 2, nitrocellulose membrane, separation gel, 6 sheets of 3MM paper pre-soaked in transfer buffer 3 (38 mM Tris, 10 mM β-alanine, 20% methanol, pH 9.4) and negative electrode.

At the completion of the electrotransfer the membrane was washed in Tris Buffer Saline (TBS; 20 mM Tris, 140 mM NaCl, pH 7.6) for 5 minutes. To eliminate non-specific binding the membrane was soaked in blocking buffer (TBS, 5% skim powdered milk, 0.1% Tween 20; 1 hour, room temp) and then washed for 3x5 minutes with TBS-T (TBS, 0.1% Tween 20). The membrane was incubated by gently shaking overnight (4°C) with primary antibody diluted in primary antibody buffer (TBS-T, 5% Fraction V BSA). The membrane was washed with TBS-T (3x5 minutes) and primary antibody detected with horse radish peroxidase (HRP) linked secondary antibody diluted in blocking buffer (1 hour at room temperature). Membrane was washed with TBS-T (3x5 minutes) before being exposed with enhanced chemiluminescence, Lumi-light Western Blotting Substrate (Roche, IN, USA) according to the manufacturer's instructions. The membrane was exposed to Hyperfilm ECL film (Amersham Bioscience) under darkroom conditions and developed in D-19 developer (1-5 minutes; Schreiber Photographics, Vic, Aust), stop solution (1 minute; Schreiber Photographics), fixative (5 minutes; Schreiber Photographics) and gently rinsed in water (5 minutes). Film was analysed using ImageQuaNT™.

2.8 Data analysis

Quantitative autoradiography tissue sections were analysed on ImageQuaNT™ (Version 4.1; Molecular Dynamics, U.S.A.) with density of binding expressed as arbitrary phosphorimager units and converted into dpm/mm^2 using [γ-^{33}P]-ATP standards (Section 2.3.2.4).

Saturation binding data from quantitative autoradiography and cellular binding assays were performed in duplicate and entered into GraphPad PRISM (GraphPad Inc., San Diego, U.S.A.). Specific binding was calculated as total binding - non-specific binding for each concentration of radioligand and the cell protein concentration was determined where applicable (Section 2.6). Data was plotted as concentration of [^{33}P]-H2RLX (M) vs specific binding as dpm/mm^2 for quantitative autoradiography or fmol/mg protein for cellular binding assays, as mean ± S.E.M. of n experiments. The radioligand affinity (pK_D) and receptor expression (B_{max}) were calculated by GraphPad PRISM.

Competition binding data from quantitative autoradiography and cellular binding assays were performed in duplicate and entered into GraphPad PRISM. The specific binding was calculated as total binding - non-specific binding for each concentration of cold relaxin analogue and was calculated as a percentage of total specific binding. The data was plotted as concentration of relaxin analogue (M) vs % specific binding, as mean ± S.E.M. of n experiments. The affinity of the relaxin analogues (pK_i) was calculated in GraphPad PRISM using the Cheng & Prusoff (1973) equation.

For the cAMP FlashPlate and cAMP AlphaScreen™ assays, the concentration of cAMP in each sample was calculated against the cAMP standard curve. The cAMP assay was performed in duplicate and data was converted to a percentage of maximum cAMP response determined by forskolin for the FlashPlate assay and determined by H2RLX for the AlphaScreen assay. Data was plotted as concentration of the agonist vs % maximal response as mean ± S.E.M. of n experiments and the pEC_{50} was calculated in GraphPad PRISM.

2.9 Drugs and reagents

The following drugs were gifts: recombinant human gene 2 RLX (H2RLX, Connetic Corporation, CA, USA); all synthetic relaxin and INSL3 analogues (Dr J. Wade, Howard Florey Institute of Experimental Physiology and Medicine, Vic, Aust); $DH_{5\alpha}$ cells (Dr D. Bulach, formerly from Department of Microbiology, Monash University); stably transfected HEK293T cells expressing the LGR7 or the LGR8 receptors (Ms S. Layfield, Howard Florey Institute of Experimental Physiology and Medicine, Vic, Aust); relaxin polyclonal antibody, relaxin and LGR7 DNA constructs (Dr R. Bathgate, Howard Florey Institute of Experimental Physiology and Medicine, Vic, Aust).

Drugs and reagents were purchased as follows: [γ-^{33}P]-ATP, dNTPs, LM-1 hypercoat emulsion, Hybond-C Extra nitrocellulose membrane, Hyperfilm ECL film, Not I restriction enzyme, Xho I restriction enzyme (Amersham Pharmacia Biotech, Uppsala, Sweden); BigDye Terminator Mix v3.1 (Applied Biosystems, CA, USA); acrylamide (30%), ammonium peroxide sulphate, TEMED (Bio-Rad Laboratories, Hercules, CA, USA); Fraction V BSA (Boehringer Manneheim, Germany); skim-milk powder (Diploma, Vic, Aust); polyethylene glycol (PEG) (ICN Biomedicals Inc, OH, USA); 3MM Whatmann paper (Interpath, West Heidelberg, Vic, Aust); DMRIE™, LIPOFECTAMINE™, OPTI-MEM®, Pfx polymerase, ProBond™ Purification System, SFV gene expression system, Sindbis expression system, SDS, X-gal, yeast extract, zeocin (Invitrogen, Mount Waverley, Vic, Aust); HRP-linked anti-rabbit antibody, Pml I restriction enzyme, Sph I restriction enzyme (New England Biolabs, Beverly, MA, USA); agar bacteriological (agar No. 1), casein hydrolysate, tryptone (Oxoid Limited, Basingstoke, Hampshire, England); liquid scintillant Microscint O (Perkin-Elmer, Mount Waverley, Vic, Aust); cAMP-dPK, RNase-free TE, Wizard PCR Preps DNA Purification System, Wizard Plus Midipreps DNA Purification System, Wizard Plus Miniprep DNA purification System (Promega, Madison, WI, USA); agarose LE, ampicillin, Lumi-light Western Blotting Substrate (Roche Diagnostics, IN, USA); nickel IDA agarose beads (Scientifix, Cheltenham, Vic, Aust); ammonium acetate (RNase free), ATP, BSA,

DMSO, eosin, Folin and Ciocalteu's phenol reagent, forskolin, glycerol, haematoxylin, IBMX, pertussis toxin, PMSF, polyethylenimine (PEI), poly-L-lysine, Sigmacote® sodium azide, Tween 20 (Sigma Chemical Company, St.Louis, MO, USA); GF/C filters and filter paper (Skudtek Scientific, Mount Waverley, Vic, Aust); PCR Script™ Amp Electroporation-Competent Cell Cloning Kit, QuikChange™ Site-directed Mutagensis Kit (Stratagene, CA, USA); αMEM media, DMEM/Hams F12 media, glutamine, penicillin/ streptomycin, RPMI 1640 media, trypan blue, trypsin/EDTA solution (Trace Biosciences, Castle Hill, NSW, Australia);

All other chemicals and reagents were of either analytical or laboratory grade.

Chapter Three

Structure-activity relationships of relaxin analogues at the human relaxin receptor

3.1 Introduction

To pharmacologically characterise the structural interaction between relaxin and its receptor, functional and binding assays were used to screen different relaxin analogues for relaxin-like activity. Historically, the most commonly used functional assay for the determination of the potency and efficacy of relaxin analogues was the mouse interpubic ligament assay where administration of relaxin caused a specific elongation of the interpubic ligament (Section 1.4.1.2). The ability of the analogues to produce relaxin-like activity was correlated with the ability of the analogues to compete with binding in mouse brain membrane preparations (Schwabe *et al.*, 1994). Examination of species homologues of relaxin showed that H2RLX and PRLX were of equal potency, while H1RLX and mouse RLX were only partially active (Schwabe *et al.*, 1994) at the mouse relaxin receptor. Interestingly, H2RLX, H1RLX and PRLX all displayed high affinity in the mouse brain receptor binding assay, while mouse RLX displayed significantly weaker affinity for the receptor (Schwabe *et al.*, 1994). Modification to the H2RLX A chain sequence reduced relaxin activity (Section 1.2.4.1), an action thought to reflect the disruption of the structural integrity of the peptide rather than a direct role of these amino acids in interacting with the receptor. Modifications to the B chain of H2RLX (Section 1.2.4.2) highlighted the importance of the conserved cysteine residues in maintaining peptide integrity and relaxin activity (Schwabe *et al.*, 1994), and the important binding motif comprising of Arg(B13)-X-X-X-Arg(B17)-X-X-Ile(B20) (Figure 1.2) positioned along the B chain α-helix (Büllesbach *et al.*, 2000; Büllesbach *et al.*, 1992). The highly conserved glycine residues immediately adjacent to the cysteine residues are critical for correct structural folding of insulin and it has been hypothesised that they maintain the same role in relaxin (Shire *et al.*, 1991).

More recently, interactions between relaxin analogues and the rat relaxin receptor have been explored using the rat atrial bioassay and quantitative autoradiography using [^{33}P]-H2RLX, and sections of rat uterine or atrial tissue. Both natural isoforms of H2RLX (B33 & B29), H1RLX (B33) and PRLX were equally effective at producing chronotropic and inotropic responses in the atrial bioassay, while RatRLX

and H1RLX (B29) were less effective (Tan *et al.*, 1998). Competition studies in sections of rat atria using quantitative autoradiography indicated that H2RLX and PRLX have equal binding affinity for the rat receptor while RatRLX had lower affinity (Tan *et al.*, 1999). From the results obtained at the mouse and rat relaxin receptors, it appears that the native ligand for each receptor appears to show the weakest interaction. Of the analogues tested, mouse RLX and RatRLX displayed the lowest affinity and efficacy at the mouse and rat receptors respectively, while H2RLX and PRLX analogues consistently produced the strongest interaction.

The important binding motif identified in the B chain, Arg(B13)-X-X-X-Arg(B17)-X-X-Ile(B20), is also found in a close relative of relaxin, INSL3. However in INSL3 the arginine residues are displaced 4 amino acids to the C-terminus, X(B13)-X-X-X-Arg(B17)-X-X-Val(B20)-Arg(B21). The Ile(B20) is not as highly conserved as the arginine residues but is only replaced with large hydrophobic residues, such as valine as seen in PRLX and also in the INSL3 sequence (Büllesbach *et al.*, 2000). As ruminants do not express a functional relaxin gene and INSL3 is thought to be a replacement for relaxin in these animals (Bathgate *et al.*, 2001), the sheep INSL3 was modified to contain a relaxin binding motif in the correct position. Four INSL3 analogues were designed and synthesised with minimal amino acid modification; INSL3-A His(ArgB13), INSL3-D His(ArgB13)-His(GluB14)-Phe(LysB15), INSL3-E His(ArgB13) & Arg(AlaB21), INSL3-G Arg(AlaB21) (Claasz *et al.*, 2001; Dawson *et al.*, 2001). At the rat relaxin receptor INSL3-A and INSL3-D containing relaxin and INSL3 arginine residues were found to have higher relaxin activity than the native INSL3 (Tan *et al.*, 2002). Interestingly, the INSL3-E analogue, containing only the relaxin positioned arginines, failed to produce a relaxin-like response in the isolated rat atrial bioassay, as did INSL3-G which contained no correctly positioned arginines. These results could indicate that INSL3 is not a replacement for relaxin in ruminants or that the binding motif in the B chain does not solely determine relaxin activity.

While the interaction between relaxin analogues and the relaxin receptor has been extensively examined in experimental animals, it has proved more difficult to investigate the characteristics of the human relaxin receptor because of the limited

availability of human tissue. However a THP-1 cell line derived from human monocyte cells was found to express a receptor that specifically bound relaxin yet had little affinity for other members of the insulin-gene super family (Parsell *et al.*, 1996). The aim of this study is to examine the interaction of various relaxin and INSL3 analogues at the human relaxin receptor expressed in THP-1 cells.

3.2 Methods

The affinities of various analogues of human relaxin, PRLX and RatRLX, as well as several sheep INSL3 analogues (Figure 3.1) were examined at the human relaxin receptor. Due to the difficulty and limited amount of INSL3 analogues synthesised, only native sheep INSL3 and the INSL3-D and INSL3-G analogues were available for investigation.

3.2.1 Development of the high through-put 96 well binding assay

3.2.1.1 Harvesting filter pre-treatment

A major problem associated with the use of [^{33}P]-H2RLX as a radioligand is high non-specific binding to the harvesting filter. The effect of several pre-binding treatments was explored on this non-specific binding, together with the use of two different binding buffers. The pre-treatments included soaking for 30 minutes in cold H2RLX (1μM), Sigmacote® (Sigma Chemical Company, MO, USA), in 0.5% PEI and a non-treated filter as a negative control. The influence of two binding buffers on non-specific binding was also examined (Tan *et al.*, 1999: 25 mM HEPES, 300 mM KCl, pH 7.2, 1 mg/ml BSA; Parsell *et al.*, 1996: 20 mM HEPES, 1.5 mM $CaCl_2$, 50 mM NaCl, pH 7.5, 1% BSA, 0.1 mg/ml Lysine, 0.01% NaN_3).

Binding experiments were conducted in siliconised 96 well plates, in 100 μL volume containing 200 pM [^{33}P]-H2RLX in binding buffer. Samples were harvested on filter plates pre-treated as described above and counted. All filter treatments reduced non-specific binding to the filter, with 0.5% PEI filter pre-treatment combined with incubation binding buffer #2 being the most effective (Table 3.1) and therefore the filter pre-treatment and binding buffer used in all following experiments. Non-specific binding was further reduced when the harvesting filter plate was pre-soaked in 0.5% PEI at 4°C overnight prior to binding experiments.

Figure 3.1

The amino acid sequences of the relaxin and INSL3 analogues.

An amino acid sequence comparison of the relaxin and INSL3 analogues examined in this study. The A chain sequence for the H1RLX, H2RLX and INSL3 analogues remains unchanged while the B chain sequence differs in the corresponding analogues. The cysteine (C) residues that form the disulphide bonds (blue) are aligned and highlighted (pink). The arginine (R) and isoleucine (I) residues important for relaxin-receptor binding (green) are situated in the B-chain. The amino acid substitutions in sheep INSL3 to form the INSL3 analogues are highlighted in the B chain (yellow).

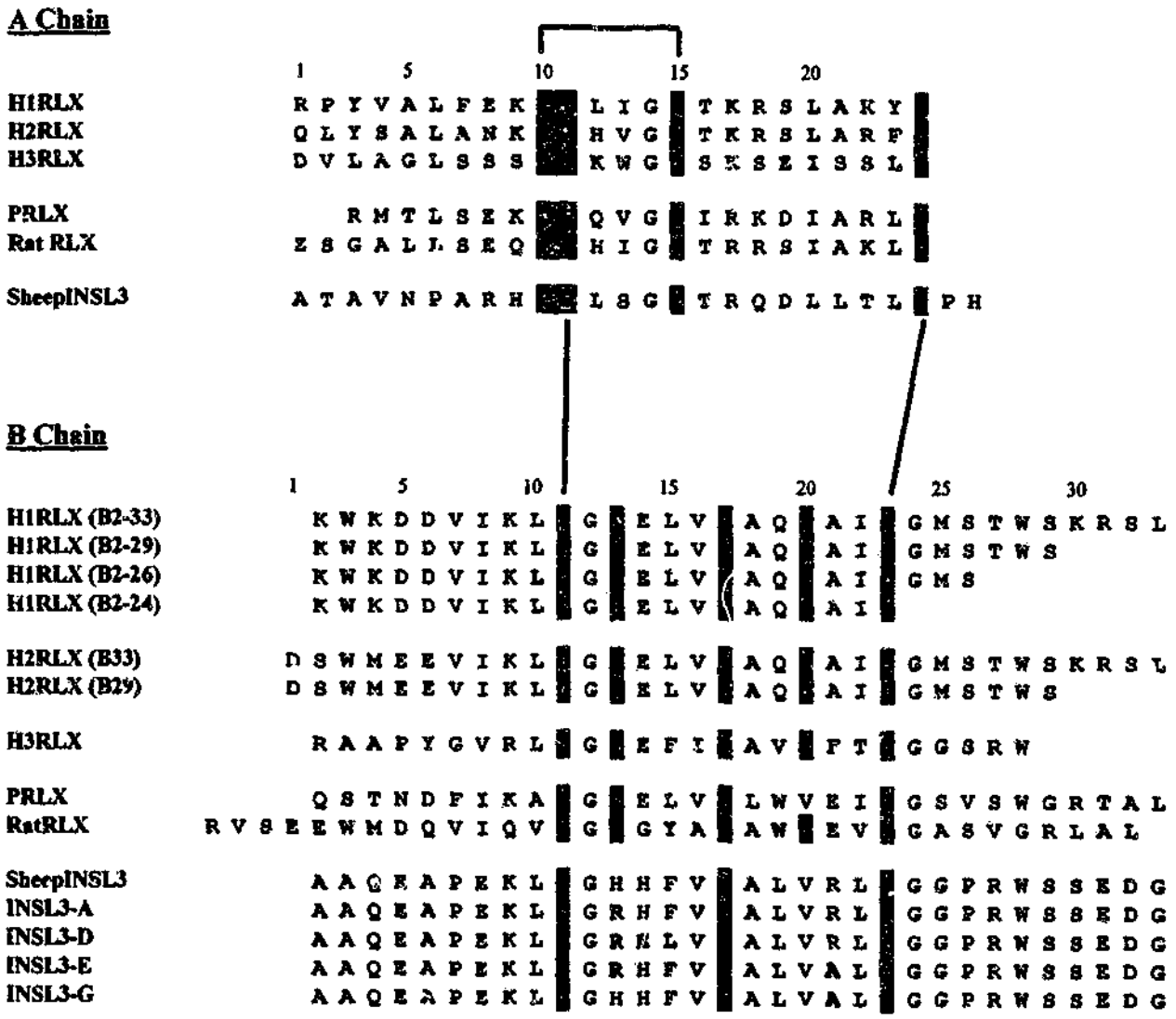

A Chain
1 5 10 15 20
H1RLX R P Y V A L F E K L I G T K R S L A K Y
H2RLX Q L Y S A L A N K H V G T K R S L A R F
H3RLX D V L A G L S S S K W G S K S E I S S L
PRLX R M T L S E K Q V G I R K D I A R L
Rat RLX E S G A L L S E Q H I G T R R S I A K L
SheepINSL3 A T A V N P A R H L S G T R Q D L L T L P H
B Chain
1 5 10 15 20 25 30
H1RLX (B2-33) K W K D D V I K L G E L V A Q A I G M S T W S K R S L
H1RLX (B2-29) K W K D D V I K L G E L V A Q A I G M S T W S
H1RLX (B2-26) K W K D D V I K L G E L V A Q A I G M S
H1RLX (B2-24) K W K D D V I K L G E L V A Q A I
H2RLX (B33) D S W M E E V I K L G E L V A Q A I G M S T W S K R S L
H2RLX (B29) D S W M E E V I K L G E L V A Q A I G M S T W S
H3RLX R A A P Y G V R L G E F I A V F T G G S R W
PRLX Q S T N D F I K A G E L V L W V E I G S V S W G R T A L
RatRLX R V S E E W M D Q V I Q V G G Y A A W E V G A S V G R L A L
SheepINSL3 A A Q E A P E K L G H H F V A L V R L G G P R W S S E D G
INSL3-A A A Q E A P E K L G R H F V A L V R L G G P R W S S E D G
INSL3-D A A Q E A P E K L G R N L V A L V R L G G P R W S S E D G
INSL3-E A A Q E A P E K L G R H F V A L V A L G G P R W S S E D G
INSL3-G A A Q E A P E K L G H H F V A L V A L G G P R W S S E D G

Table 3.1

Filter pre-treatment table

Four harvesting filter pre-treatments with two different binding buffers were examined to find the combination that would reduce non-specific [^{33}P]-H2RLX binding to the filter plate. All filter pre-treatments reduced non-specific binding with the most effective combination being the 0.5% PEI pre-treatment with binding buffer 2.

Filter Treatment	Untreated	1 μM H2RLX	Sigmacote®	0.5% PEI
Binding Buffer 1 (Tan *et al.*, 1999)	100%	20%	37%	20%
Binding Buffer 2 (Parsell *et al.*, 1996)	100%	31%	21%	**13%**

3.2.1.2 Cell number and setup

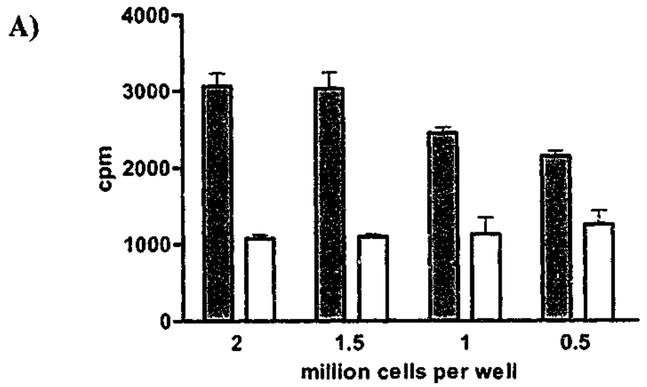
The effect of the number of THP-1 cells used per well was examined to maximise specific binding. Since many studies have used membrane preparations for binding experiments, membrane preparations from THP-1 cells and from the entire rat cortex tissue, including the cingulate, frontal, insular, motor, parietal and perirhinal cortices, were also examined. THP-1 cells were prepared as described in Section 2.3.3.1, resuspended in binding buffer and counted. THP-1 cell suspensions were split into four and diluted to achieve a final concentration to give 2, 1.5, 1 and 0.5 million cells per well in a volume of 100 µL. The four dilutions were divided into two, with one sample maintained for whole cell binding while the other was processed in a Dounce homogeniser to produce a membrane preparation. Rat cerebral cortex was obtained by anesthetising and killing a rat, and removal of the brain which was placed on ice for 5 minutes. Cortex was dissected from the remaining brain tissue, weighed and homogenised in 50x volume of Tris buffer (10 mM Tris, pH 7.4 @ 4°C, 10 µM PMSF, 250 mM Sucrose). Membrane preparations were resuspended in binding buffer and diluted to deliver 2.5, 1.25, 0.9 and 0.6 mg wet weight membrane homogenate per well in 100 µL.

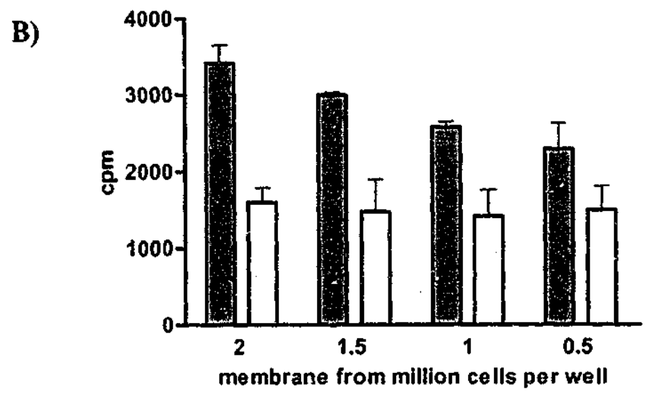
Experiments were performed in siliconised 96 well plates, total well volume 100 µL containing 200 pM [^{33}P]-H2RLX (20 µL), the cell or membrane sample (50 µL) and non-specific binding was determined with 10 µM H2RLX (10 µL). Samples were harvested onto pre-treated filter plates as described in Section 2.3.3.2, filters were dried (42°C), liquid scintillant added (30 µL/well) and plates were counted. Total binding to non-specific binding ratio with intact THP-1 cells decreased as the cell number decreased, with 2 million cells giving the optimal ratio (Figure 3.2a). THP-1 membranes displayed higher non-specific binding (Figure 3.2b), while rat cortical membranes showed very little specific binding (Figure 3.2c). 2 x 10^6 THP-1 cells/well was chosen for all further experiments.

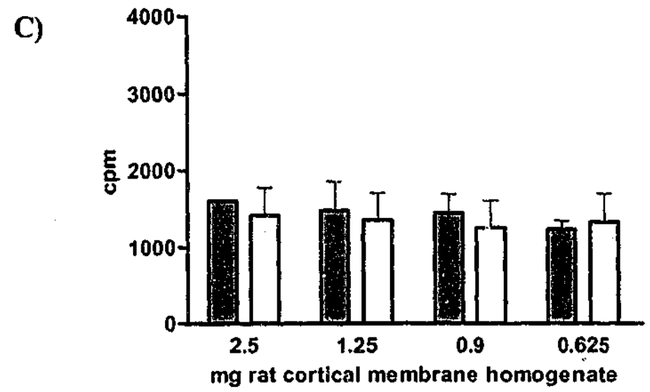
Figure 3.2

Analysis of cell number/well and cell type to obtain optimal specific binding.

The optimal number of cells and type of cell preparation was examined to obtain maximal specific binding, shown by the difference between total binding ■ and non-specific binding □. A) the whole cell preparation of THP-1 cells showed optimal specific binding at 2 million and 1.5 million cells/well. B) the THP-1 cell membrane preparation displayed higher non-specific binding compared to the whole cell preparation while C) the rat cortical membrane preparation showed no specific binding.







3.2.2 Cell culture

THP-1 cells were grown in suspension and maintained in RPMI 1640 media containing glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 μg/mL) and heat inactivated foetal bovine serum (FBS; 10% v/v). Cells were maintained within the density range of 2 x 10^5 and 1 x 10^6 cells/mL to ensure optimal growth and survival. Cells were split and cell culture performed as described in Section 2.1.1.1.

3.2.2.1 THP-1 cell preparation

THP-1 cells were prepared as described in Section 2.3.3.1 on the day of experimentation. Cells resuspended in binding buffer were counted to adjust the concentration of cells to approximately 4 x 10^6 cells/100μL to ensure that the experiments were performed with 2 x 10^6 cells/well. Cells resuspended in binding buffer were kept on ice until plated out into the 96-well plate at 50 μL/well.

3.2.3 High through-put 96 well binding assay

The THP-1 cell binding assay was set up as described in Section 2.3.3.2. The total volume used was 100 μL/well, comprising 20 μL of 200 pM [^{33}P]-H2RLX ± 10 μL unlabelled relaxin-like analogue and 50 μL cell suspension, made up to 100 μL with binding buffer. All experiments were performed in duplicate. Following a 90 minute incubation at 25°C, cells were harvested onto GF/C filter plates (Skudtek, Vic, Aust) presoaked overnight (0.5% PEI, 4°C). Filter plates were dried at 42°C, Microscint O (Perkin-Elmer, Vic, Aust) added (30 μL/well) and equilibrated overnight. Filters were counted on a Topcount scintillation counter (Perkin-Elmer, Vic, Aust) and data was analysed with GraphPad PRISM (Section 2.8).

3.2.3.1 [^{33}P]-H2RLX (B33) preparation

H2RLX (B33) was labelled with [γ-^{33}P]-ATP using a procedure based on that of Osheroff *et al* (1990) as described in Section 2.3.1.

3.2.3.2 Saturation binding protocol

Saturation binding assays were used to determine the affinity and maximum number of binding sites (B_{max}) of human relaxin receptors on THP-1 cells using [^{33}P]-H2RLX. Saturation binding assays were performed in a concentration range of 25 pM – 1 nM. Specific binding was calculated by the total binding – non-specific binding (10 μM H2RLX) at each [^{33}P]-H2RLX concentration. Protein concentrations were determined as described in Section 2.5.1 and the data was plotted as specific binding (fmol/mg protein) against concentration of radioligand used (pM) (Section 2.8).

3.2.3.3 Competition binding protocol

Competition binding assays were used to calculate the affinity of various relaxin and relaxin-like analogues for the relaxin receptor. Competition binding was performed using [^{33}P]-H2RLX at a concentration of 200 pM. As the relaxin analogues were in limited supply, limited concentration ranges were used. The concentration ranges used were; H1, H2, porcine and rat relaxin analogues 100 pM – 30 nM; H3RLX 100 pM – 300 nM; INSL3 1 nM – 300 nM and INSL3-D & -G 10 nM – 3 μM. Non-specific binding was determined using 1 μM H2RLX. Results were expressed as a percentage of maximal specific binding against concentration of the relaxin or relaxin-like analogue used (M). pK_i values were calculated (Section 2.8).

3.2.4 Analysis

Results were expressed as mean ± S.E.M. of n experiments (Section 2.8). Saturation binding data was analysed with GraphPad PRISM. Radioligand affinity (pK_D) and receptor expression (B_{max}) values were calculated and expressed in pM and fmol/mg protein respectively. Competition binding data was analysed with GraphPad PRISM and relaxin-like affinity (pK_i) values were calculated by the program using the Cheng & Prusoff (1973) equation. Statistical analysis was completed using GraphPad PRISM statistical programs with the pK_i data analysed using a Student's t-test and competition curves analysed using a two-way ANOVA. P values of less than or equal to 0.05 were considered significant.

3.2.5 Drugs and reagents

All drugs were used as described in Section 2.9. Stock solutions of H1RLX, H2RLX, H3RLX, PRLX and RatRLX and INSL3 analogues were prepared in 0.1% aqueous trifluoroacetic acid (TFA) at a concentration of 10 μM. INSL3-D & -G analogue stock solutions were at a concentration of 500 μM. All relaxin and relaxin-like analogues were diluted in binding buffer prior to use. All plastic tubes, pipette tips and binding plates used in relaxin binding experiments were coated with SigmaCoat™ (Sigma).

3.3 Results

3.3.1 Characteristics of [^{33}P]-H2RLX binding to the human relaxin receptor

[^{33}P]-H2RLX binding occurred in a saturable manner to a single binding site expressed in THP-1 cells (Figure 3.3). Expression of the binding site gave a B_{max} of 1.45 ± 0.62 fmol/mg protein in the whole cell binding assay. [^{33}P]-H2RLX showed high affinity for the human relaxin receptor with a pK_D of 9.37 ± 0.22 (n=4).

3.3.2 Competition for binding at the human relaxin receptor

3.3.2.1 Native human relaxin analogues

All products of the three human relaxin genes displayed affinity for the relaxin receptor (Figure 3.4). Both forms of H1RLX showed high affinity, however H1RLX (B2-33) appeared to have a slightly lower affinity (pK_i 8.4 ± 0.09, n=6; P<0.04) than the shorter naturally occurring form H1RLX (B2-29) (pK_i 8.8 ± 0.10, n=6) (Figure3.4a). The pattern was reversed in the H2RLX peptide (Figure 3.4b) with H2RLX (B33) displaying a slightly higher affinity (pK_i 9.1 ± 0.16, n=5; P<0.03) for the receptor compared to its shortened form, H2RLX (B29) (pK_i 8.6 ± 0.11, n=13). H3RLX displayed the lowest, though still high affinity (pK_i 8.4 ± 0.16, n=8) for the human relaxin receptor (Figure 3.4b). Interestingly H3RLX only displaced 50% of the ligand binding (P<0.0001) while both forms of H1RLX and H2RLX competed for 100% of the binding.

3.3.2.2 Species homologues of relaxin

PRLX showed high affinity for the human receptor (pK_i 8.4 ± 0.09, n=5), not significantly different from H2RLX (B29) (Figure 3.5a), and like the human relaxin completely displaced ligand binding. RatRLX did not compete as effectively, and displayed significantly lower affinity (pK_i 7.9 ± 0.09, n=7; P<0.0005) than H2RLX

(B29) (Figure 3.5b). Due to limited supply of RatRLX the concentration range could not be extended to a level that completely displaced ligand binding.

3.3.2.3 Effect of shortening the B chain on affinity of H1RLX

Shortening of the B-chain of H1RLX from B2-33 to the native B2-29 form appeared to slightly increase the affinity of H1RLX for the human relaxin receptor. Further shortening of the B chain from H1RLX (B2-29) to H1RLX (B2-26) and H1RLX (B2-24) caused a significant decrease in affinity (pK_i 8.1 ± 0.12 and approximately estimated 7.5 ± 0.17 respectively, n=6; $P<0.004$). H1RLX (B2-26) was able to compete for 100% of ligand binding (Figure 3.6a) while the concentration range for H1RLX (B2-24) could not be extended to investigate if it could compete for binding at high concentrations (Figure 3.6b).

3.3.2.4 Relaxin-like affinity of INSL3 analogues

INSL3 was able to compete for [^{33}P] H2RLX with low affinity (pK_i approximately estimated to be 5.9 ± 0.32; n=4) (Figure 3.7a). Modification of INSL3 to include a relaxin-like binding motif positioned in the B chain produced INSL3-D which displayed significantly increased relaxin-like interaction with the receptor ($P<0.0001$), however there was no significant difference in affinity values (pK_i 6.7 ± 0.11, n=4; $P>0.06$). Although INSL3-D competed for 100% of ligand binding, it was still 100 fold weaker than H2RLX (B29) (Figure 3.7b). INSL3 was modified to contain neither of the INSL3 or relaxin-like binding motifs. The INSL3-G analogue displayed similar weak relaxin-like interaction (pK_i 5.4 ± 0.13; n=4) at the human relaxin receptor compared to INSL3, resulting in 1000 fold less affinity than H2RLX (B29) (Figure 3.7c).

Figure 3.3

[^{33}P]-H2RLX saturation binding characteristics at the human relaxin receptor expressed in THP-1 cells.

The specific relaxin radioligand, [^{33}P]-H2RLX, displayed high affinity (pK_D 9.37 ± 0.22, n=4) for a single binding site on the relaxin receptor. Natural expression of the relaxin receptor in THP-1 cells gave a B_{max} of 1.45 ± 0.62 fmol/mg protein in the whole cell binding assay.

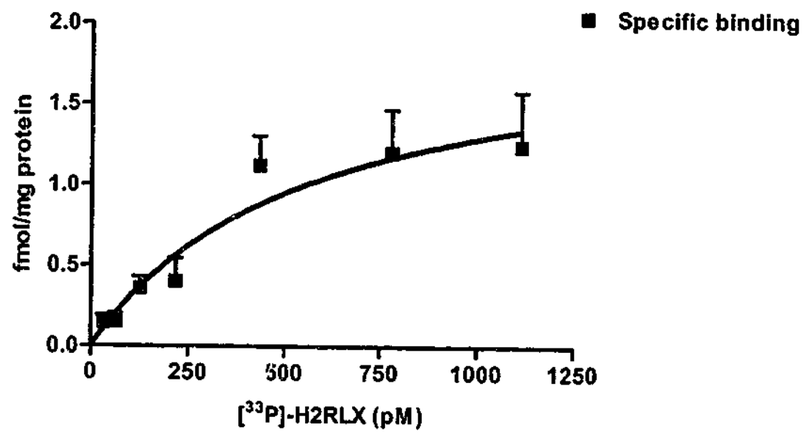
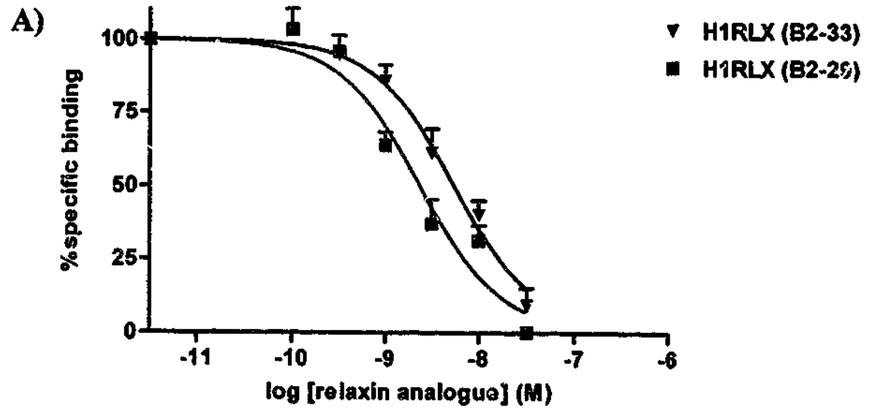


Figure 3.4

Competition for binding at the human relaxin receptor expressed in THP-1 cells by the native forms of the human relaxin analogues.

These graphs represent the pooled specific affinity data displayed by the three human relaxin genes for the human relaxin receptor. A) the two forms of human gene 1 relaxin, H1RLX (B2-33) and H1RLX (B2-29), displayed high affinity (pK_i 8.4 ± 0.09 & 8.8 ± 0.10 respectively, n=6) as did the B) two forms of human gene 2 relaxin, H2RLX (B33) (pK_i 9.6 ± 0.16, n=5) and H2RLX (B29) (pK_i 8.6 ± 0.11, n=13). The third human relaxin gene, H3RLX, displayed high affinity (pK_i 8.4 ± 0.16, n=8) although could only displace 50% of ligand binding ($P<0.0001$ cf H2RLX).



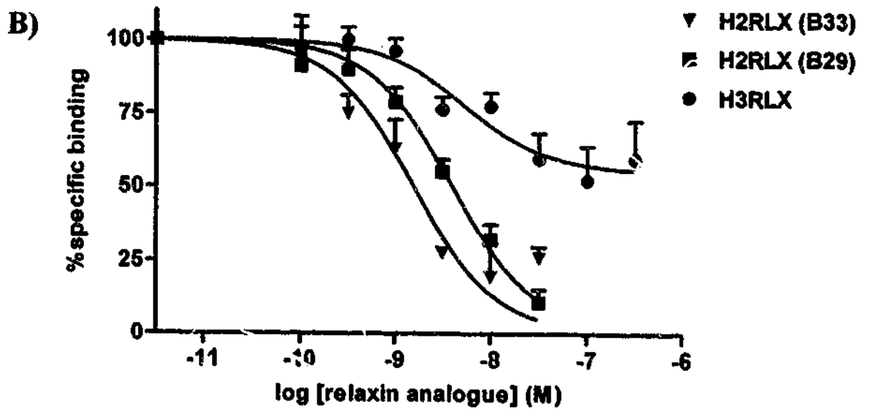
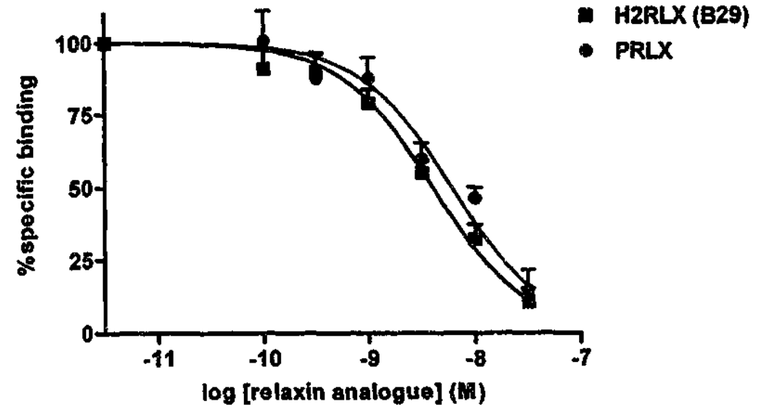


Figure 3.5

Competition for binding at the human relaxin receptor expressed in THP-1 cells by species homologues of relaxin.

These graphs represent the pooled specific affinity data displayed by porcine and rat species homologues of relaxin for the human relaxin receptor. The natural ligand for the relaxin receptor, human gene 2 relaxin (H2RLX (B29)), was used for comparison. The species homologues of relaxin showed high affinity for the human relaxin receptor with A) porcine relaxin (PRLX) having similar affinity (pK_i 8.4 ± 0.09, n=5) to H2RLX, while B) the affinity displayed by the rat homologue (RatRLX; pK_i 7.9 ± 0.09, n=7) was significantly lower ($P<0.005$).

A)



B)

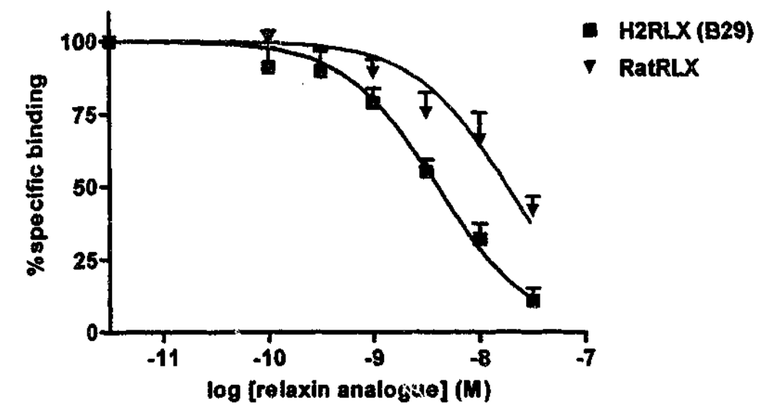
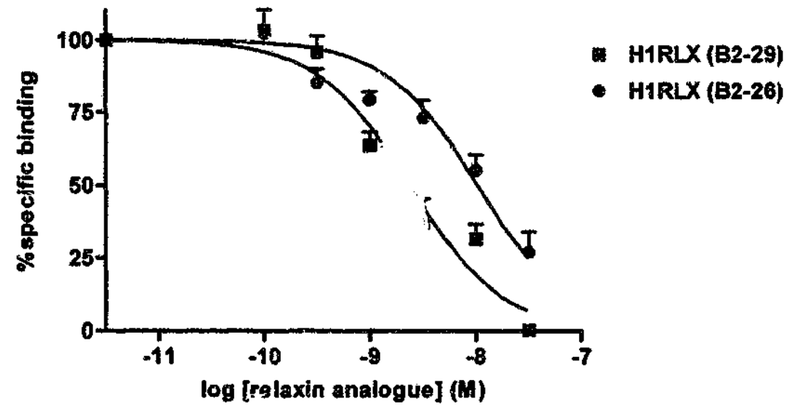


Figure 3.6

Competition for binding at the human relaxin receptor expressed in THP-1 cells by the human gene 1 relaxin analogues with shortened B chain length.

These graphs represent the pooled specific affinity data displayed by the human gene 1 relaxin analogues for the human relaxin receptor. The proposed natural form of human gene 1 relaxin (H1RLX), H1RLX (B2-29), was used for comparison. Shortening of the B chain by A) 3 amino acids, H1RLX (B2-26) reduced relaxin affinity for the receptor (pK_i 8.1 ± 0.12, n=6; P<0.004) which was further reduced with B) removal of another 2 amino acids, H1RLX (B2-24), (pK_i estimated to be 7.5 ± 0.17, n=6; P<0.004) compared to H1RLX (B2-29).

A)



B)

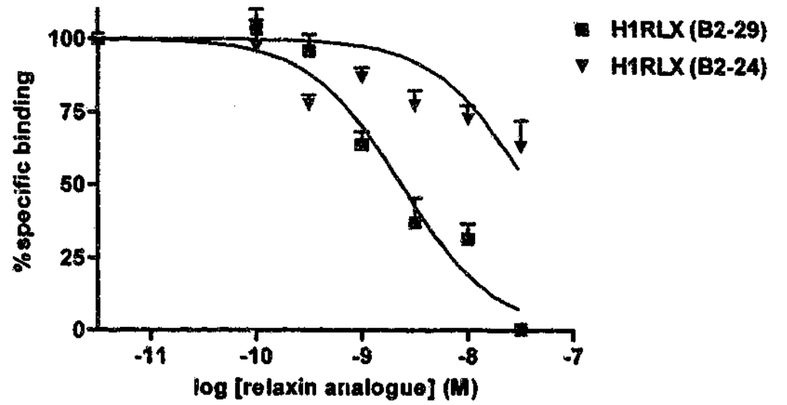


Figure 3.7

Competition for binding at the human relaxin receptor expressed in THP-1 cells by sheep insulin-like peptide 3 analogues.

These graphs represent the pooled specific affinity data displayed by the sheep insulin-like peptide 3 (INSL3) analogues for the human relaxin receptor. The natural ligand for the relaxin receptor, human gene 2 relaxin (H2RLX (B29)), was used for comparison. A) native sheep INSL3 displayed very little relaxin-like affinity (pK_i estimated to be 5.9 ± 0.32, n=4). B) insertion of the relaxin positioned arginine and intermediate residues (Figure 3.1), INSL3-D, increased relaxin-like affinity (pK_i 6.7 ± 0.11, n=4) while C) removal of arginine residues, INSL3-G, showed extremely weak relaxin-like affinity (pK_i 5.4 ± 0.13, n=4).

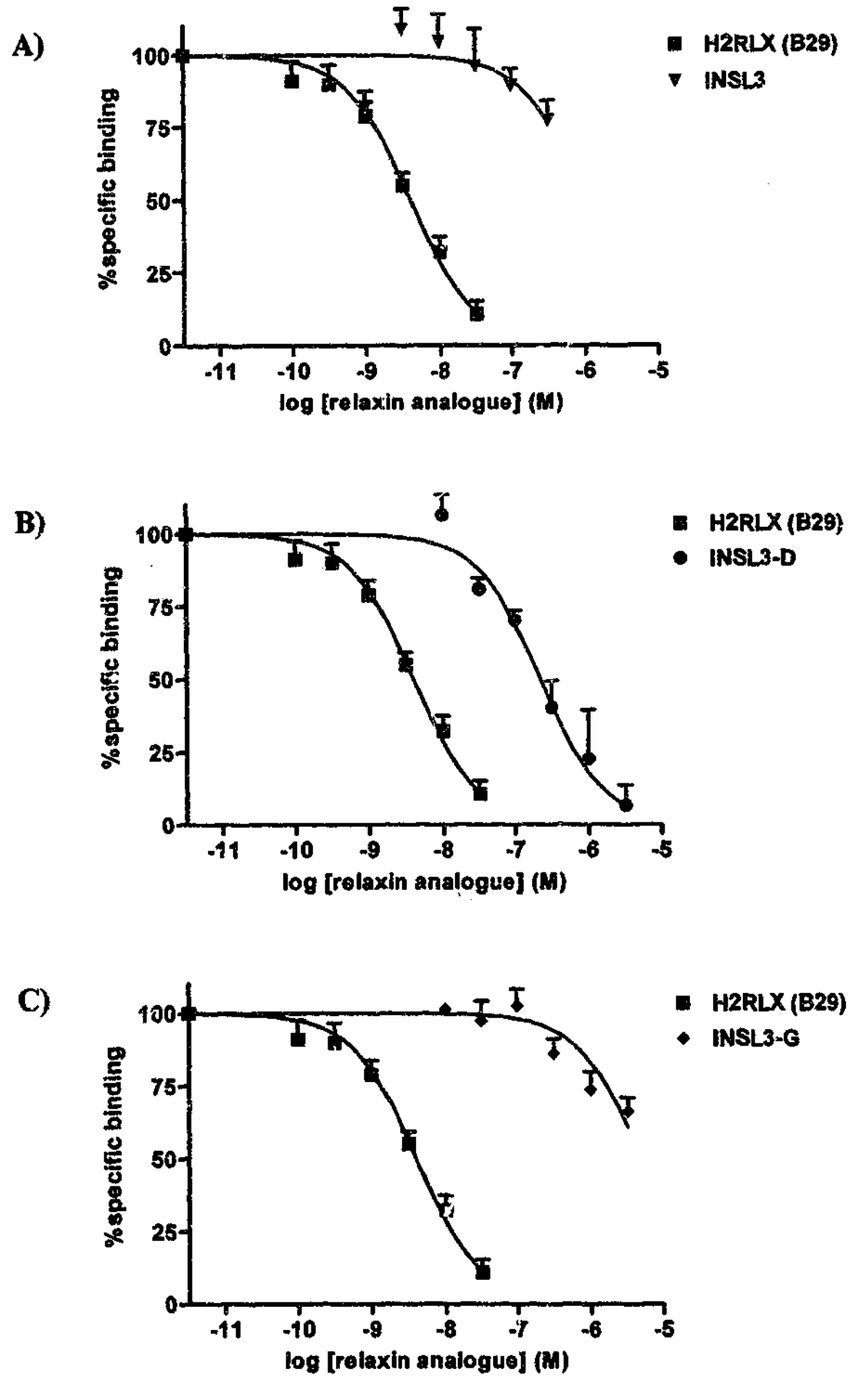


Table 3.2

Summary of the relaxin and INSL3 analogue affinities displayed for the human relaxin receptor expressed in THP-1 cells. Non-specific binding was determined using 1 μM H2RLX (B29).

* denotes P<0.05, ** P<0.005 verus H2RLX (B29); † P<0.05, †† P<0.005 verus H1RLX (B2-29).

Analogue	pK_i	n
H1RLX (B2-33)	8.4 ± 0.09 †	6
H1RLX (B2-29)	8.8 ± 0.10	6
H2RLX (B33)	9.1 ± 0.16 *	5
H2RLX (B29)	8.6 ± 0.11	13
H3RLX	8.4 ± 0.16	8
PRLX	8.4 ± 0.09	5
RatRLX	7.9 ± 0.09 **	7
H1RLX (B2-26)	8.1 ± 0.12 ††	6
H1RLX (B2-24)	7.5 ± 0.17 ††	6
INSL3	5.9 ± 0.32 **	4
INSL3-D	6.7 ± 0.11 **	4
INSL3-G	5.4 ± 0.13 **	4

3.4 Discussion

While structure-activity relationships of various relaxin analogues have been determined at mouse and rat relaxin receptors, few studies have investigated the relationships at the human relaxin receptor. This study examined the ability of various relaxins and relaxin-like analogues to interact with the human relaxin receptor endogenously expressed in THP-1 cells. The high through-put THP-1 cell binding assay allowed the interaction between peptides and the receptor to be investigated using minimal amounts of material.

[^{33}P]-H2RLX was used in this study as it has been thoroughly characterised and binds to and stimulates the relaxin receptor in a way not distinguishable from H2RLX (Osheroff *et al.*, 1990; Parsell *et al.*, 1996; Tan *et al.*, 1999). In saturation binding assays, [^{33}P]-H2RLX displayed high affinity for the human relaxin receptor, consistent with that previously reported at the human relaxin receptor (Osheroff *et al.*, 1995; Parsell *et al.*, 1996). Expression of the relaxin receptor in THP-1 cells was low, although it is difficult to make a direct comparison with binding studies using membrane preparations. Membrane preparations, in contrast to whole cells, concentrate the receptor protein. Previous reports indicate that THP-1 cells express low levels of relaxin receptor, ~275 receptors/cell (Parsell *et al.*, 1996), compared to ~1082 receptor/cell in human uterine cells, (Osheroff *et al.*, 1995).

Although H2RLX is the predominant form of relaxin and endogenous H1RLX synthesis has yet to be positively confirmed in any tissue, the H1RLX and H2RLX peptides had similar affinities. H1RLX (B2-29) and H2RLX (B33) displayed similar affinities to H1RLX (B2-33) and H2RLX (B29), revealing no further insights into the specific role of the B chain C terminal end 4 amino acids. The C terminus of the B chain varies considerably with species (Figure 1.2), with the length ranging from B28 to B40. There appears to be no direct correlation between B chain C terminus length and affinity for the receptor as H3RLX and RatRLX, with B chain C terminal lengths of B28 and B32 respectively, showed lower affinity for the receptor than H2RLX (B29 & B33) while PRLX, with a B chain of B2-33, displayed similar

affinity to H2RLX (B29) (Table 3.2). Pro-PRLX had the same affinity and efficacy as PRLX (Tan *et al.*, 1998), as did marmoset monkey pro-RLX (Zarreh-Hoshyari-Khah *et al.*, 2001) indicating that the C-peptide did not interfere with interaction with the receptor, again suggesting that the extended length of the C-terminus has little influence on relaxin function.

All but one of the relaxin analogues investigated in this study were able to displace 100% of [^{33}P]-H2RLX binding, with the H3RLX competition curve appearing to plateau at 50% of radioligand displacement. This raises an interesting issue as to the interaction of the human relaxin analogues with the relaxin receptor. While all saturation studies have indicated a single binding site, the possibility of two binding sites maintaining the same affinity for the relaxin analogues should not be dismissed. The partial displacement of [^{33}P]-H2RLX binding by H3RLX may be the result of H3RLX interacting with only one of the binding sites. An alternative explanation could be a self interaction between H3RLX peptides at high concentrations that may induce structural changes to the peptide, interfering with binding at the relaxin receptor.

The rank order of affinity of the relaxin analogues differed between the rat and human receptors. H2RLX (B29) had a higher affinity than H2RLX (B33) for the rat relaxin receptor (Tan *et al.*, 2001a), in contrast to the affinity order determined in this study. PRLX displayed similar affinity to H2RLX (B33), while RatRLX had less affinity than the other relaxin analogues at the rat relaxin receptor. The ability of these relaxin analogues to stimulate the rat receptor was demonstrated by H2RLX (B29 & B33) and H1RLX (B33) displaying similar efficacies to PRLX in the rat isolated atrial bioassay. H1RLX (B2-29) displayed significantly lower efficacy while RatRLX was the least bioactive relaxin analogue (Tan *et al.*, 1998). At the human relaxin receptor, cAMP accumulation assays using THP-1 cells reported an efficacy order of H2RLX > H1RLX > H3RLX (Bathgate *et al.*, 2002). There is no current functional data concerning the activity of PRLX and RatRLX at the human relaxin receptor in THP-1 cells.

Reducing the length of the B chain from the C-terminus has a clear negative influence on relaxin affinity with H1RLX (B2-26) and H1RLX (B2-24) displaying progressively lower affinity for the receptor. These results confirm a previous study showing reduced efficacy in the rat atrial bioassay of H1RLX (B2-26) compared to H1RLX (B2-29), while H1RLX (B2-24) had no relaxin-like activity (Tan *et al.*, 1998). Reduction of amino acids up to B24 exposes the cysteine (B23) that forms one of the interchain disulphide bonds, thus causing disruption to the structural integrity of the peptide. Removal of amino acids from the B chain C-terminus of PRLX also shows that removal to B25 has little effect on the ability of the PRLX peptide to induce relaxin-like activity, with further removal of amino acids abolishing all bioactivity (Tregear *et al.*, 1983). Since only limited amounts of H1RLX were synthesised, functional studies using THP-1 cells could not be performed.

While the extended length of the B chain has little influence on the binding capabilities of the relaxin peptide, the two highly conserved arginine residues at B13 and B17 are crucial for relaxin function (Büllesbach *et al.*, 1992). The same two arginine residues were identified in INSL3 and although these were displaced 4 amino acids towards the C-terminus, this peptide displayed very little affinity for the relaxin receptor. Modification of INSL3 so that the arginine residues resided in the relaxin binding motif position produced INSL3-D, increasing the relaxin-like interaction. However the affinity was still 100 fold less than H2RLX, indicating that factors other than the two arginines were necessary for full relaxin-like activity. A third residue critical for the relaxin binding motif, has been identified as isoleucine (B20) (Büllesbach *et al.*, 2000), but this does not explain the difference in relaxin-like affinity since INSL3 contains a compatible residue, valine B20. Functional data examining the ability of the INSL3 analogues to stimulate cAMP production in THP-1 cells showed that although the inclusion of the partial and full human relaxin binding motif in analogues INSL3-A & -D respectively (Figure 3.1) increased relaxin-like cAMP production compared to native sheep INSL3 (Claasz *et al.*, 2002) it was again 100 fold less than H2RLX. Removal of the extra INSL3 arginine in position B21 to expose the valine (B20) in INSL3-E (Figure 3.1) failed to improve

relaxin-like potency, indicating that the third arginine did not hinder relaxin-like interaction with the receptor.

The A chain of relaxin has been examined in relation to its influence in relaxin-like activity and appears to be of little significance other than maintaining peptide structure (see Section 1.2.4.1). However, not all areas important for relaxin function have been identified and these do not appear to be solely contained in the B chain. The A and B chain relaxin/INSL3 chimeras would be an invaluable tool for investigation of the roles of each chain in relaxin-like activity.

In conclusion, this study demonstrated distinct differences in the interaction between relaxin analogues and the rat and human relaxin receptors. H1 and H2RLX peptides appear to interact with the receptor in a similar manner, displacing [^{33}P]-H2RLX binding with high affinity, while H3RLX may interact slightly differently as it displayed slightly less affinity and could only displace approximately 50% of radioligand binding, possibly indicating the presence of multiple binding sites. Extension of the relaxin B chain has little influence on relaxin function whereas shortening the B chain to position B24 decreases relaxin binding. The relaxin binding motifs of Arg(B13), Arg(B17) and Ile(B20) are not the only determining factors of relaxin activity as insertion of this motif into the closely related INSL3 peptide had only limited effects on relaxin-like binding to the relaxin receptor. Further investigations using H2RLX/INSL3 chimeras may reveal novel areas important for relaxin function.

Chapter Four

Production of recombinant relaxin

4.1 Introduction

Relaxin was first isolated from sow corpora lutea (Fevold *et al.*, 1930). The peptide is present in relatively low concentrations and yields of 30-35 mg of purified PRLX peptide per kg ovary tissue were reported (Fevold *et al.*, 1930; Sherwood *et al.*, 1974). Extraction from the ovary, corpora lutea or placenta led to the successful identification and characterisation of relaxin from a variety of species, including rat, shark, whale, horse, dog and rabbit (Sherwood, 1994). Extraction from tissue could not be used to obtain all species' homologues of relaxin since the collection of large amounts of human tissues for relaxin extraction was not possible. Therefore a synthetic method of providing human relaxin peptides was developed.

The successful sequencing and subsequent synthetic production of a biologically active relaxin peptide was reported in the early 1980's. From the predetermined pro-relaxin peptide sequences of rat and porcine relaxin, a highly conserved area within the connecting C-peptide code was identified and used to identify and isolate the human relaxin gene (Hudson *et al.*, 1983). Following comparison of the human and porcine relaxin sequence (Sherwood *et al.*, 1974) and using the reported similarity between the relaxin and insulin structures (Bedarkar *et al.*, 1977), chemical synthesis of human relaxin produced a biologically active human relaxin peptide that dose-dependently inhibited rat uterine contractility (Hudson *et al.*, 1983). The successful chemical synthesis of human relaxin provided methods that allowed synthesis of otherwise unobtainable relaxin peptides and also the development of relaxin analogues to investigate the structure-activity relationships of relaxin. Studies with relaxin analogues have shown that the relaxin A chain is necessary for maintaining relaxin peptide structure while the B chain contains areas important for the interaction of relaxin with its receptor (Section 1.2.4). The pre-processed form of relaxin, pro-relaxin, retains full relaxin affinity and efficacy (Tan *et al.*, 1998; Zarreh-Hoshyari-Khah *et al.*, 2001) in contrast to insulin which only becomes active following cleavage of the connecting C-peptide (Peavy *et al.*, 1984; Yu *et al.*, 1973).

However, the synthetic production of relaxin peptides is not without problems, since low yields of peptide have been reported due to substantial loss during the combination of A and B chains (Tregear *et al.*, 1995). The complexity and variability of the individual relaxin chain sequences necessitates modifications to the synthetic conditions for each relaxin peptide (Tregear *et al.*, 2001), leading to increased cost and time to produce each relaxin analogue. These limitations, and in particular the small yield of peptides, and the increasing demand for the large scale production of human relaxin required for clinical trials, led to the development of recombinant techniques for the production of relaxin peptides.

Early investigations into recombinant methods for production of relaxin peptides involved a synthetically constructed DNA template of pro-PRLX B chain inserted into a carrier plasmid with the remaining part formed by RT-PCR and inserted with specific restriction sites (Stewart *et al.*, 1983). Analysis of the expressed peptides from an *E.coli* culture on a polyacrylamide gel showed a protein of the expected size that was identified as pro-PRLX with anti-relaxin antibodies, although further characterisation of this protein was not reported. Some years later, the successful production of recombinant pro-PRLX was reported by the insertion of the pro-PRLX DNA template into an *E.coli* expression vector, with the pro-PRLX peptide purified from an *E.coli* culture and manually refolded into the correct relaxin peptide structure (Reddy *et al.*, 1992). The recombinant pro-PRLX could be partially digested with dilute trypsin to cleave the C-peptide and form PRLX, and both forms of the recombinant peptide were shown to be biologically active in the bioassay examining the increase in cAMP accumulation in human uterine endometrial cells.

An early attempt to produce recombinant human relaxin involved the insertion of a human relaxin DNA construct with a modified connecting C-peptide into a yeast expression plasmid (Yang *et al.*, 1993). The extracted relaxin pro-peptide was partially purified and found to be biologically active, although it displayed an efficacy some 20 fold less than synthesised H2RLX in the mouse interpubic ligament bioassay. As a part of a program to develop human relaxin for therapeutic use, Genentech Inc. (CA, USA) were successful in producing a recombinant human pro-relaxin protein containing a unique "mini-C" connecting peptide between the A

and B chains of the protein (Vandlen *et al.*, 1995), a method previously employed successfully in the production of mini-C pro-insulin (Wetzel *et al.*, 1981). The shortened connecting peptide ensured efficient synthesis and extraction from the *E.coli* culture with the pro-relaxin peptide then folded and processed into the H2RLX peptide. This form of recombinant H2RLX was found to maintain full H2RLX efficacy and affinity in THP-1 cell based assays (Parsell *et al.*, 1996).

A common problem for all recombinant methods using bacteria for synthesis is that the resulting relaxin peptide has to be manually folded following extraction as the bacterial cells do not have the ability to fold a mammalian peptide into its correct structure. Recombinant pro-PRLX synthesised in a CHO cell line was biologically active following extraction without the need for manual refolding (Vu *et al.*, 1993). Although the pro-PRLX was reported to have slightly less efficacy than native PRLX in producing a cAMP response in human endometrial cells, the comparison was made between different studies completed 3 years apart (Fei *et al.*, 1990; Vu *et al.*, 1993) and the concentration of the recombinant pro-PRLX was calculated in dilutions of conditioned media.

The aim of this study was to develop a mammalian recombinant system that would allow efficient production and purification of a recombinantly synthesised and correctly folded pro-H2RLX protein. The recombinant system in conjunction with molecular cloning techniques would be used to investigate novel areas important for relaxin activity within the relaxin peptide.

4.2 Methods and Results

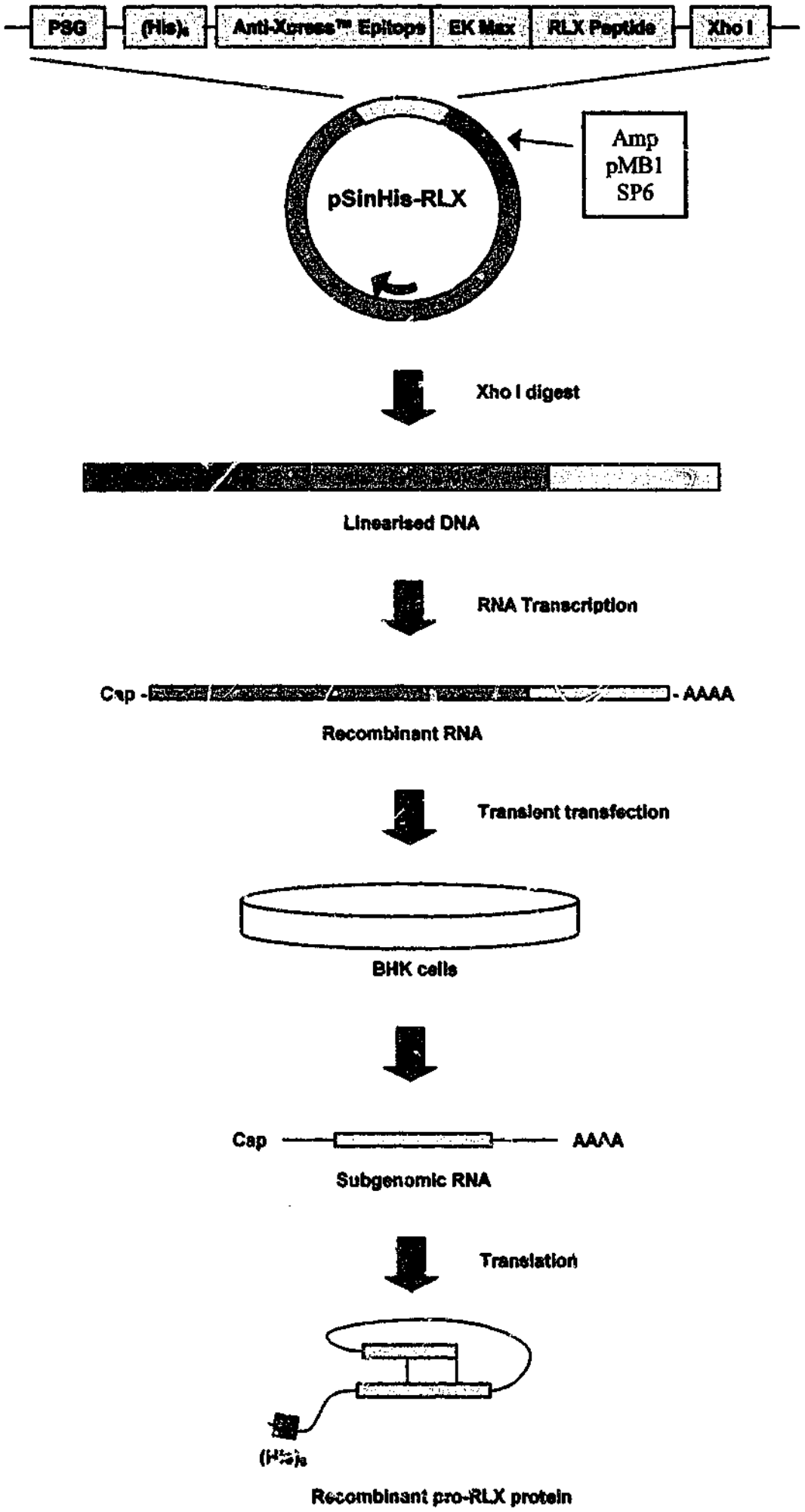
The recombinant relaxin protein system was based on a combination of the Sindbis Expression System (Invitrogen, Vic, Aust), the InvitroScript™ CAP SP6 *In Vitro* Transcription Kit (Invitrogen, Vic, Aust), the SFV Gene Expression System (Invitrogen, Vic, Aust) and the ProBond™ Purification System (Invitrogen, Vic, Aust). Briefly, the H2RLX DNA insert was ligated into the Sindbis Expression system pSinHis plasmid, linearised and used as a template for RNA transcription. The capped RNA transcript containing the relaxin coding region and a polyhistidine (His_6) tag was transiently transfected into BHK cells, the encoded protein was expressed and obtained by harvesting and lysing the cells (Figure 4.1). The relaxin protein was purified from the cell lysate through a nickel IDA bead column (Scientifix, Vic, Aust) that when activated displays high affinity for polyhistidine proteins, binding the relaxin peptide and removing all excess lysate. The relaxin peptide was cleaved from its His_6 tag with EKMax enzyme (Invitrogen, Vic, Aust) and purified from the nickel column (Figure 2.3)

Figure 4.1

Schematic diagram of the methodology to produce recombinant pro-RLX.

The RLX DNA fragment (RLX Peptide) is inserted in the pSinHis plasmid adjacent to the enterokinase cleavage site (EKMax), allowing cleavage of the Anti-Xpress™ Epitope and the N-terminal polyhistidine tag ($(His)_6$) following purification of the recombinant pro-RLX protein. Apon successful ligation of the RLX DNA into the pSinHis plasmid, the plasmid is linearised at the Xho I restriction enzyme cleavage site (Xho I) and used as a template for the transcription of recombinant RNA. *In vitro* transcription is driven by the SP6 promoter (SP6) while *in vivo* transcription, following transfection into the BHK cells, is initiated by the subgenomic promoter (PSG). The pro-RLX protein is translated and extracted from the BHK cells.

Amp = Ampicillin resistance gene ; pMB1 = pMB1 origin.


PSG
(His)6
Anti-Xpress™ Epitope
EK Max
RLX Peptide
Xho I
Amp
pMB1
SP6
pSinHis-RLX
Xho I digest
Linearised DNA
RNA Transcription
Cap
AAAA
Recombinant RNA
Transient transfection
BHK cells
Cap
AAAA
Subgenomic RNA
Translation
Recombinant pro-RLX protein

4.2.1 Ligation of the RLX insert into pSinHis plasmid

The H2RLX insert was produced by PCR using pMAL plasmid as a template (gift from Dr Ross Bathgate, Howard Florey Institute of Experimental Physiology and Medicine, Vic, Aust). The 5' and 3' primers contained a Pml I and Sph I restriction site respectively, to facilitate unidirectional cloning into pSinHis. It was necessary to introduce mutations into the multiple cloning site of pSinHis to create a Pml I restriction site (Figure 4.2)

4.2.1.1 Incorporation of Pml I restriction site into pSinHis plasmid

Primers were designed (Table 2.1) and synthesised (Invitrogen, Vic, Aust) to incorporate the Pml I restriction site into the multiple cloning site of the pSinHis plasmid using the QuikChange™ Site Directed Mutagenesis Kit (Stratagene, CA, USA). The reaction was performed as described in Section 2.2.1. Mutated pSinHis DNA was precipitated and resuspended in sterilised deionised water before being transformed into *E.coli* $DH_{5\alpha}$ cells (Section 2.2.1.1), colonies selected and DNA purified (Section 2.2.1.2). Colonies were screened using a Xho I and Pml I digest and analysed on a 1.3% agarose gel (Section 2.2.1.2). Colonies containing the pSinHis DNA with the Pml I restriction site were identified by release of a 450 bp band (Figure 4.3). Correct pSinHis DNA was amplified as described in Section 2.2.1.3.

4.2.1.2 Preparation of the RLX DNA insert

Primers were designed (Table 4.1) to generate a RLX insert that contained the EK recognition and cleavage sites of the pSinHis plasmid that would be removed with the digestion at the Pml I restriction site (Figure 2.1) and synthesised (Invitrogen, Vic, Aust). The Sph I restriction site was incorporated into the 3' end of the RLX insert. PCR reaction for production of RLX DNA fragment is described in Section 2.2.2. RLX insert was visualised as a 500 bp band on a 1.3% agarose gel (Figure 4.4) and purified from a 1.3% low melting temperature agarose gel (Section 2.2.2.1).

4.2.1.3 Ligation of RLX insert into pSinHis plasmid

The altered pSinHis plasmid (5 μg) and the relaxin fragment (2 μg) were digested separately in a reaction mixture including 1x NE 1 buffer (New England Biolabs, MA, USA), 0.01% BSA, 25 U Sph I enzyme and 100 U Pml I enzyme in a total volume of 100 μL (37°C for 2 hours). The digested DNA was loaded onto a 1.3% low melting temperature agarose gel where the relaxin DNA fragment bands (~500 bp) and linearised pSinHis DNA bands (~10 kB) were carefully cut from the gel. DNA was purified using the Wizard PCR Preps DNA Purification System (Promega, WI, USA) as described in Section 2.2.2.1. DNA was eluted with 50 μL of nuclease-free water at 70°C for the large plasmid and 60°C for the smaller DNA segment. The purified DNA concentration was calculated (Section 2.5) and was stored at -20°C.

Ligation of the RLX insert into the pSinHis plasmid occurred in a reaction mixture containing 200 ng pSinHis plasmid DNA, 40 ng RLX insert DNA, 1x One-Phor-All buffer, 1 mM ATP and 4 U T4 DNA ligase in a total volume of 50 μL. Ligation was incubated overnight at room temperature. Ligation mix was precipitated and resuspended in sterilized deionised water (10 μL). Ligated DNA was transformed into $DH_{5\alpha}$ competent cells as previously described (Section 2.2.1.1), single colonies were selected and DNA purified using the Wizard Plus Miniprep DNA Purification System (Promega, WI, USA) as previously described (Section 2.2.1.2). DNA was screened using a Pml I and Sph I digest in 30 μL reaction and DNA visualised on a 1.3% agarose gel. Unfortunately the ligation was unsuccessful as shown by a failure to produce a ~500 bp RLX insert (Figure 4.5).

Figure 4.2

Nucleotide sequence of the pSinHis and RLX insert cloning area.

The nucleotide sequence begins with the start codon (ATG) at the 5' end and includes the polyhistidine tag (orange), the Pml I restriction site (blue), the EKMax enzyme recognition site (green) followed by the start of the RLX B chain nucleotide sequence (purple). At the conclusion of the RLX A chain nucleotide sequence is the Sph I restriction site (pink) used in the insertion of the RLX fragment into pSinHis.

--- denotes that the nucleotide sequence continues.

5' — ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT **GGT**
ATG GCT AGC ATG ACT GGT GGA CAG CAC GTG GGT CGG
GAT CTG TAC GAC GAT GAC GAT AAG GAC TCA TGG ATG
GAG GAA GTT ATT AAA ----- ----- AGA TCT CTT GCT AGA
TTT TGC TGA AGC TTA GCA TGC CAC TGG CCG — 3'

Figure 4.3

Gel electrophoresis of the pSinHis plasmid mutated to include a Pml I restriction site.

Digestion of colonies containing the Pml I restriction site mutation with Xho I and Pml I restriction enzymes, resulted in the formation of a 455bp band as measured against a 100bp molecular weight marker (Lane 1). The positive samples included samples 1 and 3-6 (Lanes 2, 4-7 respectively) while sample 2 (Lane 3) failed to show a Pml I restriction site mutation.

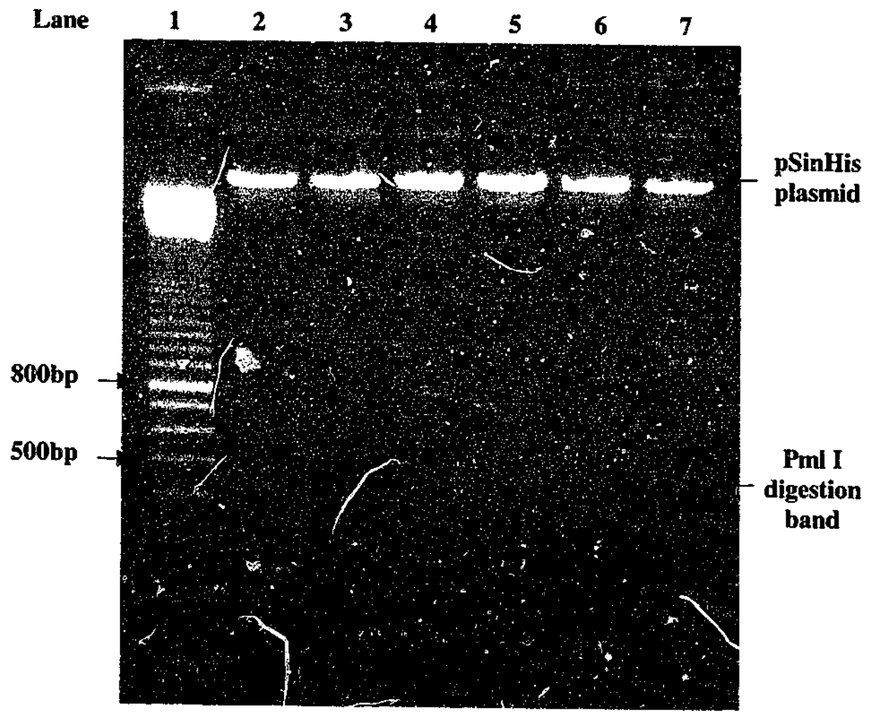


Table 4.1

The oligonucleotide primers designed for the generation of the RLX insert.

The RLX insert forward oligonucleotide primer included the Pml I restriction site (blue), the EKMax enzyme recognition site (green) and the start of the RLX B chain nucleotide sequence (purple). The RLX insert reverse oligonucleotide primer included the complementary sequence to the Sph I restriction site (pink) and the end of the RLX A chain nucleotide sequence (purple).

Primer Name	Sequence (5' ⇒ 3')
RLX insert for	CAG CAC GTG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GAC TCA TGG ATG GAG GAA G
RLX insert rev	GTG GCA TGC TAA GCT TCA GCA AAA TCT AGC

Figure 4.4

Gel electrophoresis of the PCR generated RLX insert.

The RLX construct generated by PCR from the original plasmid, pMAL plasmid, resulted in a 500bp band (Lane 2) as measured against a 100bp molecular weight marker (Lane 1)

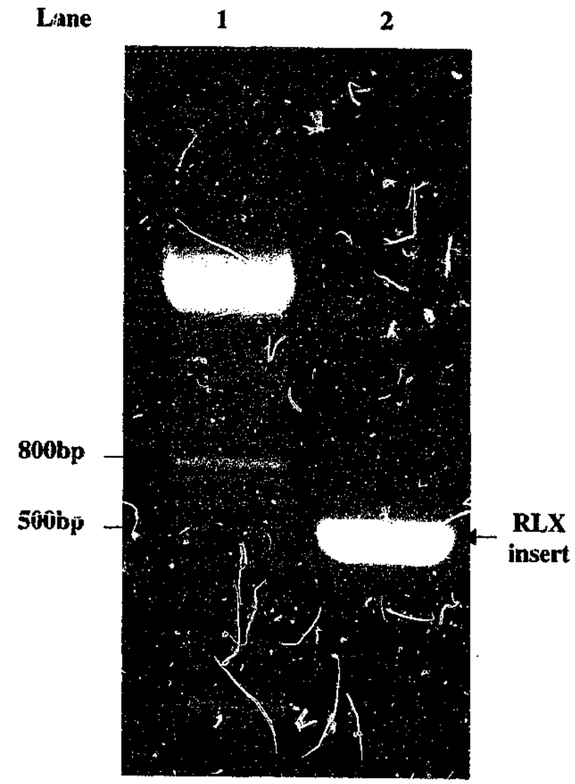
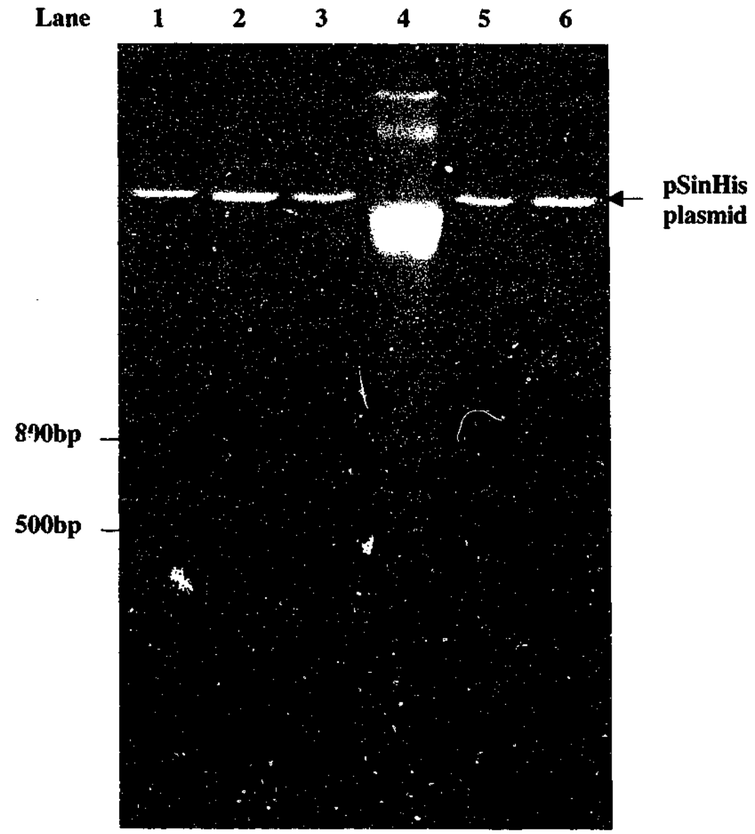


Figure 4.5

Gel electrophoresis of the pSinHis plasmid following the ligation reaction to insert the RLX fragment.

Digestion screen of selected colonies (Lanes 1-3, 5 & 6) with Pml I and Sph I restriction enzymes. None of the colonies screened contained the ligated pSinHis-RLX product that would have been represented by a 500bp band. The molecular weight marker is shown in Lane 4.



4.2.1.4 Activity of Pml I and Sph I enzymes

Due to the continued failure to demonstrate successful ligation, the restriction enzymes used in the digestion reactions were checked for enzyme activity. A digest incorporating the Sac I restriction enzyme with the Pml I and Sph I enzyme separately were designed, with successful digestion confirmed visually by bands of 2245 bp and 2190 bp respectively. The digest reaction included 1mg pSinHis DNA, 1x NE 1 buffer (New England Biolabs, MA, USA), 0.01% BSA, 10 U Sac I enzyme and either 20 U Pml I or 5 U Sph I in a total volume of 30 µL (37°C for 2 hours). The products of the digestion reaction were run on a 1.3% agarose gel (Figure 4.6) and demonstrated that both the Pml I and Sph I enzymes were working and produced the expected bands. The Pml I restriction enzyme however appeared to have lost some activity since three bands were observed, representing the expected DNA band, the fully digested pSinHis plasmid and the partially digested pSinHis plasmid.

4.2.1.5 Hybridisation screening for the RLX insert

Due to the observed decrease in Pml I enzyme activity, it was hypothesised that successful ligation may have occurred but at low efficiency. Screening large numbers of colonies by miniprep DNA purification and digestion would be time consuming and expensive, therefore a quick and efficient screen of the agar plate colonies was achieved using a [^{32}P]-RLX hybridisation probe.

4.2.1.5.1 Preparation of the RLX DNA probe

RLX insert DNA was used for the relaxin specific probe and to ensure no cross recognition of the pSinHis plasmid, the RLX insert DNA was digested with Not I enzyme to remove the pSinHis sequence at the 5' prime end of the DNA fragment. Digestion of RLX insert included 20 µg RLX insert DNA, 0.01% BSA, Not I enzyme buffer, 0.2 U Not I enzyme in a total volume of 70 µL (overnight incubation at 37°C). DNA was isolated on a 1.3% low melting temperature gel and purified as described in Section 2.2.2.1.

RLX DNA was labelled with [α^{32}P]-ATP using Ready-to-go DNA labelling beads (Amersham Pharmacia Biotech, Uppsala, Sweden). 50 ng RLX DNA was denatured at 95°C for 3 minutes, placed on ice for 2 minutes and DNA pelleted. The mixture of pelleted DNA and [α^{32}P]-ATP in a total volume of 50 µL was added to the DNA labelling beads and mixed thoroughly. Reaction was incubated at 37°C for 30 minutes and stored at -20°C. RLX probe was purified using a Sephadex G50 column. The column was equilibrated with 3 mL Tris/EDTA solution (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) prior to addition of the probe labelling reaction. RLX probe was washed through with 400 µL Tris/EDTA solution with the flow through collected at 3 drops/tube and counted with a hand-held β-counter. Tris/EDTA solution was continually added until the eluant no longer contained radioactivity. Samples containing the first peak of radioactivity were pooled and stored at -20°C.

4.2.1.5.2 Hybridisation screen

The ligated pSinHis/RLX DNA was transformed into $DH_{5\alpha}$ cells and grown on agar plates as previously stated in Section 2.2.1.1. Nitrocellulose filters cut to fit the agar plates were numbered, sparingly moistened with distilled water, carefully placed on top of the agar colonies and marked for orientation. The colony lifts were then placed, colony side up, onto 3MM paper soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 3 minutes. The colony lifts were transferred onto 3MM paper soaked in neutralizing solution (0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5) for 3 minutes, followed by 3 minutes on 2x SSC soaked 3MM paper and then air dried for 5 minutes. The colony lifts were baked in an 80°C oven for 10 minutes before being placed in a clean Petri dish.

The pre-hybridisation solution included Denhardts solution (50x; 5 mL), 20x SSC (12.5 mL), 10% SDS (2.5 mL), 100 mM ATP (50 µL), 10 mg/mL salmon sperm DNA (500 µL; denatured at 95°C, 5 minutes) in a total volume of 50 mL. The pre-hybridisation solution was placed into a Petri dish, with each individual piece of nitrocellulose being added and soaked prior to the addition of the next piece. A Petri

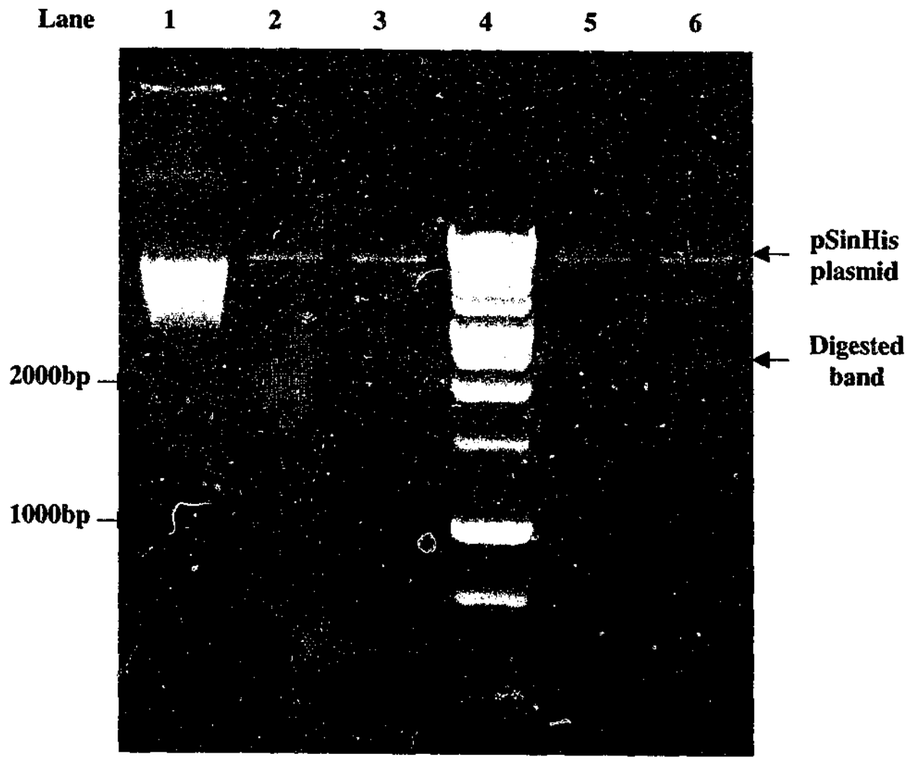
dish containing the filters and solution was wrapped in polyethylene film and incubated at 42°C for 4 hours. Filters were carefully removed from the Petri dish, excess pre-hybridisation solution removed and the [^{32}P]-RLX DNA probe added. The filters were replaced into the dishes individually, gently mixed with the probe solution (pre-boiled, 5 minutes) and incubated at 42°C overnight.

The nitrocellulose filters were washed sequentially with 2x SSC, 0.1% SDS at room temperature, at 30°C for 30 minutes, at 37°C for 5 minutes before drying at room temperature for 30 minutes. Blots were mounted onto cardboard backing, wrapped in polyethylene and exposed to film (Hyperfilm, Amersham, Sweden) overnight with orientation markers. Film was developed in D-19 developer (5 minutes; Schreiber Photographics, Vic, Aust), stop solution (1 minute; Schreiber Photographics), fixative (10 minutes; Schreiber Photographics), gently rinsed in water (10 minutes) and orientated via the markers. The film was then aligned with the original agar plates, positive colonies identified and selected for screening by Miniprep preparations (Section 2.3.1.2). All colonies were screened using a Not I digest, including 1 µg ligated DNA, 0.01% BSA, Not I enzyme buffer, 0.01% Triton X-100, 10 U Not I enzyme in a total volume of 50 µL. Not I digestion of pSinHis plasmid containing the RLX insert would result in a fragment of 845 bp with one Not I restriction site contained outside the RLX insert area of the plasmid and the other contained within the RLX insert. None of the positive colonies contained the RLX insert (Figure 4.7).

Figure 4.6

Gel electrophoresis of the pSinHis plasmid digested by Sph I/Sac I and Pml I/Sac I restriction enzymes.

Samples of pSinHis plasmid were digested with a combination of Sph I/Sac I restriction enzymes (Lanes 2 & 3) and Pml I/Sac I restriction enzymes (Lanes 5 & 6) resulting in the formation of a 2190bp and 2245bp band respectively as measured against the 100bp (Lane 1) or 1kb (Lane 4) molecular weight markers. The two pSinHis plasmid bands shown in Lanes 5 & 6 represent uncut (higher band) and cut (lower band) plasmid, indicating partial digestion of the samples.



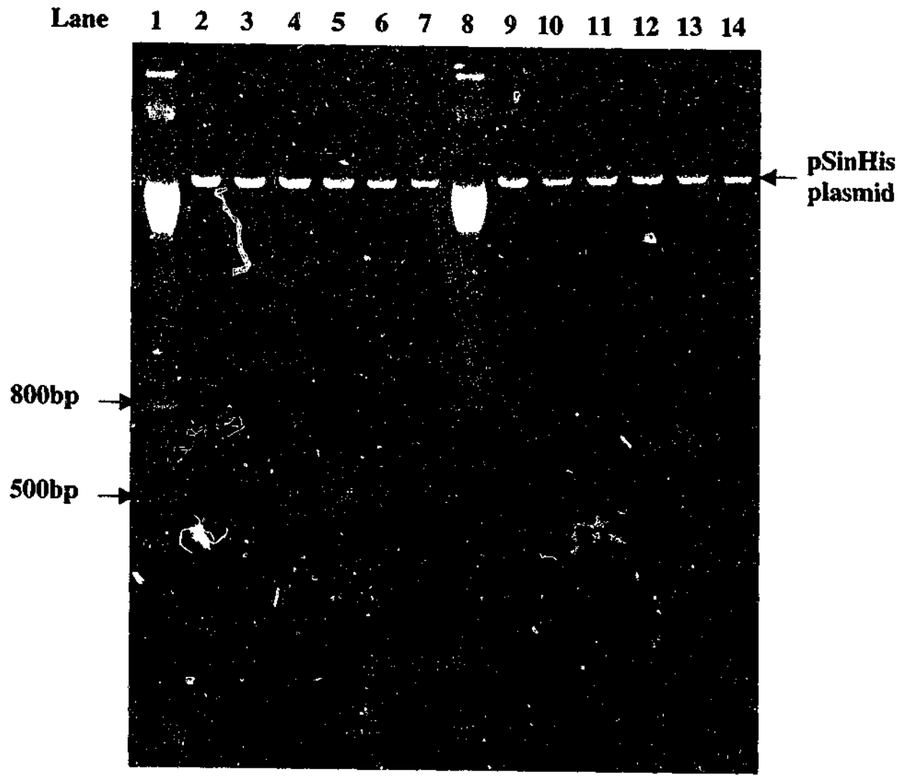


Figure 4.7

Gel electrophoresis of the colonies selected from hybridisation probe.

Positive colonies as indicated by the hybridisation probe (Lanes 2-7 & 9-14) were screen by digestion with Not I restriction enzyme. Colonies containing the ligated pSinHis-RLX plasmid were identified by a 845bp band, which was not shown by any of the samples. The 100bp molecular weight marker is shown in Lanes 1 & 8.

4.2.1.6 Regeneration of the RLX insert

The primers for the generation of the RLX insert product by PCR were redesigned (Table 2.1) to lengthen the Pml I and Sph I restriction site cleavage areas to reduce the possibility that the enzymes failed to recognise and cleave the short ends of the RLX insert. The RLX insert DNA was generated and purified as described in Section 2.2.2. The pSinHis plasmid and RLX insert DNA were digested with Pml I and Sph I, isolated, purified and ligated as described in Section 4.2.1.3. Ligated DNA was transformed in $DH_{5\alpha}$ cells and produced very small numbers of colonies on agar plates. All colonies were screened using a Not I digest as described above. The ligation failed with no DNA fragment shown when the digested DNA was visualised on a 1.3% agarose gel (data not shown).

4.2.1.7 Blunt-end ligation reaction

Digestion of DNA by the Pml I restriction enzyme results in the formation of a "blunt-end", where the DNA is cleaved cleanly with no overhanging DNA sequence. As the blunt-end could have interfered with successful ligation of the RLX DNA fragment into the pSinHis plasmid, ligation conditions were altered to accommodate a blunt-end ligation. The reaction mixture included 200 ng pSinHis DNA, 40 ng RLX insert DNA, 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mg/mL BSA, 5% PEG, 0.5 mM ATP and 4 U T4 DNA ligase in a total volume of 100 µL and was incubated overnight at room temperature. Ligated DNA was precipitated, transformed into $DH_{5\alpha}$ cells, colonies isolated and screened using a Not I restriction enzyme digestion as described in Section 4.2.1.5.2. Ligation was unsuccessful with no DNA fragment visualised on a 1.3% agarose gel (data not shown).

4.2.1.8 Generation of RLX insert from PCR script

To ensure the correct digestion of the RLX insert DNA by Pml I and Sph I enzymes, the PCR RLX insert product was directly ligated into PCR script plasmid according to the PCR-Script™ Amp Electroporation-Competent Cell Cloning Kit (Stratagene,

CA, USA). The ligation reaction included 10 ng pPCR script Amp SK(+) cloning vector, 1x PCR script reaction buffer, 0.5 mM rATP, 170 ng RLX PCR DNA product, 5 U Srf I restriction enzyme, 4 U T4 DNA ligase in a total volume of 10 µL. The ligation reaction was gently mixed, incubated at room temperature for 2 hours and the reaction halted by a 10 minute incubation at 65°C. DNA was precipitated and transformed into XLI-Blue MRF electrocompetent cells as described for $DH_{5\alpha}$ cells (Section 2.2.1.1) except that the agar plates were pre-treated with X-gal prior to plating out transformed cells. The PCR script plasmid containing the RLX insert produced white colonies due to the disruption of the β-galactosidase gene responsible for the blue colour seen in colonies that do not contain the RLX insert. Selected colonies were screened by Miniprep preparations and DNA isolated (Section 2.2.1.2). DNA was digested by Pml I and Sph I restriction enzymes, visualisation on a 1.3% agarose gel and showed that seven of the ten selected colonies contained the RLX insert (Figure 4.8).

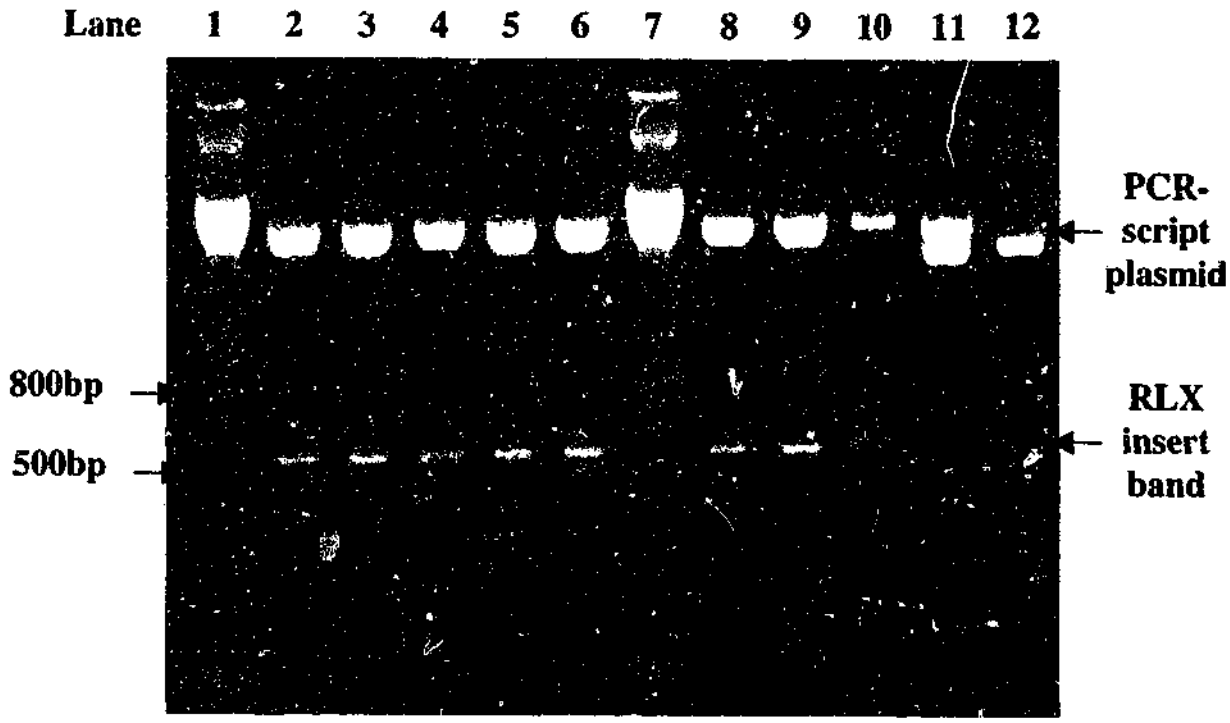
4.2.1.8.1 Sequencing of PCR script plasmid containing the RLX insert

All of the PCR script clones containing the RLX insert were sequenced to ensure no sequence anomalies had occurred during the processing of the DNA. The sequencing protocol was as described in Section 2.2.3.1, incorporating the T3 and T7 sequencing primers (Table 2.1). Results showed that PCR script-RLX clones #1, #2, #4 and #7 contained sequence errors while clones #3, #5 and #6 were correct. Clone #5 was chosen for further study. PCR-script/RLX clone #5 (2 µg) was digested with Pml I and Sph I restriction enzymes, run on a 1.3% low melting temperature gel and the RLX insert purified as previously described (Section 2.2.2.1). The RLX insert (40 ng) and the digested pSinHis plasmid (200 ng) were set up in a blunt-end ligation reaction mixture (Section 4.2.1.7) and incubated at 14°C for 4 hours. Ligated DNA was precipitated, transformed into $DH_{5\alpha}$ cells, DNA purified from selected colonies and screened using the Not I digest as previously described above. Visualisation of digested DNA on a 1.3% agarose gel showed no successful ligation of the RLX insert into pSinHis (data not shown).

Figure 4.8

Gel electrophoresis of PCR-script following ligation of RLX construct.

Selected colonies were screened (Lanes 2-6 & 8-12), for the successful ligation of the RLX insert into PCR-script, by digestion with Pml I and Sph I restriction enzymes. Positive colonies were identified by a 550bp band (Lanes 2-6, 8 & 9) as measured against the 100bp molecular weight marker (Lanes 1 & 7).


Lane 1 2 3 4 5 6 7 8 9 10 11 12
PCR-script plasmid
800bp
500bp
RLX insert band

4.2.1.9 Freeze 'n Squeeze purification and ligation of RLX insert into the pSinHis plasmid

Since the DNA fragments supported the correctly Pml I and Sph I digested ends, it was possible that there was inhibition of the ligation reaction. Therefore, instead of purifying the digested RLX insert and pSinHis plasmid DNA from the 1.3% low melting temperature gel with the Wizard PCR Preps System, it was isolated using a 'Freeze 'n Squeeze' phenol/chloroform extraction method. DNA bands were cut from the gel, weighed, cut into small pieces and place in an Eppendorf tube. An equal volume of phenol was added (0.1 g gel = 100 µL phenol), the mixture frozen at -70°C for 10 minutes and centrifuged at 4°C for 15 minutes. The aqueous phase was transferred into a clean tube, phenol added (200 µL aqueous phase to 100 µL phenol), mixed thoroughly and centrifuged for 5 minutes at 4°C. 100 µL of chloroform was added, and the mixture centrifuged for 10 minutes at 4°C. The top phase containing the isolated DNA fragments was transferred into a clean tube and kept on ice. DNA was precipitated using 1/10 volume of 3M NaOAc (pH 5.2) and 2.5x volume of absolute EtOH, storage at -20°C for 20 minutes and centrifuged for 25 minutes at 4°C (14,000g). The DNA pellet was washed twice with 70% EtOH (500 µL, 10 minute centrifugation at 4°C) before being air-dried and then redissolved in 20 µL sterilised deionised water.

The pSinHis plasmid (200 ng) and RLX insert (40 ng) DNA were set up in a standard ligation reaction that included the DNA fragments, 1 mM ATP, 1x One-Phor-All buffer and 4 U T4 DNA ligase in a total volume of 20 µL. The reaction was incubated at room temperature overnight and inactivated by heating to 65°C for 10 minutes. Ligated DNA was precipitated, transformed into $DH_{5\alpha}$ cells, DNA purified from selected colonies, screened using the Not I digest and visualised on a 1.3% agarose gel. One of twenty colonies selected was positive for RLX insert into pSinHis plasmid, clone #2 (Figure 4.9a). To check the positive clone before sequencing, clone #2 was digested with Pml I and Sph I as described above and visualised on a 1.3% agarose gel which showed the correct RLX insert dropout of

~550 bp (Figure 4.9b). The plasmid was isolated from an *E.coli* $DH_{5\alpha}$ culture and DNA purified using the Wizard Plus Midiprep DNA Purification System (Promega, WI, USA) as described in Section 2.2.1.3. Clone #2 was sequenced as described in Section 2.2.3.1.

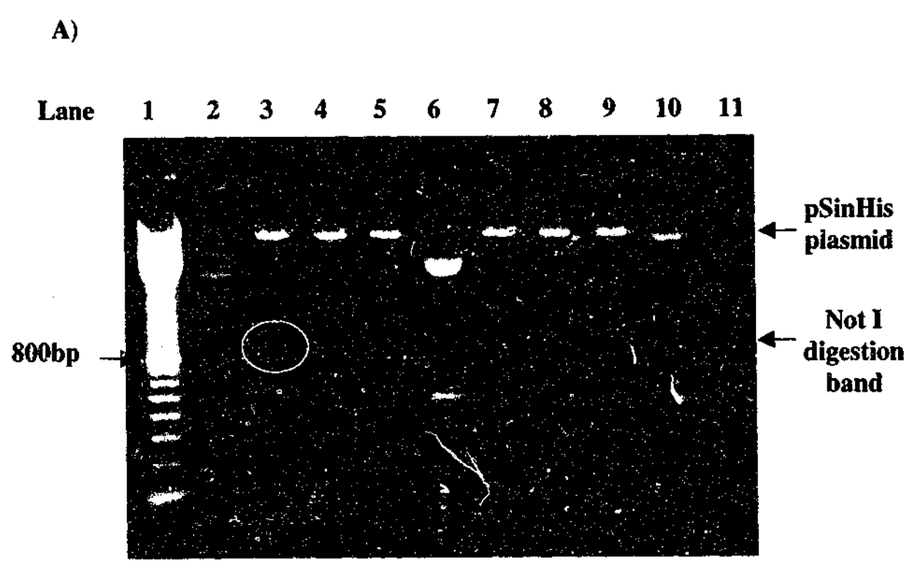
4.2.2 RNA transcription of DNA templates

To transiently express the protein in the BHK cell line, the DNA templates were used to produce RNA transcripts. The *in vitro* transcribed capped RNA is transfected into the BHK cells and is read as messenger RNA, allowing production of the protein. RNA transcripts were produced from the pSinHis-RLX plasmid and from blank pSinHis plasmid to use as an internal control for the transfection and protein extraction process. The methods for RNA transcription were as described in Section 2.2.4. Briefly, the pSinHis-RLX and pSinHis plasmid were linearised by Xho I restriction enzyme digestion, isolated on a 1.3% low melting temperature gel and purified by the 'Freeze 'n Squeeze' protocol. The linearised DNA was transcribed (Section 2.2.4), and the quality of the RNA transcripts visualised on a 1% agarose gel (Figure 4.10).

Figure 4.9

Gel electrophoresis of the pSinHis plasmid following ligation of RLX construct.

A) Selected colonies were screened (Lanes 2-11) by digestion with Not I restriction enzyme. Colonies containing the successfully ligated pSinHis-RLX plasmid were identified by a 845bp band (Lane 3, yellow circle) as measured against a 100bp molecular weight marker (Lane 1). The presence of pSinHis-RLX plasmid in Clone #2 was confirmed by B) digestion with Pml I and Sph I restriction enzymes (Lane 2), resulting in a 550bp band. The 100bp molcular weight marker is shown in Lane 1.



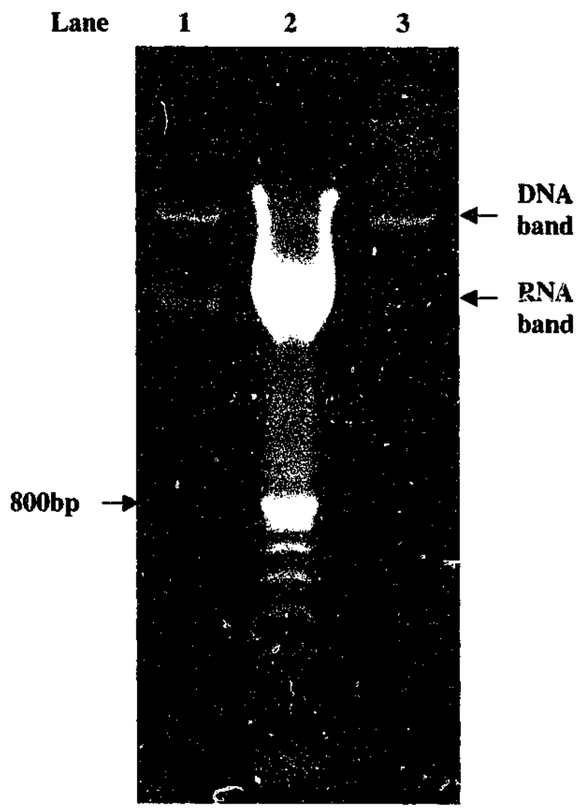


Figure 4.10

Gel electrophoresis of the RNA transcripts.

The quality of the transcribed RNA is represented by the clear band, approximately half the size of the DNA template band. The blank pSinHis plasmid is shown in Lane 1, the pSinHis-RLX plasmid is in Lane 3 while the 100bp molecular weight marker is shown in Lane 2.

4.2.3 RLX protein production and extraction

The pSinHis-RLX and pSinHis plasmid RNA transcripts were transfected into BHK cells plated in Petri dishes as described in Section 2.2.5. The transfected BHK cells were harvested at two time points, 24 and 48 hours following transfection. Protein was extracted as described in Section 2.2.6. Samples from every step were saved for analysis, including all waste and washing steps. Briefly, harvested cells were pelleted, lysed and the protein in the supernatant was separated from the cell debris. The His_6-RLX protein was purified from the cell lysate supernatant using the ProBond Purification System (Invitrogen, Vic, Aust) as described in Section 2.2.6.2. The His_6 tag attached to the RLX protein binds to the pre-equilibrated nickel bead column while the unwanted proteins and cell debris are washed away. The RLX protein is cleaved from the His_6 tag on the column by the EKMax enzyme provided with the pSinHis system, and collected from the column.

4.2.4 Analysis of recombinant protein

A sample of each extraction step was mixed with loading dye and loaded onto a 16% polyacrylamide gel as described in Section 2.7. Following electrophoresis (150V, 2 hours) proteins were visualised by staining the gel. The gel was fixed in Rapid Stain Fixing Solution (25% isopropanol, 10% acetic acid), and gently agitated for 15 minutes. The fixing solution was replaced with Rapid Coomassie Blue Stain Solution (0.06% Coomassie blue G-250, 10% acetic acid) and the gel gently agitated overnight. Excess stain was removed by washing in Destaining Solution II (7% acetic acid, 5% methanol) until the gel background was clear. Since there were few protein bands visible on the gel throughout all extraction steps (data not shown) including the cell debris pellet, it appeared that the protein in each sample was too dilute. Therefore the transfection and protein extraction was repeated using a larger scale preparation.

The process was repeated for the larger scale extraction and following electrophoresis, proteins were visualised by staining the gel with a silver staining

protocol (Silver Stain Plus kit, Bio-Rad, CA, USA), which is more sensitive than the Rapid Coomassie Blue Stain. The gel was soaked in a Fixative Enhancer Solution (50% v/v reagent grade methanol, 10% v/v reagent grade acetic acid, 10% v/v fixative enhancer concentrate) with gentle agitation for 20 minutes. The gel was rinsed with deionised water twice for 10 minutes with gentle agitation while the staining solution was prepared. Staining solution consisted of 35 mL deionised water being continually stirred while 5 mL Silver Complex Solution, 5 mL Reduction Moderator Solution and 5 mL Image Development Reagent were added. Immediately before use, 50 mL Development Accelerator Solution was added and mixed thoroughly before being poured over the gel. The gel was gently agitated in staining solution for 20 minutes or until the protein bands were clearly visible, when the reaction was stopped by transferring the gel into a 5% v/v acetic acid solution for 15 minutes. The gel was rinsed in deionised water for 5 minutes before being dried in cellophane for analysis (Figure 4.11). Results showed many protein bands throughout all extraction steps with a strong band of the RLX protein size (~17 kD) identified in the sample of the cell debris pellet. There was no visible band of the RLX protein size identified in any of the EKMax enzyme cleavage samples where the RLX protein was expected to be isolated.

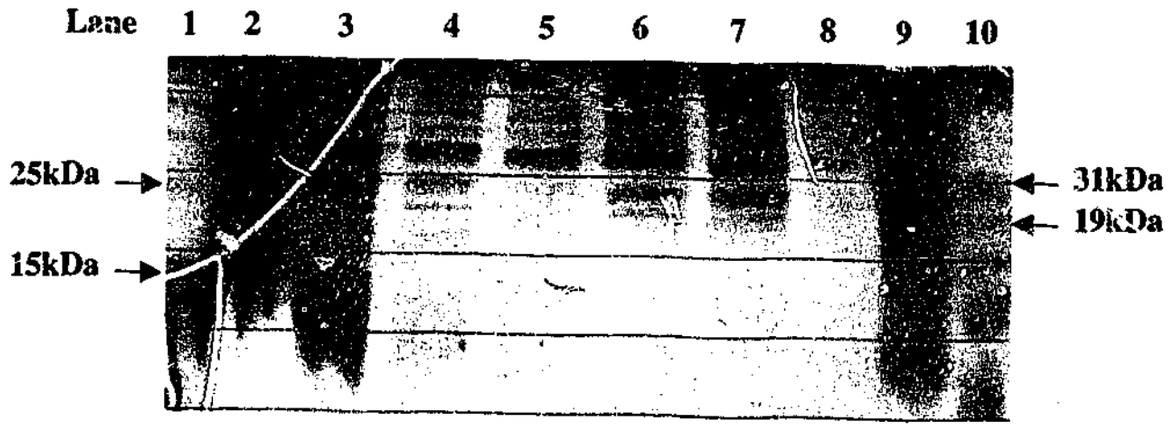
Relaxin specific antibodies were obtained (Dr R. Bathgate, Howard Florey Institute of Experimental Physiology and Medicine, Vic, Aust) to analyse the Western gel for the expressed recombinant RLX protein. Samples of the protein were loaded onto a 16% polyacrylamide gel, H2RLX protein was loaded for a positive control, and the gel processed as described in Section 2.7. Following electrophoresis the proteins were electrotransferred onto a nitrocellulose membrane which was incubated with the relaxin-specific primary antibody overnight at 4°C. The HRP-linked secondary antibody was applied to the membrane, followed by exposure to enhanced chemiluminescence (ECL) and the proteins were visualised on Hyper ECL film (Figure 4.12). The relaxin antibody clearly identified the H2RLX positive control and a band of the RLX protein size (~17 kD) in the cell debris pellet, in agreement with the results from the silver stained gel (Figure 4.11). No other protein band of appropriate size was identified in any of the other extraction step samples.

Figure 4.11

Silver stain of polyacrylamide gel showing the purification steps.

Each sample obtained from the purification step was screened for proteins of pro-RLX size, ~17 kDa. The samples from each of the purification steps (Section 2.2.6.2) included; cell debris pellet (Lane 2), raw supernatant from cell lysate (Lane 3), binding buffer washes (Lane 4 & 5), wash buffer washes (Lanes 6 & 7) binding buffer without NaCl wash (Lane 8), EKMax enzyme cleavage (Lane 9), wash buffer washes (Lane 12-14), wash buffer at pH 5.5 washes (Lane 15 & 16) and washes to strip the resin beads (Lane 17 & 18). PPS molecular weight markers are shown in Lanes 1 & 11 while kaleidoscope molecular weight markers are in Lanes 10 & 20. Lane 19 is blank. The pro-RLX protein was expected in Lane 9 and was not present, however a protein of the expected 17 kDa size was shown in the cell debris pellet and also in the raw lysate supernatant, Lanes 2 & 3 respectively.

During the drying procedure the gel cracked as seen through Lanes 1-3, 11 and 19-20.


Lane 1 2 3 4 5 6 7 8 9 10
25kDa
15kDa
31kDa
19kDa

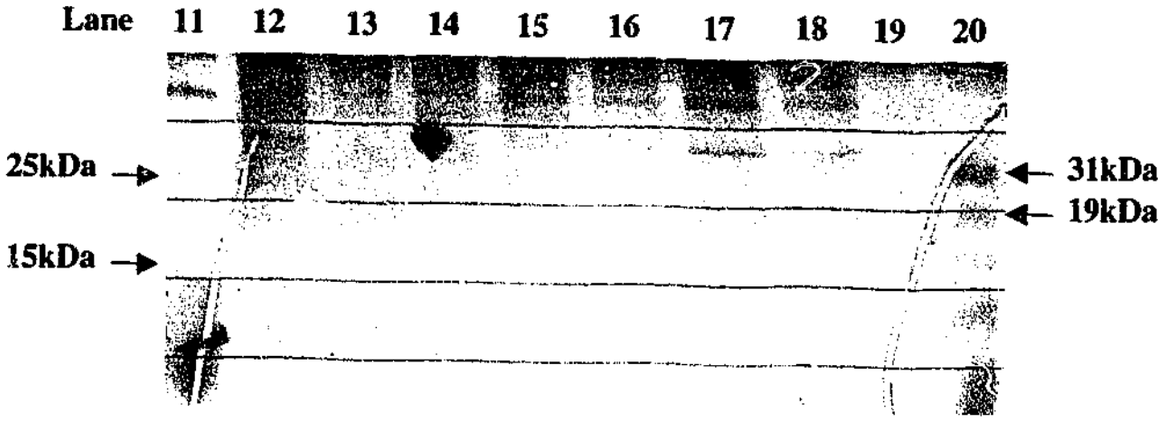
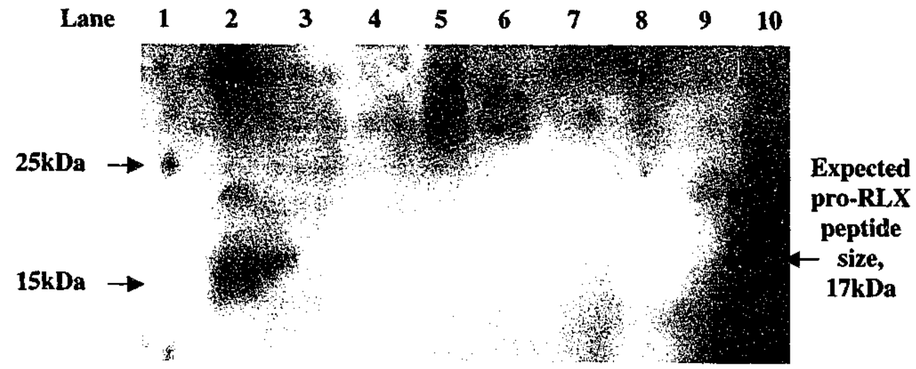

Lane 11 12 13 14 15 16 17 18 19 20
25kDa
15kDa
31kDa
19kDa

Figure 4.12

Western blot showing the purification samples probed with relaxin specific antibodies.

To probe for pro-RLX, the Western blot containing various samples from the purification steps was exposed to a relaxin-specific antibody. The purification steps (Section 2.2.6.2) included; cell debris pellet (Lane 2), raw supernatant from cell lysate (Lane 3), binding buffer wash 1 (Lane 4), EKMax enzyme cleavage (Lane 5), wash buffer wash 1 (Lane 6), and wash 1 to strip the resin beads (Lane 7). Molecular weight markers are shown in Lanes 1 & 8 with Lane 9 remaining a blank lane. Recombinant H2RLX was used as a positive control (Lane 10), however too much protein was loaded and during transfer the protein appeared to bleed into the adjoining blank lane.

The relaxin-specific antibody indicated a RLX peptide in the cell debris pellet (Lane 2) of the approritate size (17 kDa) while also appearing to bind to larger proteins of ~20 kDa and 50 kDa. The pro-RLX peptide was expected in Lane 5 and the antibody appeared to show a possible dimer form of the pro-RLX (~35 kDa).



4.3 Discussion

The aim of this study was to develop a method for the recombinant production of relaxin peptides which would encompass molecular modification of the peptide to allow further study of the structural interaction between relaxin and the relaxin receptor. The pSinHis system was chosen as the base of the recombinant methodology for two reasons: it is a mammalian system which would ensure correct folding of the human relaxin peptide and the addition of a polyhistidine tag into the protein of choice allowed for simple purification.

E.coli cultures are commonly used for recombinant protein production as they can express large amounts of protein efficiently to meet the demand placed by industry. Various methods for the recombinant production of insulin and relaxin peptides by *E.coli* cultures have been reported, including the expression of the A and B chains separately before being assembled into the native protein (Schmidt *et al.*, 1999; Vandlen *et al.*, 1995; Williams *et al.*, 1982) and the expression of the full peptide with the A and B chains connected by either the full length connecting C peptide (Reddy *et al.*, 1992; Stewart *et al.*, 1983; Williams *et al.*, 1982) or a unique "mini-C" peptide (Vandlen *et al.*, 1995; Wetzel *et al.*, 1981). The common problem and the most costly step in the production of recombinant proteins using *E.coli* cultures is the need to re-fold the peptides into the correct structure by forming the disulphide bonds following purification from the culture. *E.coli* bacteria cannot read the mammalian coding for the structural folding and therefore the peptide is expressed in a non-functional form. Further development has demonstrated success for *in vitro* folding of pro-insulin using a redox system before purification from the *E.coli* cultures (Winter *et al.*, 2002a; Winter *et al.*, 2002b), increasing the obtainable yield of the peptide.

An advantage of producing pro-RLX over pro-insulin peptides is that unlike pro-insulin, pro-RLX does not need conversion into RLX to obtain biological activity (Tan *et al.*, 1998; Vu *et al.*, 1993; Zarreh-Hoshyari-Khah *et al.*, 2001). The ultimate purpose of our recombinant system was to produce a variety of pro-H2RLX peptides

to examine the principle areas of the peptide that determine its relaxin-like activity. As only small yields of each peptide were needed for a pharmacological study, the strategy was to develop a mammalian recombinant system that allowed correctly folded RLX peptides to be isolated, as has been shown in previous studies (Kizawa *et al.*, 2003; Marriott *et al.*, 1992; Vu *et al.*, 1993). The advantage of using the pSinHis recombinant system was the synthesis of the pro-RLX peptide with a polyhistidine tag attached. The polyhistidine tag displays high affinity for activated metal cation beads, allowing efficient purification of the recombinant protein. Polyhistidine tags have been extensively used for fast and efficient extraction of recombinant proteins and receptors (Chaga *et al.*, 1999; Mukhija *et al.*, 1995; Palma *et al.*, 2001; Piatibratov *et al.*, 2000; Theis *et al.*, 2001), including recombinant human pro-insulin (Mackin, 1999). Although the polyhistidine tags do not appear to influence the activity of the recombinant peptides (Mukhija *et al.*, 1995; Piatibratov *et al.*, 2000; Theis *et al.*, 2001), the polyhistidine tag can be cleaved from the pro-RLX peptide in the pSinHis system by the EKMax enzyme, allowing a pure sample of pro-RLX to be obtained.

The analysis of the purification samples was expected to show the location of the pro-H2RLX peptide in the elutant following cleavage of the protein from the polyhistidine tag bound to the nickel beads. Unfortunately the protein staining techniques failed to identify the pro-H2RLX protein (17 kDa) in the expected extraction sample. Coomassie Blue and silver staining techniques suggested with the relaxin antibody detection that the recombinant relaxin protein at the molecular size of ~17 kDa was located within the cell debris pelleted upon removal of cell supernatant. The relaxin antibody however also identified a weak protein in the EK max cleavage sample at the molecular size of ~35 kDa. Previous relaxin recombinant studies have reported the formation of pro-RLX homodimers (Vu *et al.*, 1993) which could account for this 35 kDa protein detected by the relaxin antibodies. Although the larger form of the relaxin protein was positively identified in the correct extraction sample, the majority of the recombinant relaxin synthesised appeared to be in its monomeric form in the cell debris extraction sample.

A successful extraction of pro-RLX peptides from recombinant cultures can be achieved by the secretion of the protein into the culture media. Previous studies have shown secretion of pro-H2RLX with a shortened C-peptide from yeast cultures (Yang *et al.*, 1993), pro-PRLX from a CHO cell culture (Vu *et al.*, 1993), marmoset monkey pro-RLX from an insert cell culture (Zarreh-Hoshyari-Khah *et al.*, 2001) and H3RLX from a mouse adrenocorticotrophic hormone secreting cell line (AtT20) (Kizawa *et al.*, 2003). The addition of a signal peptide to the recombinant protein sequence encoded for the secretion the pro-H2RLX peptide into the cell culture media may prevent the pro-H2RLX peptide being retained within the cell debris pellet upon extraction.

In conclusion, the mammalian recombinant system developed to produce pro-H2RLX peptides appears to have synthesised the recombinant protein, although it seems to be retained within the cell debris pellet. Further modification of the system to allow secretion of the recombinant peptide into the culture media may allow successful isolation of pro-RLX proteins.

Chapter Five

Characterisation of the putative relaxin receptor, LGR7

5.1 Introduction

Although relaxin was one of the first reproductive hormones to be identified, the isolation and characterisation of the relaxin receptor continued to elude researchers for decades. Since relaxin is related to insulin and other members of the insulin-gene superfamily that signal through tyrosine kinase receptors, it was initially proposed that the relaxin receptor would also be a tyrosine kinase related to but distinctly different from the insulin receptor (Büllesbach *et al.*, 1995b). The relaxin receptor was reported to be comprised of two components, a 220 and a 36 kDa fragment, similar to the insulin receptor (Osheroff *et al.*, 1995; Parsell *et al.*, 1996). Binding of [^{32}P]-relaxin to human uterine cells was unaffected by GTP, indicating that the relaxin receptor was unlikely to be a G-protein coupled receptor (Osheroff *et al.*, 1995). Stimulation of relaxin receptors on lower human uterine fibroblasts caused an increase in tyrosine phosphorylation of a 220 kDa protein while failing to produce a cAMP response (Palejwala *et al.*, 1998), providing further evidence suggesting a tyrosine kinase receptor rather than a G-protein coupled receptor signalling through G_S. However, relaxin caused activation of adenylate cyclase in human myometrium membrane preparations. This response was inhibited by Tyrphostin 47, a tyrosine kinase inhibitor, and enhanced by GTP-Gpp[NH]p, a non hydrolysable guanine nucleotide analogue (Kuznetsova *et al.*, 1999). Insulin and IGF-1 stimulated adenylate cyclase activity was also regulated similarly to the relaxin response, further strengthening the idea that the relaxin and insulin receptor were similar and providing evidence of a link between a tyrosine kinase receptor and the adenylate cyclase signalling system.

However, and surprisingly given the indications noted above, the relaxin receptor was recently identified as an orphan leucine-rich repeat-containing G-protein coupled receptor (LGR). PRLX was found to interact and stimulate a cAMP response through an orphan LGR receptor, LGR7 (Hsu *et al.*, 2002), with similar potency to that observed for H2RLX acting at the relaxin receptor in THP-1 cells (Parsell *et al.*, 1996). Interestingly, relaxin also stimulated another orphan LGR receptor, LGR8 (Hsu *et al.*, 2002), but had lower potency than at the LGR7 receptor.

It was hypothesised and later confirmed that the LGR8 receptor was the INSL3 receptor rather than a second relaxin receptor (Bogatcheva *et al.*, 2003; Kumagai *et al.*, 2002), and LGR8 knockout mice displayed the same cryptorchidism phenotype as INSL3 knockout mice (Hsu *et al.*, 2002). The INSL3 and LGR8 link has also been demonstrated for human cryptorchidism (Ferlin *et al.*, 2003; Roh *et al.*, 2003).

Although the relaxin receptor did not resemble other members of the insulin gene superfamily, it does resemble other reproductive hormone receptors such as luteinising (LH) and follicle stimulating hormone (FSH) (Hsu *et al.*, 2000; Ivell, 2002). LGR receptors are large G-protein coupled receptors containing the typical seven transmembrane domains with a unique large ectodomain that includes the leucine-rich repeat (LRR) regions (Figure 1.4) (Hsu, 2003; Kajava *et al.*, 1995). The LGR family is split into three subfamilies of receptors according to their ectodomain structure (Figure 1.5); subfamily A LGRs includes the LH, FSH and the thyroid-stimulating hormone (TSH) receptors; subfamily B includes the orphan LGR4, LGR5 & LGR6 receptors; and subfamily C consists of the putative relaxin receptor (LGR7) and the INSL3 receptor (LGR8) (Hsu, 2003; Hsu *et al.*, 2000).

There is close similarity between the expression of LGR7 receptor mRNA and expression of relaxin binding sites or responses to relaxin in rat tissues. LGR7 mRNA occurs in various reproductive organs, including ovary, uterus and testis, is present in heart and brain tissue, and in low levels in the kidney, adrenal gland and small intestine (Hsu *et al.*, 2000). Relaxin binding sites are found throughout the rat reproductive system, including cervix (Kuenzi *et al.*, 1995; Weiss *et al.*, 1982) and uterus (Osheroff *et al.*, 1990; Weiss *et al.*, 1982) as well as in areas of the brain and heart (Osheroff *et al.*, 1992; Osheroff *et al.*, 1991; Tan *et al.*, 1999). Whilst no relaxin receptor has been demonstrated in the kidney, relaxin treatment has been shown to reverse the fibrotic symptoms induced by age-related fibrosis in relaxin knockout mice (Samuel *et al.*, 2003c; Samuel *et al.*, 2004).

The aim of this study was to examine the pharmacological properties of the putative relaxin receptor, LGR7, using a binding assay with the specific relaxin radioligand [^{33}P]-HRLX and a cAMP accumulation assay.

5.2 Methods

LGR7 receptors were transiently expressed in CHO K1 cells using LGR7 DNA in a pcDNA3.1-Zeo expression vector (Invitrogen, Vic, Aust). The relaxin analogues used to examine relaxin interaction with the LGR7 receptor in binding and cAMP assays included the specific relaxin radioligand, [^{33}P]-H2RLX, and the competitive relaxin analogues H2RLX, H3RLX and PRLX. RatINSL3 and human insulin were included as negative controls.

5.2.1 Cell culture

CHO K1 cells were maintained in 50:50 Dulbecco's Modified Eagle's Medium (DMEM): Ham's F-12 media containing glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 µg/mL) and heat inactivated FBS (10% v/v). CHO K1 cells were maintained below ~80% confluence and split as described in Section 2.1.1.2.

5.2.1.1 Transient transfection of CHO K1 cells

CHO K1 cells were transiently transfected with the LGR7 receptor DNA construct in the pcDNA3.1-Zeo expression vector (Invitrogen, Vic, Aust) as described in Section 2.1.4. Briefly, 18 hours before transfection, cells were harvested (Section 2.1.1.2), counted (Section 2.1.3) and plated at 8 x 10^5 cells/cm^2 in normal cell media. The transfection reaction was incubated for 30 minutes and cells incubated with the transfection mix at 37°C for 4 hours. Serum enriched medium was added and the incubation continued at 37°C for a further 20 hours. Transfection mix medium was replaced with normal media and the incubation continued at 37°C for 24 hours. Transfected cells were used 48 hours after transfection.

5.2.2 96 well binding assay

The CHO K1 cell binding assay was set up as described in Section 2.3.3.2. The total volume used was 100 µL/well, comprising 20 µL of 100 pM [^{33}P]-H2RLX ± 10 µL unlabelled relaxin analogue and 50 µL cell suspension, made up to 100 µL with binding buffer. All experiments were performed in duplicate. Following a 90 minute incubation at 25°C, cells were harvested onto GF/C filter plates (Skudtek, Vic, Aust) pre-soaked overnight (0.5% PEI, 4°C). Filter plates were dried at 42°C, Microscint O (Perkin-Elmer, Vic, Aust) added (30 µL/well) and equilibrated overnight. Filters were counted on a Topcount scintillation counter (Perkin-Elmer, Vic, Aust) and data was analysed with GraphPad PRISM (Section 2.8).

5.2.2.1 [^{33}P]-H2RLX (B33) preparation

H2RLX (B33) was labelled with [γ-^{33}P]-ATP using a procedure based on Osheroff *et al* (1990) as described in Section 2.3.1.

5.2.2.2 Saturation binding protocol

Saturation binding studies were used to determine the affinity (pK_D) of [^{33}P]-H2RLX and the maximum number of binding sites (B_{max}) of the LGR7 receptor transiently expressed in CHO K1 cells. Saturation binding studies were performed at a concentration range of 25 pM – 1 nM. Specific binding was calculated by the total binding – non-specific binding (10 µM H2RLX) for each [^{33}P]-H2RLX concentration. Protein concentrations were determined as described in Section 2.6 and the data was plotted as specific binding (fmol/mg protein) against concentration of radioligand used (pM) (Section 2.8).

5.2.2.3 Competition binding protocol

Competition binding assays were used to calculate the affinity (pK_i) of various relaxin analogues for the putative relaxin receptor, LGR7. Competition binding was performed using [^{33}P]-H2RLX at a concentration of 100 pM. To minimise the amount of relaxin analogues used, which were in limited supply, a restricted range of concentrations was used. The concentration range used for H2, H3 and PRLX analogues was 100 pM – 100 nM and a single concentration of RatINSL3 (100 nM) and human insulin (1 μM) were also examined. Non-specific binding was determined using 1 μM H2RLX. Results were expressed as a percentage of maximal specific binding against concentration of the relaxin or relaxin-like analogue used (M) and pK_i values were calculated (Section 2.8).

5.2.3 cAMP accumulation assay

The ability of the relaxin analogues to stimulate a functional response through the LGR7 receptor was examined using the Perkin-Elmer Flashplate cAMP assay (Section 2.4.1). Briefly, cells were plated into a 96-well tissue culture plate 16 hours before each experiment at 10,000 cells/well. Cells were stimulated in incubation media with 10 μL relaxin analogue (10 pM – 30 nM), forskolin (1 μM) for the reference response or diluting vehicle (PBS) for basal levels for 30 minutes at 37°C. Stimulation was halted by the addition of 0.5M HCl (25 μL/well) and the plate stored at -20°C. cAMP samples were analysed using a NEN FlashPlate RIA (Perkin-Elmer, Vic, Aust) according to the manufacturers instructions (Section 2.4.1). Radioactivity was measured on a Topcount scintillation counter (Perkin-Elmer, Vic, Aust) and data analysed against a cAMP standard curve using GraphPad PRISM (Section 2.8).

5.2.4 Analysis

Results are expressed as mean ± S.E.M. of n experiments (Section 2.8). Saturation binding data was analysed with GraphPad PRISM. Radioligand affinity (pK_D) and LGR7 receptor expression (B_{max}) values were calculated and expressed in pM and fmol/mg protein respectively. Competition binding data was analysed with GraphPad PRISM and the relaxin analogue affinity (pK_i) values were calculated using the Cheng & Prusoff (1973) equation. cAMP accumulation data was analysed with GraphPad PRISM, calculated as pM cAMP per well, expressed as a percentage of maximal cell response as determined by forskolin stimulation and analogue potency (pEC_{50}) values calculated. Statistical analysis was completed using GraphPad PRISM statistical programs with competition binding and functional response curves analysed using a two-way ANOVA. pK_i values were analysed using a Student's t-test. P values of less than or equal to 0.05 were considered significant.

5.2.5 Drugs and reagents

All drugs were used as described in Section 2.9. Stock solutions of H2, H3, PRLX analogues and RatINSL3 were prepared in 0.1% aqueous trifluoroacetic acid (TFA) at a concentration of 10 μM. Human insulin (Humulin® R) was supplied by Aza Research Pty. Ltd. (NSW, Aust) at a concentration of 100 units/mL. All drugs were diluted in binding or stimulation buffer prior to use. All plastic tubes, pipette tips and binding plates used in relaxin experiments were coated with Sigmacote® (Sigma Chemical Company, MO, USA).

5.3 Results

5.3.1 Characteristics of [^{33}P]-H2RLX binding to the LGR7 receptor

Specific [^{33}P]-H2RLX binding occurred in a saturable manner to a single population of binding sites on the LGR7 receptor expressed in CHO K1 cells (Figure 5.1). Determination of the B_{max} value indicated that the density of LGR7 receptors was 336 ± 73 fmol/mg protein in the whole cell binding assay. [^{33}P]-H2RLX showed high affinity for the LGR7 receptor with a pK_D of 9.33 ± 0.23 (n=4).

5.3.2 Competition for [^{33}P]-H2RLX binding at the LGR7 receptor

Two forms of human relaxin displayed high affinity for [^{33}P]-H2RLX binding at the LGR7 receptor (Figure 5.2a) with H2RLX having the highest affinity with a pK_i of 9.2 ± 0.19 (n=6) while H3RLX was significantly lower (pK_i 7.4 ± 0.18, n=5; P<0.004). PRLX also displayed high affinity for the human LGR7 receptor (Figure 5.2b) with a pK_i of 8.5 ± 0.13 (n=6; P<0.03 cf H2RLX) while both RatINSL3 and Human Insulin failed to compete for [^{33}P]-H2RLX binding (n=6 & 5 respectively; Figure 5.2c).

Figure 5.1

[^{33}P]-H2RLX saturation binding characteristics at the human LGR7 receptor transiently expressed in CHO-K1 cells.

The specific relaxin radioligand, [^{33}P]-H2RLX, displayed high affinity (pK_D 9.33 ± 0.23; n=4) for a single binding site on the LGR7 receptor. Transient expression of the LGR7 receptor in CHO K1 cells gave a B_{max} of 336 ± 76 fmol/mg protein.

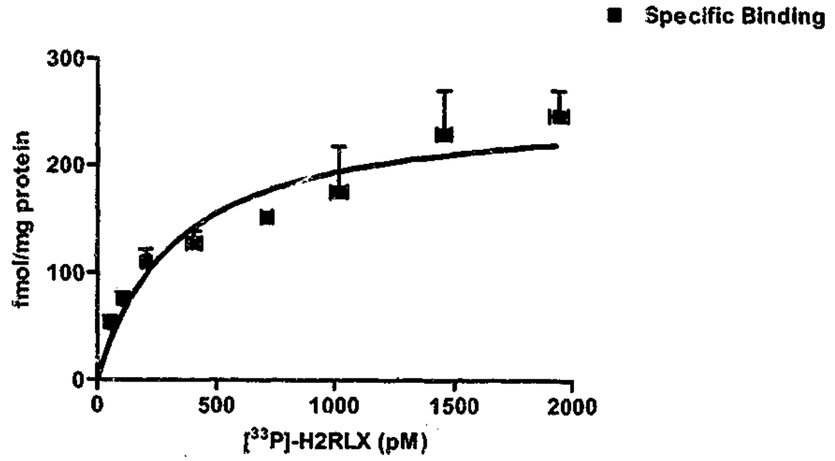
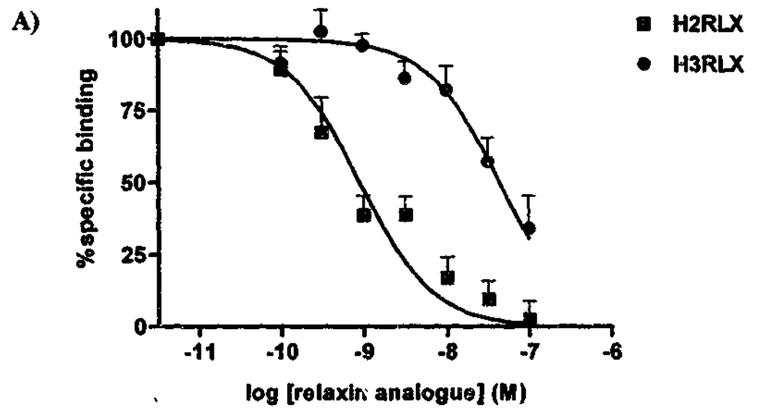
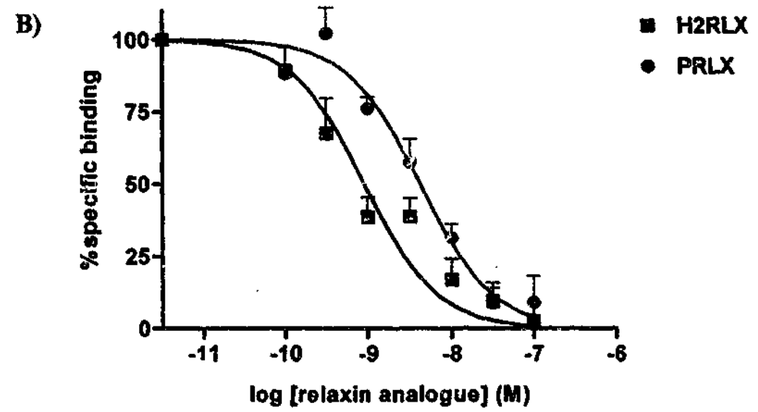


Figure 5.2

Competition for binding at the human LGR7 receptor transiently expressed in CHO-K1 cells.

These graphs represent the pooled specific affinity data displayed by human and species homologues of relaxin for the human relaxin receptor. Both forms of human relaxin, human gene 2 (H2RLX) and human gene 3 (H3RLX) relaxin, displayed specific affinity for the LGR7 receptor with H2RLX having a higher affinity (pK_i 9.2 ± 0.19, n=6) than A) H3RLX (pK_i 7.4 ± 0.18, n=5; P<0.004). B) the porcine relaxin homologue (PRLX) showed high affinity for the human LGR7 receptor (pK_i 8.5 ± 0.13, n=6; P<0.03 cf H2RLX) while C) structurally related peptides, rat insulin-like peptide 3 (RatINSL3; n=6) and Human Insulin (n=5), failed to compete.





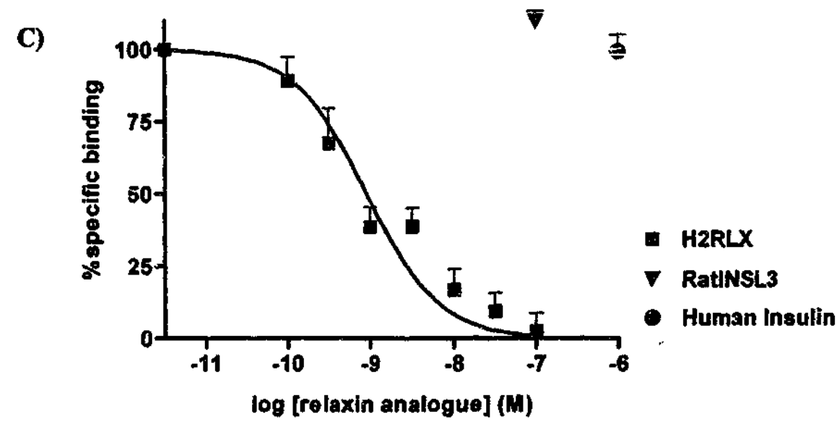


Table 5.1

Comparison of relaxin and relaxin-like analogue affinity for the naturally expressed human relaxin receptor in THP-1 cells and the putative human relaxin receptor LGR7 transiently expressed in CHO K1 cells. * denotes P<0.05, ** P<0.005 verus H2RLX. N/D denotes affinity not determinable, as the analogue did not compete for binding.

Analogue	THP-1 pK_i (n)	CHO-LGR7 pK_i (n)
H2RLX	8.6 ± 0.11 (13)	9.2 ± 0.19 (6)
H3RLX	8.4 ± 0.16 (8)	7.4 ± 0.18 (5)**
PRLX	8.4 ± 0.09 (5)	8.5 ± 0.13 (6)*
SheepINSL3	5.9 ± 0.32 (4)**	
RatINSL3		N/D (6)
Human Insulin		N/D (5)

5.3.3 Stimulation of cAMP accumulation by the LGR7 receptor

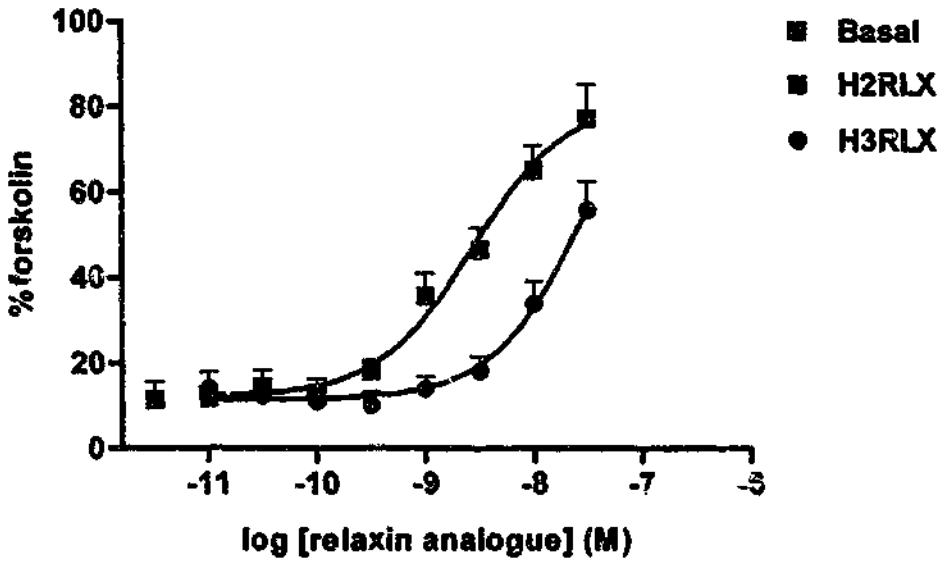
The relaxin analogues that competed with high affinity for binding of [^{33}P]-H2RLX at the LGR7 receptor also stimulated a cAMP response. PRLX was the most potent (Figure 5.3b) with a pEC_{50} of 9.3 ± 0.08 (n=4), followed by H2RLX 8.6 ± 0.12 (n=4; P<0.03 cf PRLX) and H3RLX 7.7 ± 0.10 (n=4; P<0.03 cf H2RLX) (Figure 5.3a). Interestingly the order of affinity obtained in the [^{33}P]-H2RLX binding studies is somewhat different from the order of potency for stimulation of cAMP accumulation with PRLX displaying lower affinity at the LGR7 receptor compared to H2RLX while displaying a higher potency for stimulation of a cAMP response. RatINSL3 and Human Insulin failed to induce a cAMP response above basal levels (n=4; Figure 5.3c)

Figure 5.3

Stimulation of cAMP accumulation through the human LGR7 receptor transiently expressed in CHO-K1 cells.

These graphs represent the pooled cAMP accumulation data stimulated by the human and species homologues of relaxin through the human relaxin receptor. cAMP accumulation was stimulated by all relaxin analogues with human gene 2 relaxin (H2RLX; pEC_{50} 8.6 ± 0.12, n=4) being more potent than A) human gene 3 relaxin (H3RLX; pEC_{50} 7.7 ± 0.1, n=4; $P<0.03$). B) The porcine homologue of relaxin (PRLX) was the most potent relaxin analogue (pEC_{50} 9.3 ± 0.08, n=4), being significantly stronger than H2RLX ($P<0.03$). C) the structurally related peptides, rat insulin-like peptide 3 (RatINSL3; n=4) and Human Insulin (n=4) failed to produce a cAMP response above basal levels.

A)


100
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% forskolin
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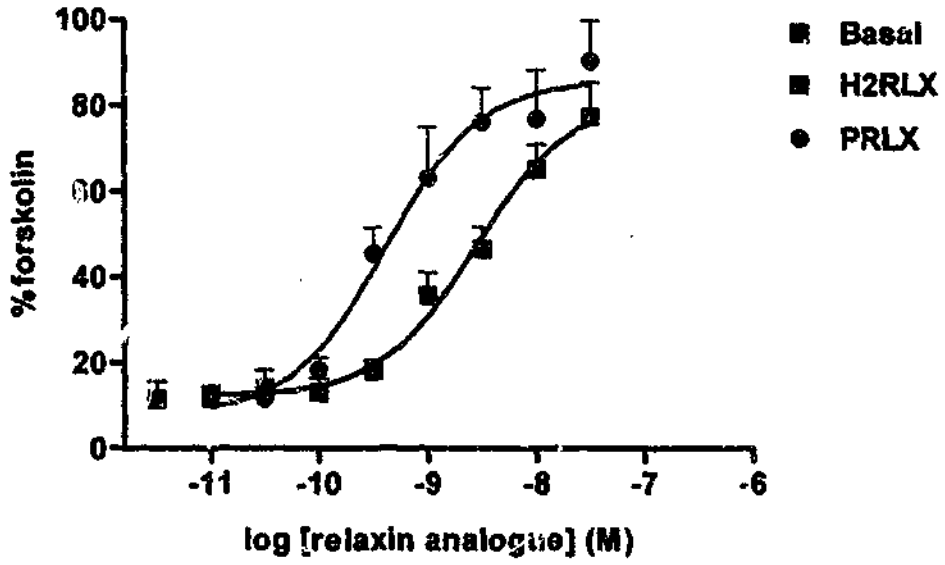
log [relaxin analogue] (M)
Basal
H2RLX
H3RLX

B)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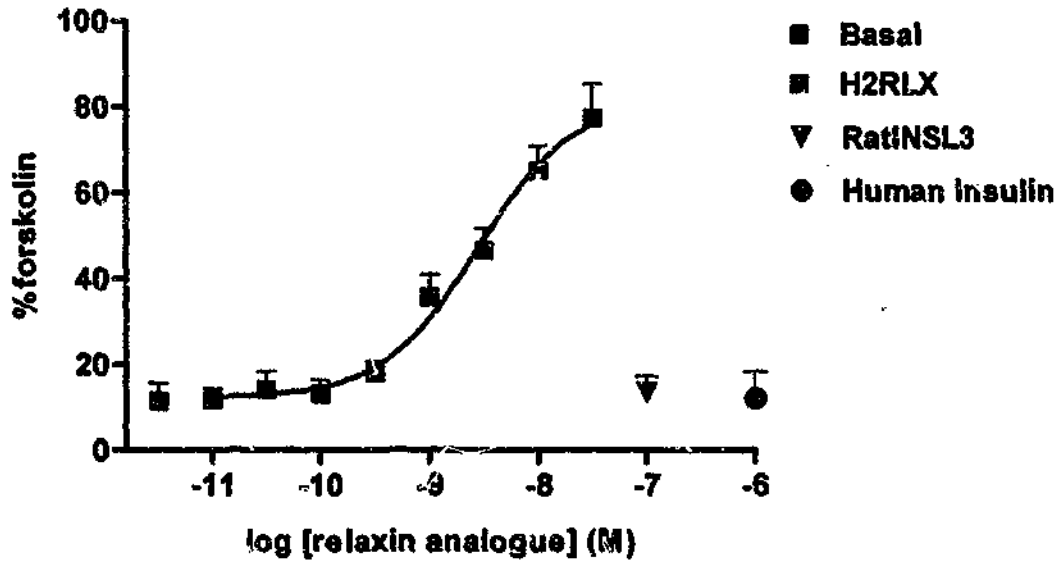
log [relaxin analogue] (M)
Basal
H2RLX
PRLX

C)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log [relaxin analogue] (M)
Basal
H2RLX
RatINSL3
Human insulin

5.4 Discussion

The relaxin receptor has recently been identified as an orphan G-protein coupled receptor, LGR7 (Hsu *et al.*, 2002). The aim of this study was to examine the pharmacological properties of the LGR7 receptor by examining the interaction of several relaxin analogues with the human LGR7 receptor transiently expressed in CHO K1 cells.

The LGR7 receptor displayed high affinity for the relaxin radioligand, [^{33}P]-H2RLX. The pK_D value of 9.33 ± 0.23 for the binding of [^{33}P]-H2RLX to the LGR7 receptor was almost identical to the affinity shown in the THP-1 binding assay of 9.37 ± 0.22. This is consistent with previously published reports using THP-1 cells (Parsell *et al.*, 1996) and a human uterine cell line (Osheroff *et al.*, 1995). The binding characteristics of the relaxin receptor do not appear to change markedly with expression levels as THP-1 cells express very low levels of the relaxin receptor with a B_{max} of 1.45 ± 0.62 fmol/mg protein (Section 3.3.1) or ~275 receptors/cell (Parsell *et al.*, 1996), and the human uterine cell line expresses low levels of receptors with approximately 1082 receptors/cell (Osheroff *et al.*, 1995) while our transient expression system appeared to express the LGR7 receptor with a B_{max} of 336 ± 73 fmol/mg protein. However, these assays were conducted as whole cell assays rather than using membrane preparations, therefore the protein levels reflect the entire cell protein content, diluting the receptor expression values.

Interestingly, the affinity of the relaxin analogues appeared to vary between the LGR7-CHO cell system and the THP-1 cells. Although H2RLX showed a greater affinity for the LGR7 receptor than that shown in the THP-1 binding assay (Table 5.1), H3RLX displayed a lower affinity for the LGR7 receptor than that shown in THP-1 cells. PRLX displayed high affinity for the human LGR7 receptor, similar to the affinity displayed for the relaxin receptor expressed in the THP-1 cell line (Table 5.1), and as previously shown non-relaxin members of the insulin gene superfamily failed to interact with LGR7, as neither RatINSL3 nor human insulin could displace radioligand binding.

Not only did the affinities vary slightly, but H3RLX effectively competed for 100% of binding at the LGR7 receptor whereas it only competed for 50% in the THP-1 binding assay. A possible explanation for this is that low levels of relaxin receptor naturally expressed in the THP-1 cells may be accompanied by expression of other receptors in the cell that may also interact with the relaxin analogues, such as the LGR8 receptor, as both LGR7 and LGR8 mRNA have been identified in THP-1 cells (Nguyen *et al.*, 2003). Our radioligand, [^{33}P]-H2RLX, can interact with both the LGR7 and LGR8 receptors (see Chapter 6) while it has been reported that H3RLX displays preferential interaction with LGR7 while displaying no affinity for the LGR8 receptor (Sudo *et al.*, 2003). The transfection of the LGR7 receptor into CHO cells allows the specific interaction between the LGR7 receptor and the relaxin analogues to be examined as the over expression of the LGR7 receptor is likely to overshadow any small interaction with other naturally expressed receptors.

Another interesting finding was that PRLX was more potent than the native ligand, H2RLX, for cAMP accumulation while displaying a lower affinity for the LGR7 receptor. This is the first study to demonstrate this discrepancy between the relaxin analogues. A previous study has examined both H2RLX and PRLX (Sudo *et al.*, 2003) and reported an equal potency for the two relaxin analogues at the LGR7 relaxin receptor expressed in HEK293 cells. The conflicting results may reflect slight differences in the interaction of the relaxin analogues at the receptor that is augmented by differences in the signal transduction mechanisms of the two cell lines. A difference in the ability to inhibit cAMP signalling has previously been shown between CHO K1 and HEK 293 cells. The cAMP response in CHO K1 cells was inhibited with greater potency than the response in HEK293 cells (Zhang *et al.*, 1995), possibly indicating a difference in strength of coupling to the cAMP pathway between the two cell lines. H3RLX was the weakest of the relaxin analogues in stimulating a cAMP response, in accordance with published reports (Sudo *et al.*, 2003). Also in accordance with the binding studies, neither RatINSL3 nor human insulin could stimulate a cAMP response through the LGR7 receptor.

In conclusion, the relaxin receptor has been positively identified as the LGR7 receptor. H2RLX and PRLX displayed high affinity and potency for the LGR7 receptor, while H3RLX interaction was significantly weaker. Rat INSL3 and human insulin failed to maintain either affinity or efficacy for the relaxin LGR7 receptor. These results correlate well with previous studies using THP-1 cells that have been found to express the LGR7 receptor (Nguyen *et al.*, 2003).

Chapter Six

Characterisation of LGR7 & LGR8 receptors

6.1 Introduction

The protracted search for the relaxin receptor concluded with the characterisation of the relaxin LGR7 receptor (Hsu *et al.*, 2002). However, it was also demonstrated that relaxin interacted with a second receptor, LGR8. Relaxin displayed higher affinity for LGR7 than LGR8 but the interaction with LGR8 was shown to be a specific relaxin interaction that could not be replicated by insulin, IGF's or glucagon (Hsu *et al.*, 2002; Sudo *et al.*, 2003). The LGR7 receptor behaved in the same manner and showed the same distribution as the relaxin receptor (Chapter 5) leading to the conclusion that LGR7 was the classical relaxin receptor as defined by functional and binding studies, leaving the relevance of the interaction between relaxin and the LGR8 receptor unclear.

The LGR7 receptor was isolated from EST sequences and classified as a new subclass of LGR. Subfamily C LGR's are characterised by a unique low-density lipoprotein (LDL) domain within the N-terminus of the ectodomain (Hsu, 2003; Hsu *et al.*, 2000). LGR7 has approximately 24% homology with Subfamily A LGR's, including the well characterised LH, FSH and TSH receptors, and only slightly higher homology (< 50%) with the Subfamily B LGR's, three orphan receptors known as LGR4, LGR5 and LGR6 (Hsu, 2003). Recently, another orphan LGR isolated from EST sequences was found to have > 90% homology with the LGR7, including the characteristic Subfamily C LDL domain. The new receptor became the second Subfamily C LGR, and was chronologically labelled the LGR8 receptor (Hsu, 2003; Hsu *et al.*, 2002).

Further analysis of the LGR8 sequence positively identified it as the human homologue to the mouse GREAT receptor (Hsu *et al.*, 2002). GREAT receptor gene knockout mice (Gorlov *et al.*, 2002; Overbeek *et al.*, 2001) displayed the phenotype of abnormal testis decent, uncannily similar to INSL3 knockout mice (Adham *et al.*, 2000; Nef *et al.*, 1999; Zimmermann *et al.*, 1999). The relaxin-related peptide, INSL3, was found to be another ligand for the LGR8 receptor in both binding and functional studies (Bogatcheva *et al.*, 2003; Kumagai *et al.*, 2002; Sudo *et al.*, 2003).

All three species homologues of INSL3, human, porcine and rat INSL3, as well as biotinylated porcine INSL3 and PRLX were capable of stimulating an LGR8 cAMP response with similar potency (Kumagai *et al.*, 2002). At the LGR7 receptor however only PRLX produced a cAMP response and INSL3 analogues had no efficacy. However not all relaxin analogues interact with LGR8 as one study has shown the preferential interaction of H3RLX with LGR7 while displaying no affinity or potency at LGR8 (Sudo *et al.*, 2003).

The confirmation of LGR7 as the relaxin receptor, LGR8 as the INSL3 receptor and the differential interaction displayed by human relaxin analogues raised interesting questions. Do other relaxin analogues interact differentially with the LGR7 and LGR8 receptors and does interaction translate into functional signalling? The aim of this study was to investigate the binding characteristics of and functional response to various species homologues of relaxin at the LGR7 and LGR8 receptors.

6.2 Methods

LGR7 and LGR8 receptors were transfected and stably expressed in an adherent cell line. The relaxin analogues used included H2RLX, PRLX, RatRLX and rhesus monkey relaxin (RMRLX), as well as the human homologue of INSL3 (HINSL3).

6.2.1 Production of a cell line stably expressing the LGR7 and the LGR8 receptors

Transfection of the LGR7 and LGR8 receptor cDNA in the pcDNA3.1-Zeo expression vector (Invitrogen, CA, USA) into the CHO K1 cell line was preformed using a LIPOFECTAMINE™ transfection method as described in Section 2.1.4. Development into a stable cell line was as described in Section 2.1.5. Screening of the CHO K1 cell colonies for expression levels of the LGR7 and LGR8 receptors showed extremely low levels of receptor expression (data not shown), resulting in colonies that were unusable for further investigation.

HEK293 cell lines stably expressing the LGR7 and LGR8 receptors were successfully produced as described in Section 2.1.5 and kindly provided by our collaborator Sharon Layfield at the Howard Florey Institute of Experimental Physiology and Medicine, Vic, Aust.

6.2.2 Cell Culture

HEK293 cells stably expressing either the LGR7 or LGR8 receptors were maintained in RPMI 1640 media containing glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 µg/mL), heat inactivated FBS (10% v/v) and zeocin (200µM). All tissue culture plates and flasks were coated with poly-L-lysine (0.1 mg/mL) prior to cell culture to ensure cell adhesion. HEK293 cells were maintained below ~80% confluence and split as described in Section 2.1.1.2.

6.2.3 High through-put 96 well binding assay

The HEK293 LGR7/LGR8 cell binding assay was set up as described in Section 2.3.3. The total volume used was 100 µL/well, comprising 20 µL of [^{33}P]-H2RLX ± 10 µL unlabelled relaxin or relaxin-like analogue and 50 µL cell suspension, made up to 100 µL with binding buffer. All experiments were performed in duplicate. Following a 90 minute incubation at 25°C, cells were harvested onto GF/C filter plates (Skudtek, Vic, Aust) pre-soaked overnight (0.5% PEI, 4°C). Filter plates were dried at 42°C, Microscint O (PerkinElmer, Melb, Aust) added (30 µL/well) and equilibrated overnight. Filters were counted on a Topcount scintillation counter (PerkinElmer, Melb, Aust) and data was analysed with GraphPad PRISM (Section 2.8).

6.2.3.1 [^{33}P]-H2RLX (B33) preparation

H2RLX (B33) was labelled with [γ-^{33}P]-ATP using a procedure based on Osheroff *et al* (1990) as described in Section 2.3.1.

6.2.3.2 Saturation binding protocol

Saturation binding studies were used to determine the affinity (pK_D) of [^{33}P]-H2RLX and number of LGR7 and LGR8 binding sites (B_{max}) expressed in HEK293 cells (Section 2.8). Saturation binding studies were performed using a concentration range of 50 pM – 2.5 nM. Specific binding was calculated by the total binding – non-specific binding (10 µM H2RLX) at each [^{33}P]-H2RLX concentration. Protein concentrations were determined as described in Section 2.5.1 and the data was plotted as specific binding (fmol/mg protein) against concentration of radioligand used (pM).

6.2.3.3 Competition binding protocol

Competition binding assays allowed the calculation of the affinity (pK_i) of various relaxin analogues for the LGR7 and the LGR8 receptors expressed in HEK293 cells (Section 2.8). Competition binding was performed using [^{33}P]-H2RLX at a concentration of 100 pM for LGR7 competition studies and two concentrations, 100 pM and 1 nM, for the LGR8 competition studies. The concentration range used for H2RLX, PRLX, RatRLX, RMRLX and HINSL3 analogues was 1 pM – 100 nM, with the exception of a single concentration of HINSL3 (100 nM) examined at the LGR7 receptor. Non-specific binding was determined using 1 μM H2RLX for the LGR7 receptor and 1 μM HINSL3 for the LGR8 receptor. Results were expressed as a percentage of maximal specific binding and pK_i values were calculated (Section 2.8).

6.2.4 cAMP accumulation assay

The ability of the relaxin analogues to stimulate a functional response through the LGR7 or LGR8 receptors was examined with the AlphaScreen™ cAMP accumulation assay (Section 2.4.2). Briefly, cells were plated into a 96-well tissue culture plate 24 hours before each experiment at 10,000 cells/well. Cells were incubated in stimulation buffer containing 10 μL relaxin analogue (10 pM – 1 μM) or vehicle (PBS) for 30 minutes (37°C). Stimulation mix was removed, the cells lysed and stored in lysis buffer (-20°C). cAMP samples were analysed using an AlphaScreen™ cAMP detection kit (PerkinElmer, Melb, Aust) according to the manufacturers instructions (Section 2.4.2). Detection plates were read by Fusion™ α microplate reader (PerkinElmer, Melb, Aust) and data were analysed against a cAMP standard curve with GraphPad PRISM (Section 2.8).

6.2.5 Analysis

Results are expressed as mean ± S.E.M. of n experiments (Section 2.8). Saturation binding data were analysed with GraphPad PRISM. Radioligand affinity (pK_D) and LGR7 or LGR8 receptor expression (B_{max}) values were calculated and expressed in pM and fmol/mg protein respectively. Competition binding data were analysed with GraphPad PRISM and relaxin analogue affinity (pK_i) values were calculated using the Cheng & Prusoff (1973) equation. cAMP accumulation data were analysed with GraphPad PRISM and analogue potency (pEC_{50}) values calculated (Section 2.8). Statistical analysis was completed using GraphPad PRISM statistical programs with competition binding and functional response curves analysed using a two-way ANOVA. pK_i and pEC_{50} values were analysed using a Student's t-test. P values of less than or equal to 0.05 were considered significant.

6.2.6 Drugs and reagents

All drugs were used as described in Section 2.9. Stock solutions of H2RLX, PRLX, RMRLX, RatRLX and HINSL3 analogues were prepared in 0.1% aqueous trifluoroacetic acid (TFA) within the concentration range of 10-100 μM. All drugs were diluted in binding or stimulation buffer prior to use. All plastic tubes, pipette tips and binding plates used in relaxin experiments were coated with Sigmacote® (Sigma Chemical Company, MO, USA).

6.3 Results

6.3.1 Characteristics of [^{33}P]-H2RLX binding

6.3.1.1 [^{33}P]-H2RLX binding characteristics of the LGR7 receptor

[^{33}P]-H2RLX binding occurred in a saturable manner to a single population of sites on the LGR7 receptor (Figure 6.1). Analysis of the binding data gave a B_{max} of 6.7 ± 0.21 fmol/mg protein in the whole cell binding assay, indicating that the LGR7 receptor was expressed at higher levels than in THP-1 cells (B_{max} 1.45 ± 0.62 fmol/mg protein; Chapter 3). [^{33}P]-H2RLX showed high affinity for the LGR7 receptor with a pK_D of 9.5 ± 0.15 (n=4)

6.3.1.2 [^{33}P]-H2RLX binding characteristics of the LGR8 receptor

[^{33}P]-H2RLX binding occurred in a saturable manner to the LGR8 receptor (Figure 6.2a), with a calculated pK_D of 9.0 ± 0.10 (n=6) and B_{max} of 13.3 ± 1.5 fmol/mg protein. Although the data conformed to a single binding site model, closer analysis of the data revealed a two site binding pattern. Conversion of the data into a Scatchard plot (Figure 6.2b) resulted in two affinity sites, a higher affinity site (pK_D 9.3) and a lower affinity site (pK_D 8.1). However the affinity values could only be calculated from the grouped data (n=6) as many of the individual data sets would not allow the calculation of the two affinity sites. This can probably be explained by the concentration range of [^{33}P]-H2RLX used in some experiments which was not high enough to clearly define the second binding site, but also to variability within the data sets. The two binding sites resulted in two B_{max} values of 4.35 and 29.34 fmol/mg protein.

Figure 6.1

[^{33}P]-H2RLX saturation binding characteristics at the LGR7 receptor.

The specific relaxin radioligand, [^{33}P]-H2RLX, displayed high affinity (pK_D 9.5 ± 0.15; n=4) for a single binding site on the LGR7 receptor. Stable expression of the LGR7 receptor in HEK293 cells gave a B_{max} of 6.7 ± 0.21 fmol/mg protein.

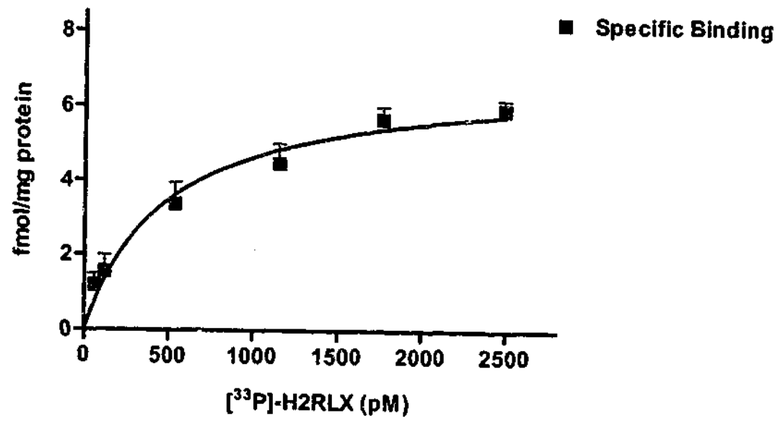
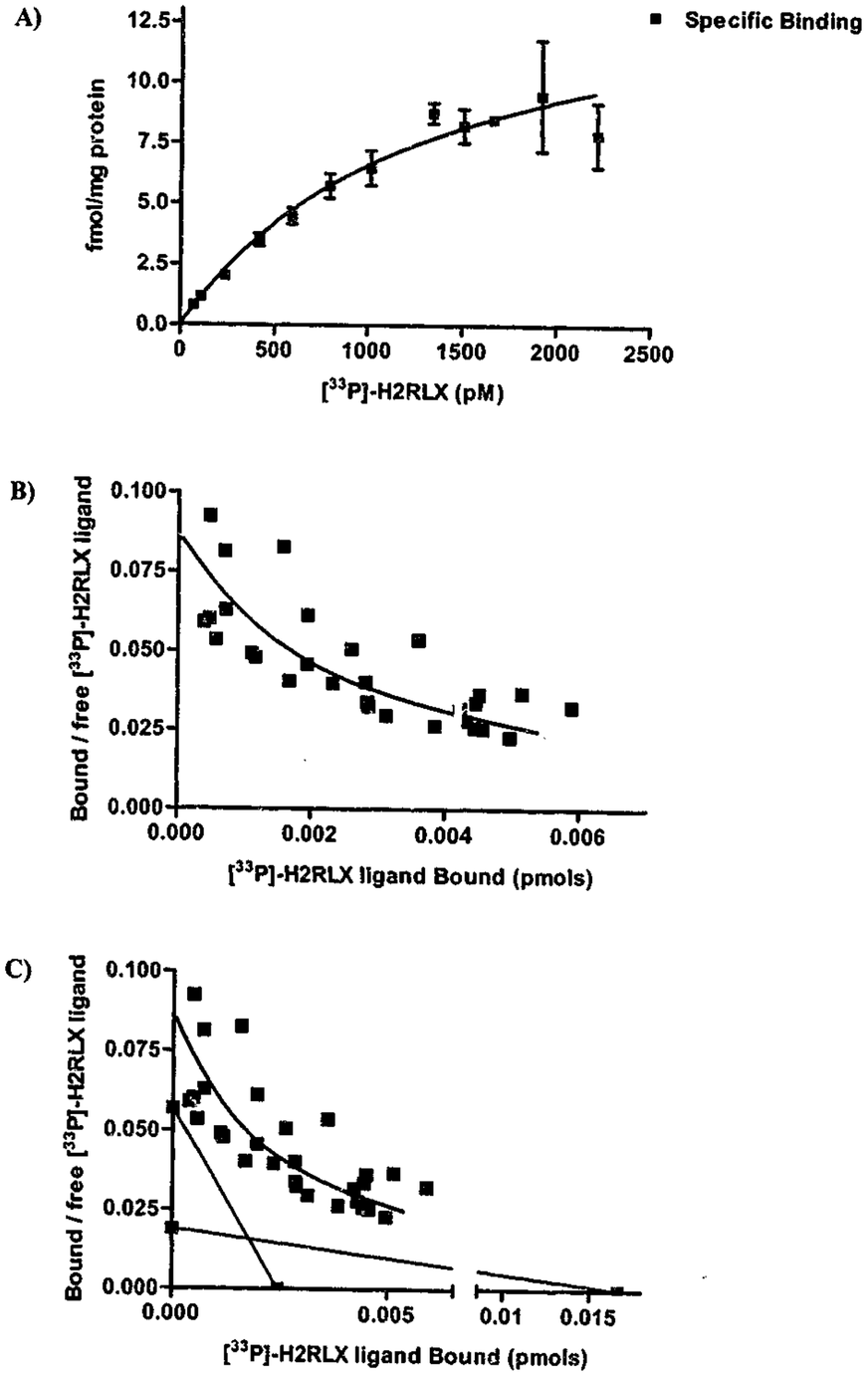


Figure 6.2

[^{33}P]-H2RLX saturation binding characteristics at the LGR8 receptor.

A) the LGR8 receptor stably expressed in HEK293 cells displayed high affinity for [^{33}P]-H2RLX with a pK_D 9.0 ± 0.10 (n=6) and B_{max} of 13.3 ± 1.5 fmol/mg protein. Conversion of the data B) into a Scatchard plot revealed the presence of two binding sites (n=6). Analysis of the grouped data showed C) a higher and lower affinity binding site, with pK_D values of 9.3 & 8.1 and B_{max} values of 4.35 & 29.34 fmol/mg protein respectively.



6.3.2 Competition binding characteristics

6.3.2.1 Competition for binding at the LGR7 receptor

All relaxin analogues competed for [^{33}P]-H2RLX binding and had high affinity for the LGR7 receptor. The order of affinity of RLX analogues for the LGR7 receptor was H2RLX (pK_i 9.4 ± 0.10, n=6) ≥ RMRLX (pK_i 9.3 ± 0.10, n=6; Figure 6.3b) ≥ PRLX (pK_i 9.0 ± 0.12, n=6; Figure 6.3a) >> RatRLX (pK_i 7.2 ± 0.18, n=6; P<0.002 cf H2RLX) (Figure 6.3c) whereas HINSL3 failed to compete for binding (n=4; Figure 6.3c).

6.3.2.2 Competition for binding at the LGR8 receptor

Competition for binding at the LGR8 receptor was examined using two concentrations of [^{33}P]-H2RLX with the aim of further exposing the two LGR8 binding sites. 100 pM [^{33}P]-H2RLX was used to examine the higher affinity binding sites while 1 nM was used to expose binding at a larger proportion of the lower affinity sites. The order of affinity at LGR8 (Figure 6.4) was HINSL3 (pK_i 9.7 ± 0.15, n=5; P<0.004 cf H2RLX) > H2RLX (pK_i 8.6 ± 0.14, n=6) ≥ PRLX (pK_i 8.2 ± 0.10, n=5) > RMRLX (pK_i 7.3 ± 0.20, n=4; P<0.01 cf H2RLX). RatRLX showed no affinity for the LGR8 receptor (n=4). Increasing the concentration of [^{33}P]-H2RLX failed to significantly alter the affinity of the competitive analogues (Figure 6.5; Table 6.1), although there was a trend for a decrease in the ability of H2RLX (Figure 6.5a) and HINSL3 (Figure 6.5d) to compete for [^{33}P]-H2RLX binding.

Figure 6.3

Competition for binding at the LGR7 receptor

All species homologues of relaxin displayed high affinity for the LGR7 receptor. Similar affinity was shown by H2RLX (pK_i 9.4 ± 0.10, n=6), A) PRLX (pK_i 9.0 ± 0.12, n=6) and B) RMRLX (pK_i 9.3 ± 0.10, n=6). C) RatRLX displayed the lowest relaxin-like affinity (pK_i 7.2 ± 0.18, n=6; P<0.002 cf H2RLX) while HINSL3 failed to compete for the LGR7 receptor (n=4).

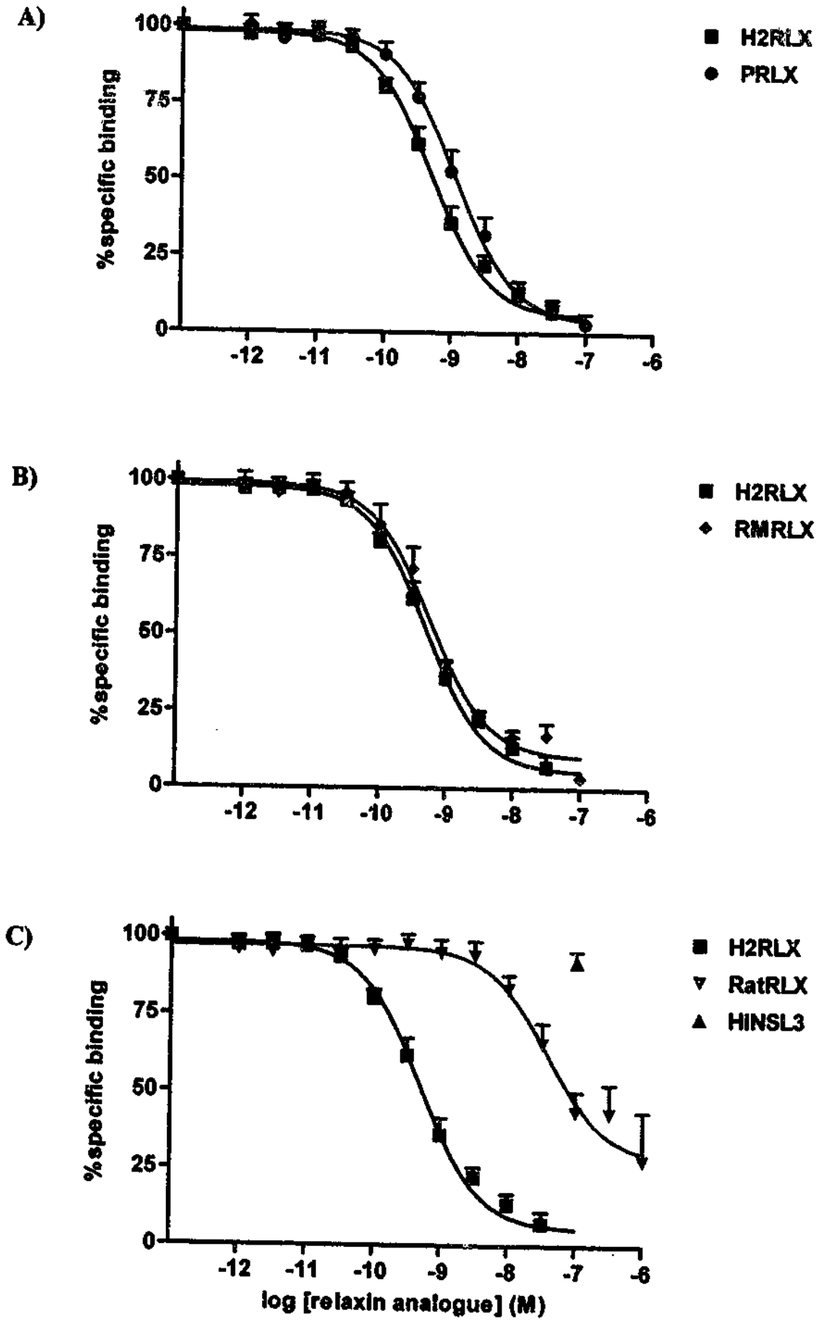


Figure 6.4

Competition for [^{33}P]-H2RLX (100pM) binding at the LGR8 receptor

H2RLX and A) PRLX analogues displaying significant lower affinity for the LGR8 receptor, pK_i 8.6 ± 0.14 (n=6; P<0.004 cf HINSL3) and 8.2 ± 0.10 (n=5) respectively. B) RMRLX displayed the lowest affinity, pK_i 7.3 ± 0.20 (n=4; P<0.01 cf H2RLX), while C) RatRLX failed to compete (n=4). C) HINSL3 displayed the highest affinity with a pK_i of 9.7 ± 0.19 (n=5).

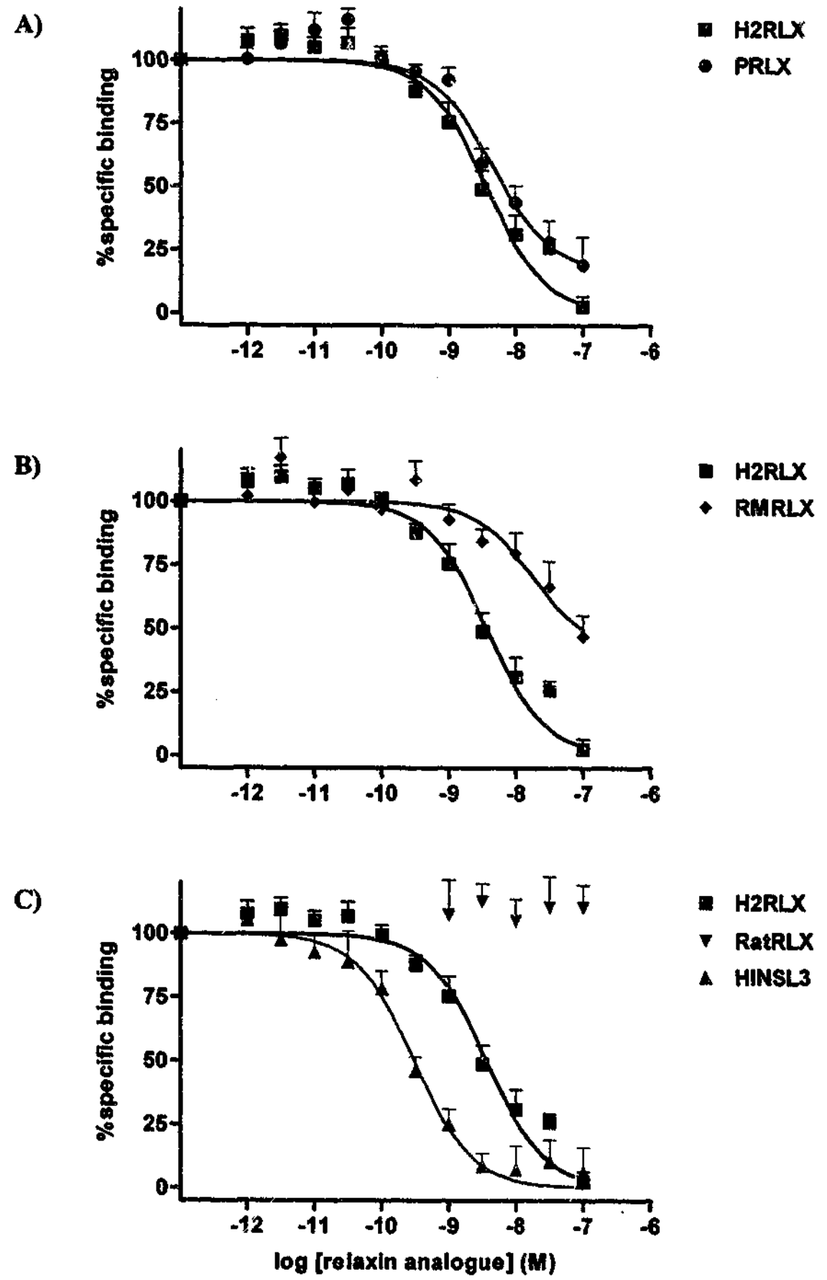


Figure 6.5

Competition for [^{33}P]-H2RLX binding at the LGR8 receptor using high and low ligand concentrations.

The affinity of A) H2RLX (n=6), B) PRLX (n=5-6), C) RMRLX (n=4) and D) HINSL3 (n=5) for the LGR8 receptor was not significantly altered by an increase in radioligand concentration. Competition for 100 pM [^{33}P]-H2RLX is shown by closed symbols while competition for 1 nM [^{33}P]-H2RLX binding is shown by open symbols.

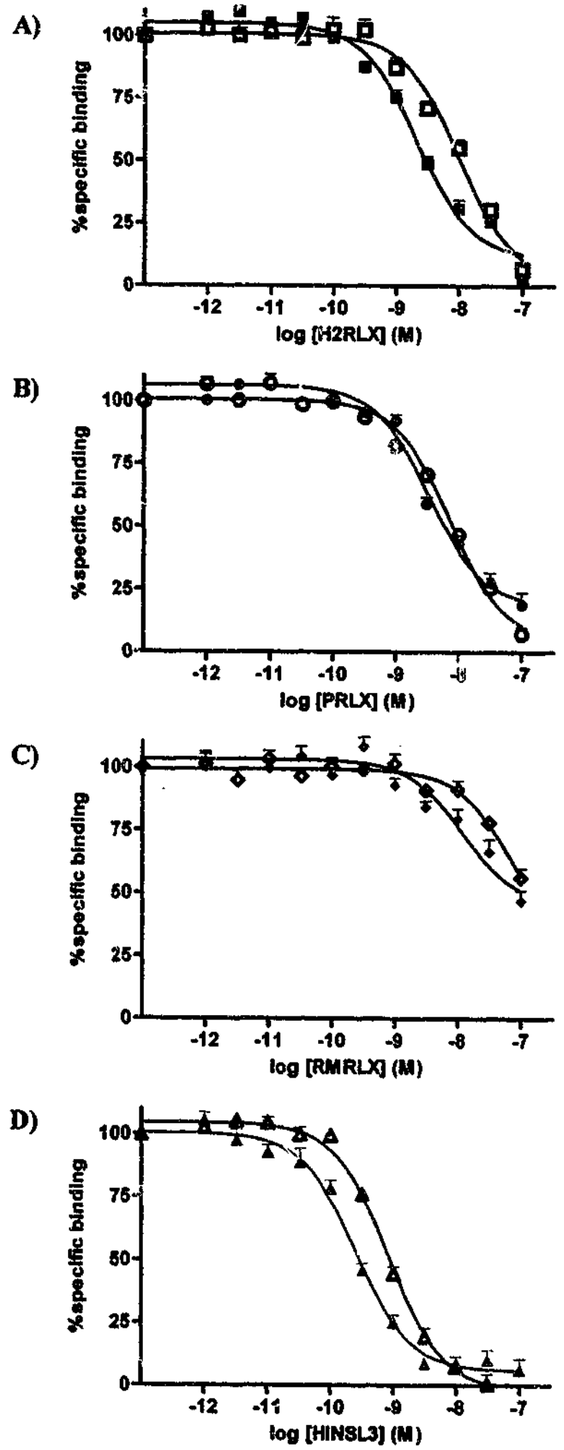


Table 6.1

The affinity (pK_i) of relaxin and HINSL3 analogues for the LGR7 and LGR8 receptors stably expressed in HEK293 cells. Competition for binding at LGR7 occurred at a single site, while affinity for the LGR8 receptor was examined using two concentrations of [^{33}P]-H2RLX (100pM and 1nM) with the aim of further exposing the two LGR8 binding sites. * denotes P<0.01, ** P<0.005 versus H2RLX (unpaired t-test). N/D denotes affinity not determinable, as the analogue did not compete for binding.

Analogues	LGR7-HEK pK_i (n)	LGR8-HEK 100pM pK_i (n)	LGR8-HEK 1nM pK_i (n)
H2RLX	9.4 ± 0.10 (6)	8.6 ± 0.14 (6)	8.2 ± 0.18 (6)
PRLX	9.0 ± 0.12 (6)	8.2 ± 0.10 (5)	8.2 ± 0.09 (6)
RatRLX	7.2 ± 0.18 (6)**	N/D (4)	N/D (5)
RMRLX	9.3 ± 0.10 (6)	7.3 ± 0.20 (4)*	6.9 ± 0.21 (4)*
HINSL3	N/D (4)	9.7 ± 0.19 (5)**	9.1 ± 0.11 (5)**

6.3.3 cAMP accumulation assay

6.3.3.1 Stimulation of cAMP production by activation of the LGR7 receptor stably expressed in HEK293 cells

All species homologues of relaxin activated the LGR7 receptor and stimulated a cAMP response. Initial analysis of the data with a sigmoidal dose-response curve (GraphPad Prism) showed that H2RLX (pEC_{50} 9.4 ± 0.42, n=4) and PRLX (pEC_{50} 9.4 ± 0.30, n=4) displayed the highest potency at the LGR7 receptor (Figure 6.6a), followed by RMRLX with a pEC_{50} 8.9 ± 0.16 (n=3; Figure 6.6b). However there appeared to be anomalies between the data points and the generated curve of best fit, therefore the data was re-analysed and found to conform to a two site model (Figure 6.7). The re-calculation resulted in two pEC_{50} values for each analogue; H2RLX, 10.2 ± 0.11 & 8.1 ± 0.19 (n=4); PRLX, 9.8 ± 0.14 & 8.0 ± 0.36 (n=4); RMRLX, 9.9 ± 0.21 & 8.0 ± 0.15 (n=3); with a high affinity state fraction of 0.7, 0.75 and 0.51 respectively. Interestingly RatRLX conformed to a single site model only, displaying the weakest potency with a pEC_{50} of 8.2 ± 0.34 (n=4; Figure 6.6c). HINSL3 failed to activate LGR7 and cause a significant cAMP response (n=4; Figure 6.6c). .

Figure 6.6

Stimulation of cAMP production by activation of the LGR7 receptor

cAMP accumulation was stimulated by all relaxin analogues with H2RLX (pEC_{50} 9.4 ± 0.42; n=4) and A) PRLX (pEC_{50} 9.4 ± 0.30; n=4) displaying the highest potency, followed by B) RMRLX (pEC_{50} 8.9 ± 0.16; n=4) and C) RatRLX (pEC_{50} 8.2 ± 0.34; n=4). HINSL3 (n=4) failed to produce a cAMP response above basal levels.

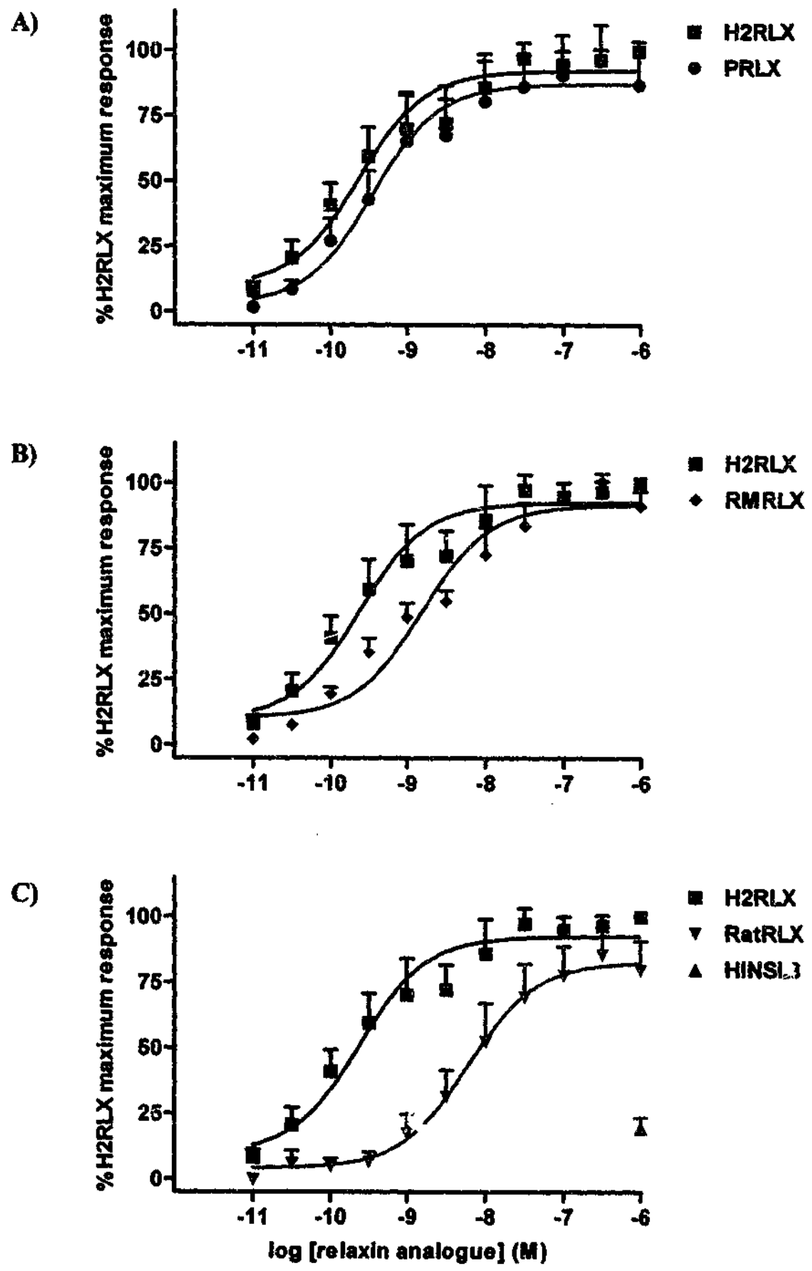
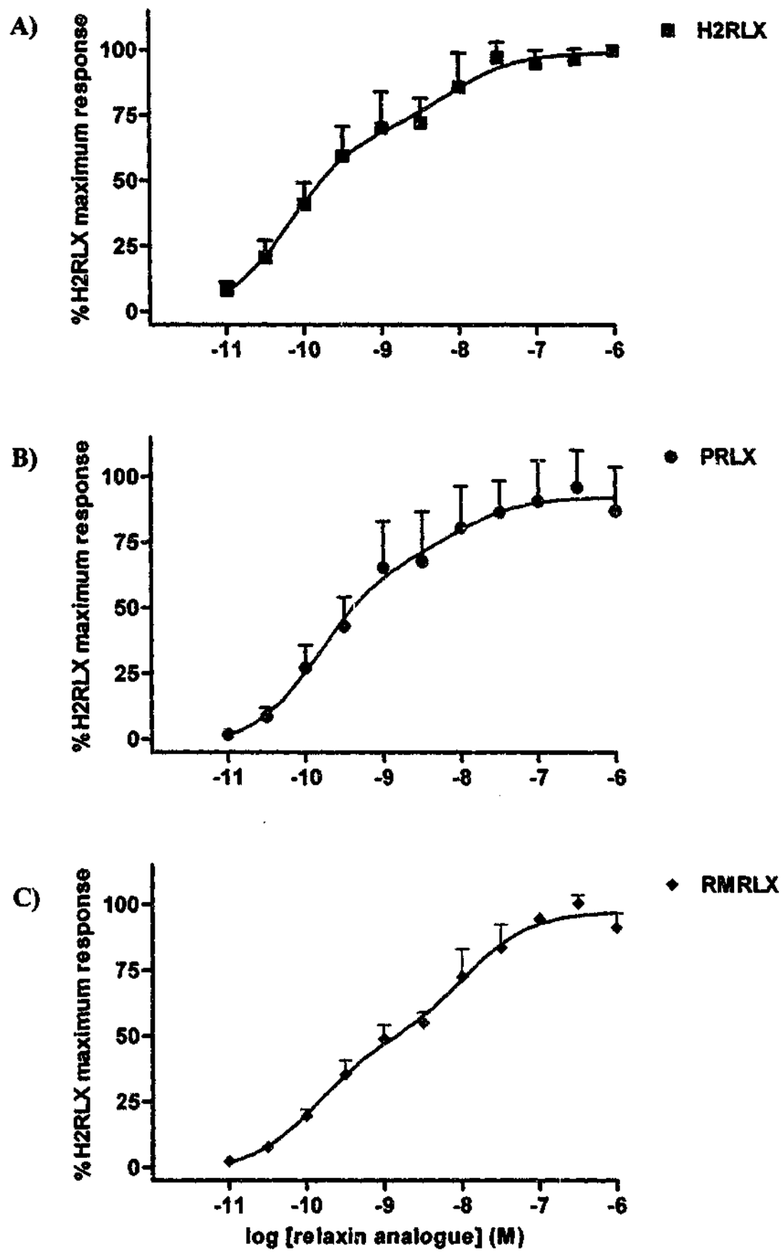


Figure 6.7

Stimulation of cAMP production at the LGR7 receptor

Further analysis of the cAMP response produced by the relaxin analogues at the LGR7 receptor showed stimulation in a biphasic manner. A) H2RLX (pEC_{50} 10.2 ± 0.11 & 8.1 ± 0.19, n=4); B) PRLX (pEC_{50} 9.8 ± 0.14 & 8.0 ± 0.36, n=4); C) RMRLX (pEC_{50} 9.9 ± 0.21 & 8.0 ± 0.15, n=3).



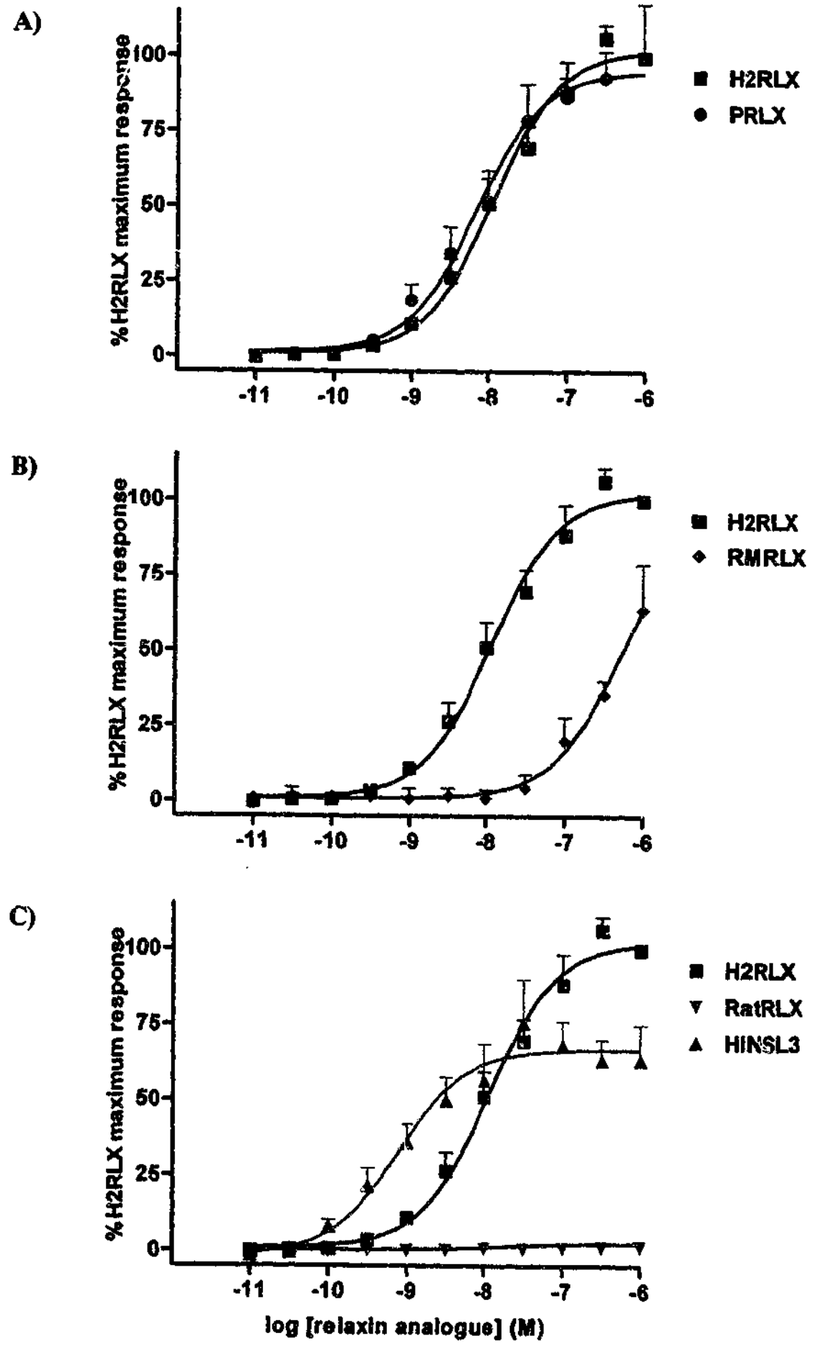
6.3.3.2 Stimulation of cAMP production by activation of the LGR8 receptor stably expressed in HEK293 cells

HINSL3 displayed the highest potency at the LGR8 receptor (pEC_{50} 9.1 ± 0.17, n=4; Figure 6.8c) with H2RLX (pEC_{50} 7.9 ± 0.16, n=4; P<0.03) and PRLX (pEC_{50} 8.1 ± 0.21, n=3; P<0.03) (Figure 6.8a) displaying significantly lower potency. The potency of RMRLX was dramatically reduced at the LGR8 receptor with a pEC_{50} of 6.4 ± 0.01 (n=3; P<0.001, Figure 6.8b) while RatRLX failed to stimulate a cAMP response (n=4; Figure 6.8c). Although HINSL3 was the most potent, it failed to produce a maximal cAMP response with the dose-response curve reaching a plateau at approximately 70% of the maximal response to H2RLX.

Figure 6.8

Stimulation of cAMP production by activation of the LGR8 receptor

The rank order of potency for cAMP accumulation was similar to the rank order of affinity with H2RLX (pEC_{50} 7.9 ± 0.16, n=4) and A) PRLX (pEC_{50} 8.1 ± 0.21, n=4) displaying high potency, followed by B) RMRLX (pEC_{50} 6.4 ± 0.01, n=4). C) RatRLX (n=4) failed to produce a cAMP response above basal levels while HINSL3 displayed the highest potency (pEC_{50} 9.1 ± 0.17, n=4; P<0.03 cf H2RLX).



6.4 Discussion

While the relaxin receptor was identified as the LGR7 receptor, the same study also reported an interaction between relaxin and the LGR8 receptor (Hsu *et al.*, 2002). The native ligand for the LGR8 receptor was later shown to be the INSL3 peptide (Bogatcheva *et al.*, 2003; Kumagai *et al.*, 2002), further highlighting the unexpected interaction between this receptor and relaxin. The aim of this study was to examine the interaction between various relaxin analogues and HINSL3 with the LGR7 and LGR8 receptors.

[^{33}P]-H2RLX binding was saturable to a single population of sites on the LGR7 receptor whereas the radioligand appeared to bind to two sites at the LGR8 receptor. This was the first indication of a difference between the actions of relaxin at the two receptors. The appearance of two LGR8 binding sites did not result from over-expression of the receptor compared to the LGR7 receptor as the B_{max} values revealed similar levels of receptor expression. [^{33}P]-H2RLX binding to LGR7 and LGR8 was of high affinity, comparable with previously determined [^{33}P]-H2RLX affinity for LGR7 receptors transiently expressed in a CHO-K1 cell system (pK_D 9.33 ± 0.23; Chapter 5), and naturally expressed in THP-1 cells (pK_D 9.37 ± 0.22; Chapter 3). While the LGR8 saturation curve was able to conform to a two site Scatchard plot, it was difficult to show a difference between the sites in competition binding at the two concentrations of radioligand used in this study. The relative lack of difference displayed by the relaxin analogues in the competition assays could be attributed to the unselective labelling of the two sites by [^{33}P]-H2RLX. Using the affinity and B_{max} values calculated from the two site LGR8 saturation binding model, an estimated fraction of occupancy (FO) was determined for each of the affinity sites. The equation for FO was taken from GraphPad PRISM; FO x B_{max} = (B_{max} x [radioligand])/(K_D + [radioligand]). Competition binding experiments conducted with 100pM radioligand should occupy 16% of the higher affinity sites while labelling only 1% of the lower affinity sites. Increasing the concentration of radioligand to 1nM increased the FO to 67% for the high affinity site and 11% for the low affinity site, so that 10% of the total number of sites occupied would be low

affinity sites. To occupy greater than 80% of the low affinity sites, the concentration of the radioligand would need to be increased to 40 nM.

The generation of LGR7/LGR8 receptor chimeras (Sudo *et al.*, 2003) containing the ectodomain of LGR7 attached to the transmembrane domain of LGR8, LGR7/8 receptor, and the complementary LGR8/7 receptor, have been used to show two separate LGR8 binding sites. Differences in the affinities displayed by relaxin analogues in competition binding studies at the chimeric receptors suggested that a higher affinity LGR8 binding site resided in the receptor ectodomain while a lower affinity site appeared to be located in the transmembrane domain (Halls, 2003; Halls *et al.*, 2003). The ectodomain of the receptor seemed to dictate the majority of the receptor properties with relaxin analogues interacting at LGR7/8 with similar affinity and potency to LGR7 while LGR8/7 followed LGR8 properties. The transmembrane domain does maintain a role in determining the receptor characteristics as further manipulation of the LGR7/8 chimera to contain the individual extracellular loops of LGR7, as well as the ectodomain, revealed the importance of extracellular loop 2 for maintaining H3RLX interaction at LGR7 (Sudo *et al.*, 2003).

While the binding studies showed that LGR7 displayed a single affinity site, the cAMP stimulation assay appeared to conform to a biphasic pattern. The relaxin induced cAMP accumulation is reportedly a biphasic response, involving a rapid increase in cAMP by the classical G_S stimulated pathway followed by a slightly delayed and sustained increase by a phosphoinositide 3-kinase (PI3K) dependent pathway (Nguyen *et al.*, 2003). Interestingly, not all relaxin analogues appear to stimulate in a biphasic manner as shown by the monophasic response to RatRLX. A possible explanation is that the biphasic response is the result of ligand directed signalling (Kenakin, 1995), where the individual relaxin analogues can differentially direct the coupling of the receptor to various signalling pathways involved in the production of cAMP. This could also explain the difference seen in the maximal responses produced by the relaxin analogues and HINSL3 at the LGR8 receptor. Relaxin signalling through LGR8 may involve coupling to a pathway that INSL3 fails to activate. Further experiments are needed to confirm these results and to

explore the mechanisms involved in relaxin and INSL3 signalling through the LGR7 and LGR8 receptors.

Research is showing that not only do relaxin and INSL3 interact differently at LGR7 and LGR8, but relaxin analogues also interact differently with the receptors. While H2RLX and PRLX showed strong affinity for both LGR7 and LGR8, RatRLX, and to a lesser degree RMRLX, displayed preferential interaction with LGR7 over LGR8. H3RLX has also been reported to interact with the LGR7 receptor while having no affinity for the LGR8 receptor (Sudo *et al.*, 2003). This study also showed that while extracellular loop 2 is important for H3RLX interaction with LGR7, H2RLX and PRLX show equal preference for the extracellular loops of LGR7 and LGR8, as interchanging the LGR7/LGR8 extracellular loops of the LGR7/8 chimera had no affect on either affinity or efficacy for the receptor. Examination of the importance of the extracellular loops to RatRLX may identify a common factor with H3RLX that dictates the selectivity for the LGR7 receptor.

The ability of the human relaxin analogues to interact differentially with receptors has been further highlighted with the identification of two new receptors that display high affinity for H3RLX. The orphan receptors GPCR135 (Liu *et al.*, 2003b) and GPCR142 (Liu *et al.*, 2003a) bind H3RLX but have no affinity for PRLX or other members of the human insulin-relaxin superfamily, including insulin, IGF I & II, INSL3, INSL4 & INSL6 peptides. The GPCR135 and GPCR142 receptors are Type I GPCR's with a short N terminal extracellular domain and couple primarily to the G_i subunit, completely different to the LGR7 and LGR8 Type III GPCR's with the large N terminal extracellular domain and primarily G_S coupling. The interaction of H1RLX and H2RLX at the two new receptors was not completely explored although preliminary data suggests that only H3RLX interacts with these receptors (Liu *et al.*, 2003b). An interesting detail to note was that while separated A and B chains of H2RLX fail to interact with the relaxin LGR7 receptor, the separated H3RLX B chain displays affinity for the GPCR135 and GPCR142 receptors, although at some 3 log units lower affinity than the full peptide.

It is difficult to predict the physiological consequences of relaxin interacting at the LGR7 and LGR8 receptors due to the uncertainty of the specific physiological actions of relaxin and INSL3 in the human. Although it has been established that relaxin maintains a role in reproduction that is likely to involve implantation (Chapter 7) and that INSL3 is critical for testicular development (Ferlin *et al.*, 2003; Roh *et al.*, 2003), the consequences of a cross-interaction between the relaxin peptides and the LGR8 receptor is still unknown. Human tissues reported to express LGR8 receptor mRNA transcripts include the brain, kidney and uterine tissues (Hsu *et al.*, 2002) while relaxin mRNA transcripts have been identified in human uterine tissues (Yki-Jarvinen *et al.*, 1983). Although there is little information concerning the expression of relaxin mRNA transcripts in human brain and kidney tissues, studies have shown relaxin mRNA expression in the rat brain and kidney tissues (Burazin *et al.*, 2002; Gunnersen, 1995). While the co-localised expression of relaxin and LGR8 mRNA does allow for cross-interaction, further investigation is needed however to establish that relaxin does interact with the LGR8 receptor *in vivo*, rather than just in the *in vitro* situation.

In conclusion, H2RLX and PRLX analogues could interact with high affinity and potency at both the LGR7 and LGR8 receptors. RMRLX and RatRLX analogues interacted selectively with the LGR7 receptor, while HINSL3 selectively interacted with the LGR8 receptor. The ability of the relaxin analogues to interact selectively with the two receptors could be further developed as a useful research tool to examine the individual physiological properties of the LGR7 and LGR8 receptors.

Chapter Seven

LGR7 expression in the human uterus

7.1 Introduction

Relaxin is primarily produced by the corpus luteum or placenta and affects connective tissue remodelling in the cervix and vagina in preparation for parturition (Sherwood, 1994). Studies in relaxin knockout mice have also demonstrated an essential role for the peptide in mammary gland development and nipple growth (Zhao *et al.*, 1999). In humans, however, the physiological role of H2RLX is associated more with degradation of foetal membrane collagen at term (Bogic *et al.*, 1997) rather than remodelling of the cervix. Moreover, labour is not prolonged in pregnant women who lack circulating relaxin following ovum donation (Emmi *et al.*, 1991)

In contrast to many other mammalian species, there is no evidence of a preterm surge in serum relaxin in pregnant women. Maximum concentrations of the hormone (0.8 ng/ml) are measured in the first trimester of pregnancy, after which there is a steady decline to term (Eddie *et al.*, 1986). Low plasma relaxin levels are also detected during the luteal phase of the menstrual cycle, with small increases 6-7 days after the LH surge (Stewart *et al.*, 1990). The source of H2RLX in normally cycling women is the corpus luteum (Goldsmith *et al.*, 1995; Ivell *et al.*, 1989; Khan-Dawood *et al.*, 1989), with substantial gene expression observed in the late luteal phase of the menstrual cycle. However, it has been recently shown that local synthesis of H2RLX occurs in human endometrial stromal and glandular epithelial cells taken from both proliferative and secretory phases of the menstrual cycle (Palejwala *et al.*, 2002). Immunoreactive relaxin has also been localized to decidualised stromal cells in the late secretory phase and in early pregnancy (Bryant-Greenwood *et al.*, 1993) implying that the endometrium provides an additional local source of RLX in women.

Several studies show that relaxin has a broad range of effects on the human endometrium and may mediate stromal cell differentiation and/or vascularization. Relaxin stimulates the production of a number of factors from endometrial cells *in vitro* including prolactin (Telgmann *et al.*, 1998), insulin-like growth factor binding protein-1 (IGFBP-1) (Bell *et al.*, 1991), glycodelin (Tseng *et al.*, 1999) and vascular

endothelial growth factor (VEGF) (Palejwala *et al.*, 2002; Unemori *et al.*, 1999). The effects of relaxin on VEGF secretion differs depending on the stage of the menstrual cycle, with a negative influence observed in the proliferative phase and a stimulatory effect in the secretory phase (Palejwala *et al.*, 2002). Of particular interest is the ability of relaxin to stimulate VEGF expression in the secretory phase of the cycle and the suggestion that the increased incidence of menometrorrhagia in relaxin-treated patients is due to increased VEGF production (Unemori *et al.*, 1999). Although relaxin clearly stimulates cAMP production in cultured human endometrial cells (Fei *et al.*, 1990), little is known about the expression of relaxin receptors and their intracellular signalling pathways. Using biotinylated PRLX, binding sites were demonstrated in both the endometrium and myometrium of women, with substantial binding observed in the luminal and glandular epithelium (Kohsaka *et al.*, 1998). Specific relaxin binding has also been shown in marmoset endometrial stromal cells, with weak signals observed in the epithelium (Einspanier *et al.*, 2001). Biotinylated relaxin-binding is highest in the endometrial stroma during the secretory phase of the cycle and in early pregnancy.

To date the relaxin LGR7 receptor gene transcript has been identified in the human uterus (Hsu *et al.*, 2000) but no assessment of gene expression or receptor localization has been conducted throughout the human menstrual cycle. The aim of this study was to examine and characterise the relaxin receptor, correlating expression with LGR7 gene expression in human uterine tissue throughout the menstrual cycle.

7.2 Methods

7.2.1 Human tissue collection and preparation

Full thickness endometrial tissue was collected from 27 hysterectomy operations performed on ovulating women suffering from menorrhagia (age range 34-46) as described in Section 2.3.2.1.

7.2.1.1 Preparation of slide mounted uterus sections

Tissue blocks containing the intact endometrial layer with several mm of myometrium attached were frozen in liquid nitrogen and stored at –80°C. Frozen blocks were oriented so that sections would be cut at right angles to the endometrial luminal surface, sectioned in a cryostat at -18°C and collected on precleaned microscope slides coated with poly-l-lysine. Following routine haematoxylin and eosin staining, tissue samples were sorted into 7 groups according to the phases of the menstrual cycle by an experienced histopathologist using established criteria for the normal menstrual cycle (Noyes *et al.*, 1950). The 7 phases of the menstrual cycle were early proliferative (EP), mid proliferative (MP), late proliferative (LP), early secretory (ES), mid secretory (MS), late secretory (LS) and menstrual (M). Frozen sections (10μm) were then used for quantitative autoradiography to determine expression levels and location of the relaxin receptor.

7.2.1.2 RNA extraction from human uterus tissue samples

RNA samples were prepared by Dr Gareth Weston (Monash Medical Centre, Vic, Aust). Total RNA was extracted from thin sections of the frozen specimens homogenized in TRIZOL reagent (Invitrogen, Mt. Waverley, Vic, Australia) according to the manufacturer's protocol. Purity of the RNA was enhanced by precipitating the aqueous phase of the TRIZOL preparation with an equal volume of 100% ethanol, followed by a second extraction through a QIAGEN RNeasy column (Qiagen, Hilden, Germany). After resuspension in RNase-free water, RNA was precipitated overnight with ethanol as previously described (Weston *et al.*, 2002).

7.2.2 Expression and localisation of the relaxin receptor using [^{33}P]-H2RLX

7.2.2.1 [^{33}P]-H2RLX (B33) preparation

H2RLX was labelled wi[illegible]^{33}P]-ATP using a procedure based on that of Osheroff *et al* (1990) as describe[illegible]ction 2.3.1.

7.2.2.2 Relaxin receptor expression throughout the phases of the menstrual cycle

Slide-mounted uterus sections were processed as described in Section 2.3.2.2. Briefly, slides were preincubated in 100µL of binding buffer (25mM Hepes and 300mM KCl, pH 7.2) containing phenylmethylsulphonylflouride (PMSF, 1µM) for 30min in a humidity chamber (25°C). The binding buffer was removed and slides were incubated for 90mins with 100µL of binding buffer with BSA (1mg/mL), containing 100 µL [^{33}P]-H2RLX. Slides for determining non-specific binding were incubated with H2RLX (1µM). Labelled sections were washed, briefly rinsed in distilled water, dried and apposed to a phosphorimager plates for 3 days with [γ-^{33}P]-ATP reference standards (Section 2.3.2.4). Plates were scanned with PhosphorImager SI™ (Molecular Dynamics, U.S.A.) and images were analysed with ImageQuaNT™ (Version 4.1; Molecular Dynamics, U.S.A.). Receptor expression images were expressed as arbitrary phosphorimager units that were converted into dpm/mm^2 using the [γ-^{33}P]-ATP reference standards. Uterus block numbers were then organised according to menstrual phase and data grouped using PRISM (GraphPad Inc., San Diego, U.S.A.).

7.2.2.3 Localisation of the relaxin receptor using high resolution autoradiography

Slide-mounted uterus sections were processed as described in Section 7.2.2.2. Slides were stored overnight in a vacuum-sealed container with silica gel before

commencing the emulsion protocol (Section 2.3.2.5). LM-1 hypercoat emulsion (Amersham Pharmacia Biotech, U.S.A.) was incubated at 42°C (under darkroom conditions) for 1 hour and then transferred to a dipping chamber. Each slide was dipped into the emulsion twice, drained and allowed to dry in a light-proof box overnight. Slides were then transferred into a slide box, wrapped in foil to prevent exposure to light and stored at 4°C for 3 weeks. Prior to development, slides were equilibrated at room temperature for 1 hour. Emulsion was developed in Kodak D-19 developer (5mins), Kodak stop solution (1min), Kodak fix solution (10mins) and gently rinsed in running water (15mins). Slides were air-dried and examined under a dark-field microscope. For histological analysis, slides were stained with Gill's haematoxylin (distilled water 730mL, ethylene glycol 250mL, haematoxylin 2g, sodium iodate 17.6g and glacial acetic acid 20mL) and Eosin (1% solution).

7.2.3 Characterisation of [^{33}P]-H2RLX binding in the proliferative and secretory phases.

Slide-mounted uterus sections from the proliferative and secretory phase were processed as described in Section 2.3.2.2. Briefly, slides were preincubated in 100µL of binding buffer containing PMSF (1µM) for 30min in a humidity chamber (25°C). The binding buffer was removed and slides were incubated for 90mins with 100µL of binding buffer with BSA (1mg/mL), containing 50-1500pM [^{33}P]-H2RLX for saturation binding or 100pM [^{33}P]-H2RLX in the absence or presence of increasing concentrations of unlabelled H2RLX or PRLX (10nM to 10µM) for competition binding. All non-specific binding was defined with H2RLX (1µM). Labelled sections were processed and analysed as described in Section 7.2.2.2. Saturation binding images were expressed as arbitrary phosphorimager units that were converted into dpm/mm^2 using [γ-^{33}P]-ATP reference standards (Section 2.3.2.4), while competition binding was expressed as a percentage of total specific binding.

7.2.4 Analysis of relaxin, LGR7 and related gene expression

RT-PCR experiments were performed and analysed by Dr Chrishan Samuel and Ms Chongxin Zhao (Howard Florey Institute of Experimental Physiology and Medicine, Vic, Aust). RLX (H1 & H2), LGR7 and $VEGF_{165}$ gene expression were first assessed by RT-PCR. Analysis of H3RLX was not conducted in this study because there was no known human tissue that expressed H3RLX and hence no positive control cDNA. Total RNA (1μg) was used for synthesis of first strand cDNA in a 20μl reverse transcription reaction using a RT kit (Promega Corporation, Annandale, NSW, Australia) and random hexamer primers. Between 1-2μg of this cDNA template was used in touch-down PCR reactions (50μl volume) with 0.2U Taq DNA polymerase (Promega) and 100ng/μl of human gene-specific oligonucleotide primers (Geneworks Pty Ltd, Adelaide, Australia). Human LGR7 primers were designed from the human genome sequence (NCBI, Accession No. AF190500), whereas all other relaxin oligonucleotide primers were obtained from previously published sequences; H1RLX (Hudson *et al.*, 1983), H2RLX (Hudson *et al.*, 1984) and human $VEGF_{165}$ (Houck *et al.*, 1991). The relaxin and LGR7 oligonucleotide primers used were designed to span intron-exon junctions to distinguish between PCR products derived from mRNA and those derived from contaminating DNA, while the $VEGF_{165}$ primers were kindly provided by Dr Elaine Unemori (Connetics Corporation, Palo Alto, CA, USA). All PCR reactions were carried out in a Perkin Elmer Gene Amplifier with an initial denaturation step at 94^0C (3 minutes) and annealing temperatures as shown in Table 7.1. GAPDH was used in separate PCR reactions to control for quality and equivalent loading of the cDNA. Aliquots of the PCR products were electrophoresed on 2-4% (w/v) agarose gels stained with ethidium bromide and photographed.

Real-time PCR experiments were conducted and analysed by Dr Laura Parry and Ms Helen Gehring (University of Melbourne, Vic, Aust). Real-time PCR was used to quantify H2RLX, LGR7, $VEGF_{165}$ and $VEGF_{121}$ gene expression using human gene-specific primers and FAM-labelled probes (Keystone Division, Biosource International, Foster City, CA) designed with the Primer Express™ computer

software (Applied Biosystems, Foster City, CA). The human VEGF primers and probes were kindly provided by Dr Elaine Unemori (Connetics Corporation). All primers were designed to span introns to distinguish between PCR products derived from mRNA and those derived from contaminating DNA. For each sample, 1μg total RNA was reverse transcribed in a 30μL reaction containing 1xTaqMan® buffer (5.5mM $MgCl_2$, 500μM dNTPs, 2.5μM oligo d(T), 0.4μL RNase inhibitor and 1.25U/μL MultiScribe™ reverse transcriptase; Applied Biosystems). For the LGR7 cDNA, 2.5μM random hexamers replaced the oligo d(T). A second reaction mix using 30ng total RNA from each sample and a series of endometrium RNA dilutions (100-0.001ng) was prepared for the endogenous reference 18S ribosomal RNA (18S) PCR reactions and to generate the 18S standard curves respectively. First strand cDNA synthesis for all samples was carried out simultaneously at 25°C for 10min, 42°C for 45min and 95°C for 10min with a final cooling temperature a 4°C before storage at -20°C. The PCR reactions were conducted in triplicate using 96-well optical reaction plates (Applied Biosystems), in 25 μl volumes consisting of 1xTaqMan® Universal PCR Master Mix, 0.8 μM forward and reverse primers, 0.4 μM probe and 2.5 μl cDNA template. An ABI PRISM 7700 Sequence Detector (Applied Biosystems) was used with the following conditions: 50°C for 10 min, 95 °C for 10 min followed by 40 cycles of 95°C for 15 sec and 58°C for 1 min. Negative template samples were included in each plate as controls. The relative C_T standard curve method was used in this study, where C_T is the cycle number at which DNA amplification is first detected. In the relative standard curve method, specific gene and 18S (endogenous reference) expression are assessed in separate PCR reactions and the C_T values for both genes are related to those of the endogenous reference standard curve. The amount of specific gene expressed in each sample is calculated by dividing the log (gene of interest concentration) by the log (18S concentration) using the following formula: log (gene concentration) = $(C_T - b/a)$ where b = y-intercept of the standard curve and a = slope of the standard curve. In this experiment the regression line for the 18S standard curve was $y = -3.398x + 14.235$, $R^2=0.998$. The same 18S standards were used on every plate, with inter-plate variation 2.2 % (1ng standard, n=4 plates).

Table 7.1

Primers, probes and conditions used for RT/PCR

Primer Sequences:	Annealing temperature(s) used:	Product size (bp)
H1RLX forward primer: 5'GTCGCGGCCAAATGGAAGGA3' H1RLX reverse primer: 5'TTGCCTCTCAGATAGGGCTGC3'	58^0C(3 cycles), 57^0C(3), 56^0C(3), 55^0C(31)	250
H2RLX forward primer: 5'GAACCAATTTTCCAGAGCAGTCG3' H2RLX reverse primer: 5'TGTAGAGTTGTCTCTTTTTTCGAG3'	55^0C(2), 54^0C(2), 53^0C(2), 52^0C(2), 51^0C(2), 50^0C(30)	445
Human LGR7 forward primer: 5'GCGCAGCTGTGGATGGAGAG3' Human LGR7 reverse primer: 5'GGTCTTGTGGTCAGAGTATAG3'	58^0C(2), 57^0C(2), 56^0C(2), 55^0C(2), 54^0C(2), 53^0C(2), 52^0C(2), 51^0C(2), 54^0C(25)	634
Human $VEGF_{165}$ forward primer: 5'CCAGCACATAGGAGAGATG3' Human $VEGF_{165}$ reverse primer: 5'GGAACATTTACACGTCTGC3'	59^0C(5), 58^0C(5), 57^0C(30)	140

7.2.5 Statistical Analysis

Data was analysed as described in Section 2.8. Quantitative autoradiography results were analysed using one-way ANOVA with a Newman-Keuls post-test. The pK_D, B_{max} and pK_i data was analysed using a Student's t- Test. Data for mRNA concentrations did not show homogeneity of variance and were log transformed before analysis by one-way ANOVA (SPSS 10.0). The Least Squares Difference method tested for significant differences at the 95% confidence level between stages of the menstrual cycle.

7.3 Results

7.3.1 Changes in binding of [^{33}P]-H2RLX with the phases of the menstrual cycle.

Slide mounted sections of uterus from the seven phases of the menstrual cycle were incubated with [^{33}P]-H2RLX to determine receptor binding levels and to determine any changes that may occur during the menstrual cycle. Initial studies were conducted blind with a variety of sections from different phases of the cycle and showed strong binding in some sections but not others. Sections with high levels of binding were subsequently shown to be from the secretory phase of the cycle and those with little binding from the proliferative phase. Systematic studies of uterine sections from all proliferative phases showed very little [^{33}P]-H2RLX binding (EP 1.3 ± 0.51, n=4; MP 2.1 ± 0.39, n=5; LP 2.6 ± 1.12 dpm/mm^2, n=3; Figure 7.1), whereas those from the secretory phase showed a large amount of binding mainly confined to the endometrium (ES 11.6 ± 2.33, n=5, $P < 0.05$ cf all proliferative phases; MS 10.0, n=2; LS 6.5 ± 0.73, n=6; and M 5.6 dpm/mm^2, n=2; Figure 7.2). Binding levels were markedly increased in the early secretory phase and then progressively declined towards the menstrual phase although these levels were still higher than in the early proliferative stage.

7.3.2 Localisation of the relaxin receptor in uterine sections using emulsion autoradiography

To identify the cells that express the relaxin receptor, [^{33}P]-H2RLX labelled uterine sections were coated with silver grain hypercoat emulsion and exposed for 3 weeks. Low levels of silver grains appeared over the endometrium of sections from the E-MP phase with no significant silver grains overlying the myometrium (Figure 7.3a). In contrast, in sections from E-MS, an abundance of silver grains was highly localised to sites in the endometrium, with non-specific levels of silver grains associated with the myometrium (Figure 7.3b). Comparison of corresponding dark

field and light field images showed that the silver grains in the endometrium were localised to the glandular epithelial cells and the luminal epithelium.

7.3.3 Characterisation of [^{33}P]-H2RLX binding in the proliferative and secretory phases.

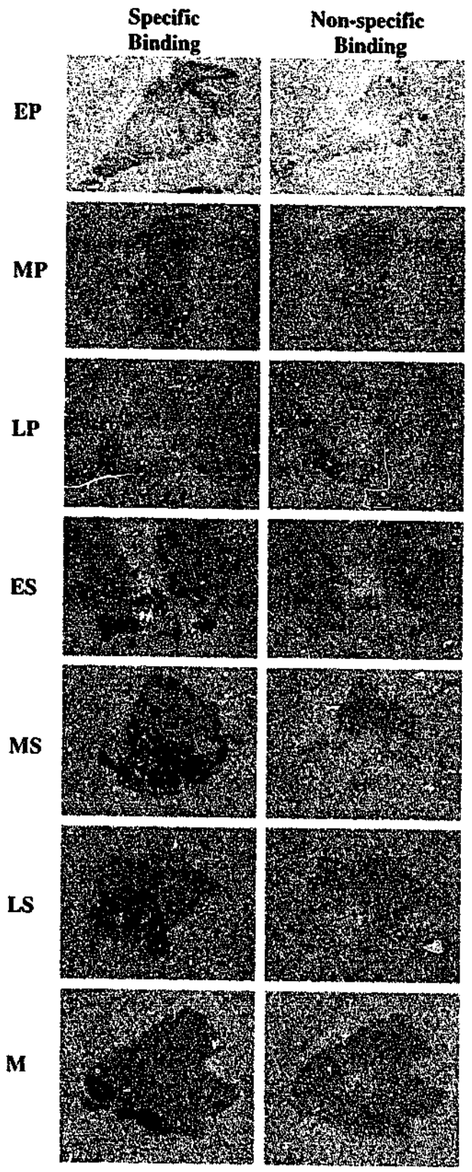
The saturation and competition binding properties of [^{33}P]-H2RLX to sections from the early-mid proliferative (E-MP) and early-mid secretory (E-MS) were studied to determine if the changes in intensity were related to an alteration in receptor number or affinity. Binding to sections from both the E-MP and E-MS phases was saturable to a single population of binding sites (Figure 7.4) which displayed no significant difference in the binding affinity (E-MP pK_D 9.16 ± 0.17, n=4; E-MS pK_D 9.16 ± 0.06, n=4). However, the number of binding sites in E-MS was significantly ($P<0.05$) higher (B_{max} 160 ± 32; n=4) than in the E-MP sections (B_{max} 15 ± 4; n=4).

Competition studies were conducted in uterine sections to examine the ability of H2RLX and PRLX to compete for [^{33}P]-H2RLX binding. pEC_{50} values were calculated from competition curves fitted using Prism and pK_i values calculated using the Cheng & Prusoff (1974) equation. Sections from the E-MP showed that H2RLX (pK_i 8.0 ± 0.06, n=4) had a significantly higher affinity for the relaxin receptor in the endometrium than PRLX (pK_i 7.4 ± 0.21, n=4, $P < 0.05$). The same pattern was observed in the E-MS with H2RLX (pK_i 8.3 ± 0.11, n=4) having a significantly higher affinity for the receptor than PRLX (pK_i 7.3 ± 0.08, n=4, $P < 0.05$; Figure 7.5). There was no significant difference however in the affinity of either H2RLX or PRLX determined in the E-MP or E-MS phase.

Figure 7.1

Autoradiographic localisation of [^{33}P]-H2RLX binding to slide-mounted sections of human uterus at 7 different phases of the menstrual cycle.

The left panels are representative slides of the total binding and right panels of non-specific [^{33}P]-H2RLX binding from each of the phases of the menstrual cycle.



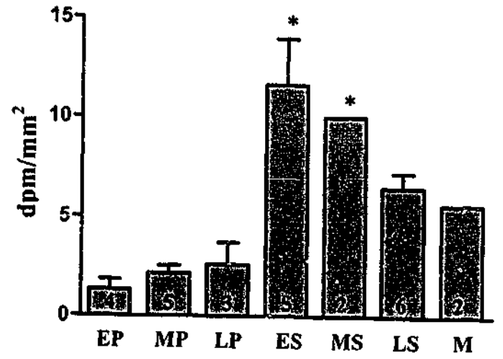


Figure 7.2

Quantitation of the autoradiographic localisation of [^{33}P]-H2RLX binding to slide-mounted sections of human uterus at 7 different phases of the menstrual cycle.

The levels of specific binding to the endometrial layer as determined by quantitative autoradiography from each of the phases in the menstrual cycle (mean ± SEM). The 7 phases of the menstrual cycle include: early proliferative (EP), mid proliferative (MP), late proliferative (LP), early secretory (ES), mid secretory (MS), late secretory (LS) and menstrual (M). Note the relatively low levels of binding in the proliferative stages, the significant increase that occurs at the start of the secretory phase (* $P<0.05$ cf all proliferative stages) and the gradual reduction towards the menstrual phase.

Figure 7.3

Qualitative autoradiographic localisation of [^{33}P]-H2RLX binding to slide mounted sections of human uterus using photographic emulsion.

Light field (LF) and dark field (DF) images of A) myometrium (M) and endometrium (E) showing silver grains abundant in the glandular epithelial cells of the endometrium (x2 magnification), and B) the endometrial luminal epithelium (x10 magnification).

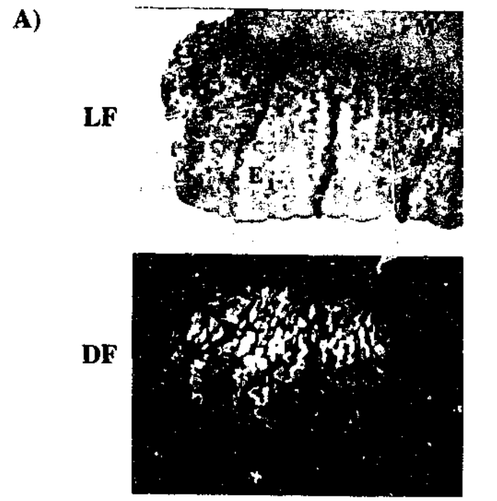


Figure 7.4

Relaxin receptor characterisation determined using quantitative autoradiography saturation binding of [^{33}P]-H2RLX to slide-mounted sections of human uterus.

Sections of uterus tissue were obtained from the A) proliferative (E-MP) and B) secretory stages (E-MS) of the cycle. Saturation binding isotherms show that the binding of [^{33}P]-H2RLX is saturable to a single population of sites

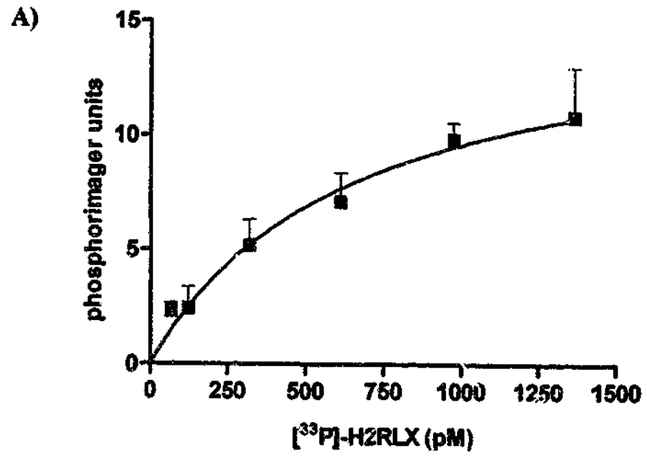
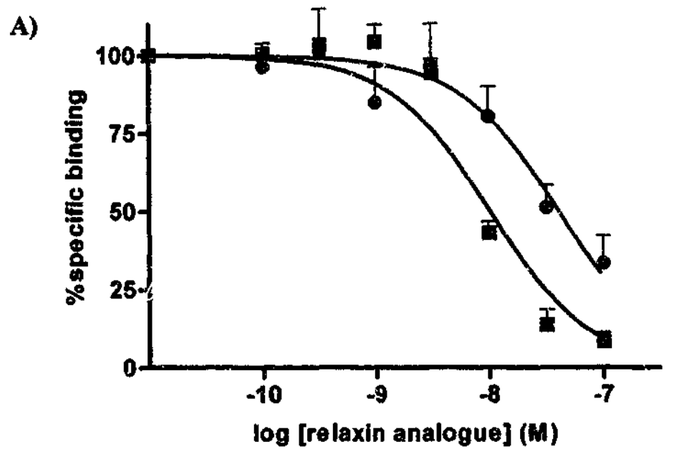


Figure 7.5

Relaxin receptor characterisation determined using quantitative autoradiography competition binding of H2RLX and PRLX to slide-mounted sections of human uterus.

Sections of uterus tissue were obtained from the A) proliferative (E-MP) and B) secretory stages (E-MS) of the cycle. Competition binding isotherms show competition for [^{33}P]-H2RLX binding by H2RLX ■ and PRLX ●.



7.3.4 Analysis of relaxin, LGR7 and related gene expression

H1RLX gene transcripts were only weakly expressed in two samples of the proliferative phase of the cycle (Figure 7.6). Gene transcripts of H2RLX were detected at relatively weak but consistent levels in both the proliferative and secretory stages of the cycle and appear to be the predominant form of relaxin in the human endometrium. Of the two bands observed, the 445bp band corresponds to the expected product for H2RLX while the 330bp band represents an unrelated fragment of DNA. H2RLX was highly expressed in the corpus luteum as expected (Figure 7.6), however no direct comparison can be made between the endometrium and the ovarian samples as different amounts of RNA were used in the first strand cDNA synthesis.

An LGR7 gene transcript (634bp) was detected in the human endometrium, with stronger expression observed in those samples obtained from the secretory phase of the cycle (Figure 7.6). $VEGF_{165}$ gene expression was also higher in the secretory phase of the cycle, with seemingly stronger expression observed in the late secretory endometrium (Figure 7.6). An additional positive control was not included as the endometrium is generally considered to be a major source of VEGF.

Quantitative analysis by real-time PCR confirmed that there was a significant ($P<0.01$ ANOVA) increase in the endometrial LGR7 mRNA concentrations in the early secretory phase compared with all other stages of the cycle (Figure 7.7a). Although LGR7 gene expression decreased in the mid-secretory endometrium, mRNA concentrations remained significantly ($P<0.05$) higher in the mid secretory, late secretory and menstrual phases compared with the proliferative phase of the cycle. This pattern of LGR7 gene expression paralleled that of RLX binding in the endometrium assessed by quantitative autoradiography (Figure 7.2). The real-time PCR for H2RLX demonstrated a low level of gene expression in the endometrium with C_T values of between 38-40 (data not shown). Thus, it was not possible to show differences for H2RLX between the phases of the cycle.

A comparison of $VEGF_{165}$ and $VEGF_{121}$ mRNA concentrations in the endometrium throughout the cycle showed an identical pattern of gene expression for both isoforms (Figure 7.7b & c). Concentrations were low in the proliferative phase of the cycle and did not differ significantly ($P<0.05$) compared with early and mid secretory endometrium. Expression of both VEGF isoforms increased significantly ($P<0.05$) in the late secretory phase and remained high in the menstrual phase.

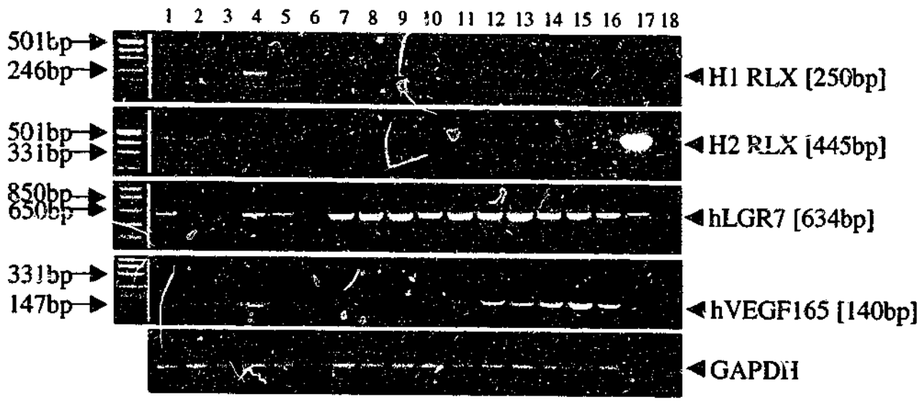


Figure 7.6

Expression of RLX (H1 & H2), LGR7 and $VEGF_{165}$ mRNA using RT-PCR on RNA extracted from the human endometrium at various phases of the menstrual cycle.

The first lane is the molecular weight marker, followed by samples from separate patients in the proliferative phase (lanes 1-5), early secretory phase (lanes 6-8), mid secretory phase (lanes 9-11), late secretory phase (12-14) and menstrual phase (lanes 15-16) of the cycle. RNA from human prostate was used as a positive control for H1RLX PCR, human corpus luteum for H2RLX PCR and THP-1 cells for the LGR7 PCR (all lane 17). Water replaced the cDNA in negative control reactions for each PCR (lane 18).

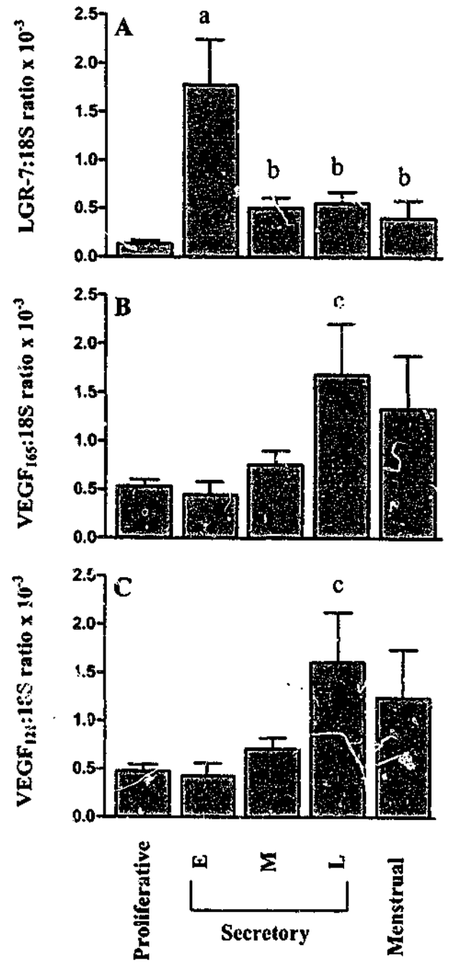
Data obtained from Dr C Samuel, Howard Florey Institute of Experimental Physiology and Medicine, Vic, Aust.

Figure 7.7

A) LGR7, B) $VEGF_{165}$ and C) $VEGF_{121}$ mRNA concentrations in the endometrium at five phases of the menstrual cycle.

Data are the mean ± SEM gene/18S ratio x 10^3. (a) significantly ($P<0.05$) higher compared with all other phases; (b) significantly ($P<0.05$) higher compared with the proliferative phase; (c) significantly ($P<0.05$) higher compared with the proliferative phase and early secretory phase.

Data obtained from Dr L Parry, Dept of Zoology, University of Melbourne, Vic, Aust.



7.4 Discussion

The aim of the present study was to investigate the sites of expression and the characteristics of the relaxin receptor in the human uterus at different phases throughout the menstrual cycle. While binding sites for biotinylated PRLX have previously been identified in the human uterus (Kohsaka *et al.*, 1998), until recently no studies had been carried out to identify changes in relaxin receptor expression during the menstrual cycle. The relaxin peptide has previously been identified in human endometrial tissue in the secretory phase however no relaxin peptide was detectable in the proliferative phase (Yki-Jarvinen *et al.*, 1983). Relaxin has been shown to differentially influence VEGF secretion in isolated uterine cells taken both from the proliferative and secretory phases (Palejwala *et al.*, 2002), although it was unclear whether the differential regulation occurs at the level of the receptor or the signalling cascade.

The results reported in this study indicate that expression of the relaxin receptor varies in a cyclical manner through the menstrual cycle. A low level of receptor binding was demonstrated during the proliferative stages with a dramatic increase in binding at the time of ovulation. The high levels of binding were maintained during the early secretory (days 15-18 of a standardised 28 day cycle) and mid secretory phase (days 19-24) and then decreased through the late secretory and menstrual phases (days 25-28). Results from the gene expression studies supported the autoradiography and showed a 13-fold increase in LGR7 mRNA following ovulation, which subsequently decreased in the mid-late secretory and menstrual stages of the cycle. The more rapid decline in the LGR7 mRNA compared to the binding probably reflects a more rapid turnover of RNA than receptor protein. This is the first study to identify a cyclic expression of the relaxin receptor in the human uterus. Two studies have appeared while our work was under review. Immunohistochemical staining of endometrial sections from 3 patients showed little if any staining in sections from patients in the proliferative or early secretory phase but intense staining in one patient in the mid-secretory phase (Ivell *et al.*, 2003). Although the other study reported no consistent regulation of LGR7 mRNA during the phases of the menstrual cycle (Luna *et al.*, 2004) the expression levels are

expressed as a ratio with GAPDH which is known to be sex-steroid dependent (Wu *et al.*, 2003). Little detail is provided about the patients recruited for these studies, however all patients were normally ovulating women not suffering from endocrine complaints and tissue samples collected were screened by histological examination, similar to the criteria followed in our study, therefore it is unlikely that the conflicting results are due to patient selection. In the present study the quantitative PCR was carried out using 18S RNA as the standard. Given our finding that relaxin receptor is elevated in the secretory phase, and the observation that plasma relaxin levels increase from day 5 to peak at day 10 post ovulation (Stewart *et al.*, 1990), it is possible that a role exists for relaxin in the process of embryo implantation. Analysis of gene expression of the relaxin peptides indicated that the endometrium is not a major source of circulating relaxin, with low to undetectable levels of relaxin mRNA present throughout all stages of the menstrual cycle. Our data are in agreement with recent work (Palejwala *et al.*, 2002) that demonstrated expression of relaxin in the human endometrium using a nested PCR method. The major source of circulating relaxin is the corpus luteum, since it is highly expressed in this tissue (Ivell *et al.*, 1989), high levels of the hormone are found in the ovarian vein (Weiss *et al.*, 1976) and women without ovaries who become pregnant by oocyte donation have no circulating relaxin (Emmi *et al.*, 1991; Johnson *et al.*, 1991). Local production of relaxin from the endometrium may therefore play a significant role in the establishment or maintenance of early pregnancy.

The binding characteristics of the relaxin receptor remained unaltered between the proliferative and the secretory phases, with the pK_D values for the [^{33}P]-H2RLX ligand and the pK_i values for H2RLX and PRLX being not significantly different with the changing stages of the cycle. The values determined were comparable with those obtained with previous binding studies using THP-1 cells (Tan *et al.*, 2001a), that endogenously express the human relaxin receptor (Parsell *et al.*, 1996), or human uterine cells (Osheroff *et al.*, 1995; Palejwala *et al.*, 1998) indicating that THP-1 cells and the uterus express the same receptor. The consistency in the binding characteristics between the different menstrual cycle phases indicates that the variations in relaxin function during the menstrual cycle, such as its potential influence on VEGF secretion (Palejwala *et al.*, 2002), are not due to changes in receptor conformation or affinity of the relaxin receptors expressed. Relaxin

receptor expression in the endometrium during the secretory phase was confined specifically to glandular and luminal epithelial cells. The binding of [^{33}P]-H2RLX was appropriate for binding to the relaxin receptor since it was competitively inhibited by both H2RLX and PRLX with high affinity. In contrast to previous studies that showed binding of biotinylated PRLX to blood vessels and myometrium in addition to glandular and luminal epithelium (Kohsaka *et al.*, 1998), the present studies provided no evidence for binding of [^{33}P]-H2RLX to these tissues. The differing results may relate to the concentrations of labelled relaxin used in the two studies. In the present study we used [^{33}P]-H2RLX at a concentration of 100pM, similar to the known pK_D value for relaxin at the receptor (Tan *et al.*, 1999) whereas the study using biotinylated PRLX used a concentration of approx 0.7μM, several orders of magnitude higher than the pK_D. It is possible that the use of high labelling concentrations could result in binding to low affinity sites such as the related LGR8 receptor which is known to bind relaxin (Hsu *et al.*, 2002). However the lack of binding to stromal cells was surprising given the demonstration of functional responses in these cells in culture (Bell *et al.*, 1991; Einspanier *et al.*, 2001; Palejwala *et al.*, 2002). The reason for the difference may relate to the different conditions experienced by cells *in situ* and those in culture since human endometrial stromal cells fail to produce a cAMP response to relaxin when first isolated but the response appears gradually over the first 11 days of culture and then increases dramatically from 11-30 days (Tseng *et al.*, 1995). As with stromal cells, studies with human myometrial cell lines (PHM1-41) isolated from term pregnant myometrium show clear evidence of relaxin stimulated cAMP accumulation indicating the presence of relaxin receptors (Dodge *et al.*, 1998). This may also relate to the expression of relaxin receptors in an immortalised cell line under cell culture conditions. In contrast, functional studies of the effects of relaxin in the human uterus show that human myometrium is much less able to respond *in vitro* to relaxins than either pig or rat myometrium. Porcine relaxin had little relaxant effect on myometrium from pregnant or non-pregnant women (MacLennan *et al.*, 1986a). Later studies with recombinant H2RLX showed little or no relaxant effect on human myometrium excised at hysterectomy or at caesarian section (MacLennan *et al.*, 1991) or on uterine fundus or isthmus at term (Petersen *et al.*, 1991). Studies of spontaneous contractions of human myometrium demonstrated small reductions in

amplitude to relaxin-rich extracts of corpus luteum (Szlachter *et al.*, 1980) or of amplitude and frequency to H2RLX in some oestrogen-primed tissues (MacLennan *et al.*, 1991). This is in direct contrast to studies in pig myometrium or rat uterus where complete inhibition of contraction was observed with H2RLX, PRLX and H1RLX (MacLennan *et al.*, 1991; MacLennan *et al.*, 1995; MacLennan *et al.*, 1986b; Petersen *et al.*, 1991). The lack of significant [^{33}P]-H2RLX binding to relaxin receptors in the human myometrium reported here is in accord with the poor relaxant responses to relaxin observed in this tissue.

Immunohistochemical studies demonstrated relaxin in endometrium in the secretory, but not in the proliferative phase nor in myometrium from either phase (Yki-Jarvinen *et al.*, 1983). The hormone has also been localised to decidualised stromal cells in the late secretory phase and in early pregnancy (Palejwala *et al.*, 2002). Recent studies have established that relaxin is synthesised in the endometrium since relaxin mRNA was present in cultured endometrial stromal and glandular epithelial cells with stronger bands seen in cells from the secretory phase and immunoreactive relaxin was detected in culture medium bathing the cells (Palejwala *et al.*, 2002). As there was a lack of evidence for relaxin receptors associated with the endometrial vasculature, our study does not support a direct role for relaxin in regulating endometrial vascular function. However, relaxin does increase VEGF expression in glandular epithelial and stromal cells from the secretory phase (Palejwala *et al.*, 2002) suggesting that it may have a role in endometrial vascularisation. The significant increase in relaxin binding in the early secretory phase, and the retention of the elevated binding throughout the secretory phase, suggests a role for relaxin in implantation. Support for this hypothesis comes from a study showing that high levels of relaxin produced in granulosa lutein cell cultures are associated with 100% implantation success rates with IVF, while low levels resulted in an implantation rate of 13% (Stewart *et al.*, 1999). Although this would indicate that the production of relaxin is linked to successful implantation, circulating relaxin is clearly not obligatory for this process since implantation can occur in women without ovaries (Emmi *et al.*, 1991; Johnson *et al.*, 1991). This may indicate that local relaxin may be important, that it is a marker for other factors important in implantation or merely that it is a marker of oocyte quality. VEGF is known to have an important role in the proliferation and vascularization of the uterus lining and while relaxin has been

found to stimulate VEGF secretion in glandular epithelial and stromal cells (Palejwala *et al.*, 2002), the VEGF responsible for uterus growth has been identified in intravascular neutrophils rather than from the glandular epithelial cells (Gargett *et al.*, 2001). The increase in $VEGF_{165}$ and $VEGF_{121}$ gene expression during the mid-late secretory phases supports previous reports showing an increase in VEGF mRNA during this stage of the cycle (Sugino *et al.*, 2002).

In conclusion, we have localised binding sites for relaxin in the human uterus and have demonstrated that they are primarily confined to the glandular and luminal epithelium in the secretory stage of the cycle. Furthermore, the levels of relaxin receptor expression change dramatically during the different phases of the menstrual cycle. The binding characteristics of the receptor were appropriate for binding to the relaxin receptor (LGR7) and were unchanged with differing expression levels. Thus any difference in relaxin function between the phases is a reflection of changes in the intracellular properties of the receptor and not due to changes in receptor characteristics. The location and regulation of relaxin receptor expression that occur during the menstrual cycle suggest a role in the implantation process, although further studies are needed to confirm this hypothesis.

7.5 Acknowledgements

I would like to acknowledge the contribution of; Assoc Prof Peter Rogers and Ms Fiona Ledermann (Centre for Women's Health Research, Monash University, Vic, Aust) for the provision of human uterus samples used in the study, Drs Caroline Gargett and Gareth Weston (Centre for Women's Health Research, Monash University, Vic, Aust) for the provision of the human endometrial mRNA samples, Dr Chrishan Samuel and Ms Chongxin Zhao (Howard Florey Institute of Experimental Physiology and Medicine, Vic, Aust) for conducting and analysing the RT-PCR experiments, Dr Laura Parry and Ms Helen Gehring (University of Melbourne, Vic, Aust) for conducting and analysing the real-time PCR experiments.

This study is currently under review for publication.

Chapter Eight

General Discussion

8.1 General Discussion

The interaction of relaxin with its receptor has been, and continues to be, extensively studied in experimental animals such as mice and rats. While such investigations are vital for obtaining information regarding the physiological and pharmacological properties of relaxin, the investigation of these properties at the human relaxin receptor is also important. The work presented in this thesis examines the pharmacological properties of the human relaxin receptor by investigating the interaction with various relaxin analogues in binding and cAMP assays using cell lines endogenously and recombinantly expressing the human relaxin receptor. Investigation into the properties of the physiologically expressed relaxin receptor were also explored in human uterine tissue, with the aim of gaining further insight into the role of relaxin in human reproduction.

The lack of available human tissue for research no longer restricted investigations at the human relaxin receptor as the characterisation of the human monocyte cell line, THP-1, showed endogenous expression of the relaxin receptor (Parsell *et al.*, 1996). The successful development of a high through-put binding assay (Chapter 3) allowed examination of the interaction between various relaxin analogues and the human relaxin receptor. The greatest advantage of using this binding assay over other assays was that very little peptide was needed to complete a binding study, an important factor when only limited amounts of the synthetic relaxin analogues were available.

Work presented in this thesis has shown several factors important in determining relaxin-like affinity for the human relaxin receptor (Chapter 3). The extended length of the relaxin B chain does not influence affinity, however shortening of the relaxin B chain reduces affinity for the relaxin receptor, similar to the reduced efficacy displayed at the rat relaxin receptor (Tan *et al.*, 1998). The reduction in affinity is likely due to instability of the structural integrity of the peptide rather than the loss of particular amino acids. Shortening the B chain to within one amino acid of the cysteine important in the formation of a linking disulphide bond may cause peptide

instability, similar to that previously shown with the substitution of amino acids adjacent to a disulphide bond (Büllesbach *et al.*, 1995). Another factor important in determining relaxin-like affinity is the relaxin B chain binding motif (Büllesbach *et al.*, 2000), however this does not solely determine affinity as shown by the INSL3 analogues synthesised with the relaxin binding motif. Affinity for the relaxin receptor was increased by the insertion of the binding motif into the INSL3 peptide, however this interaction was still very weak, indicating either the absence of critical factors yet to be determined or the presence of unknown factors in the INSL3 peptide that hinder interaction with the relaxin receptor. Unfortunately due to the limited amounts of the peptides synthesised, examination of the relaxin and INSL3 analogues in a functional study could not be completed at this time.

Further analysis of the structural-activity properties of relaxin was also hindered by the inability to synthesise novel relaxin analogues, modified to explore new areas. In an attempt to overcome this obstacle, a mammalian recombinant system was developed with the aim of producing various relaxin analogues modified by molecular cloning techniques (Chapter 4). While the recombinant system appeared to synthesis the pro-HRLX peptide, it was not successfully isolated from the cell debris following lysis of the expressing cells. Due to my lack of experience as a peptide chemist, there was a possibility that further attempts to successfully isolate a bioactive recombinant relaxin protein would be very time consuming and little research would be accomplished by the completion of the thesis candidature. Therefore, further development of a recombinant system was temporarily cancelled until completion of this thesis.

The identification of the relaxin receptor as the orphan LGR7 receptor (Hsu *et al.*, 2002) midway through the candidature of this thesis allowed the specific interaction between relaxin and the human relaxin receptor to be investigated in a recombinant system. An important advantage of investigating receptor pharmacology in a cell line recombinantly expressing the relaxin receptor, rather than a cell line that naturally expresses the receptor, is the ability to alter to the expression level of the LGR7 receptor. Increasing the expression of LGR7 above the naturally expressed receptors in the cell line allows a specific examination of the interaction between

relaxin and LGR7 to be completed with minimal interference by other receptors. This was particularly noticeable when comparing the ability of H3RLX to displace [^{33}P]-H2RLX binding to the relaxin receptor expressed in THP-1 cells, to the LGR7 receptor transiently expressed in CHO K1 cells. While appearing to displace all radioligand binding in the CHO-LGR7 system (Chapter 5), H3RLX could only displace approximately 50% of binding to THP-1 cells (Chapter 3), possibly indicating that H3RLX could not compete for [^{33}P]-H2RLX binding to another receptor expressed in THP-1 cells. THP-1 cells have been shown to express both LGR7 and LGR8 receptor mRNA transcripts (Nguyen *et al.*, 2003) and while our radioligand displays high affinity for both receptors (Chapter 6), H3RLX displays no affinity for LGR8 (Sudo *et al.*, 2003). Although RatRLX also displayed no affinity for LGR8 (Chapter 6), it appeared to be able to displace all radioligand binding in THP-1 cells (Chapter 3), however experiments using higher concentrations of RatRLX are needed to investigate the maximal displacement of [^{33}P]-H2RLX binding in THP-1 cells.

Even though INSL3 has the highest homology of the insulin-like peptides to relaxin, studies have consistently reported that INSL3 displays no significant affinity for the relaxin receptor (Claasz *et al.*, 2002; Smith *et al.*, 2001; Tan *et al.*, 2002) (Chapter 3, 5 & 6). In light of these results, the report that relaxin displayed high affinity for the INSL3 receptor was surprising (Bogatcheva *et al.*, 2003; Hsu *et al.*, 2002). Closer analysis of relaxin analogue interaction at LGR7 and LGR8 (Chapter 6) revealed the ability to interact at LGR8 is not universally shared by all relaxin analogues. H2RLX and PRLX displayed high affinity for both receptors (Hsu *et al.*, 2002; Sudo *et al.*, 2003), while RatRLX and H3RLX (Sudo *et al.*, 2003) failed to display affinity for the LGR8 receptor. RMRLX was able to bind to and activate LGR8, however it preferentially interacted with the LGR7 receptor. Work presented in this thesis showed that relaxin analogue binding to LGR7 occurred at a single population of sites while binding to LGR8 appeared to be a two site interaction. Analysis of chimeric LGR7/LGR8 receptors also reported relaxin interacting at two LGR8 binding sites. This was shown by differences in affinity of relaxin analogues for the separated LGR8 ectodomain and transmembrane domain in the chimeric receptors (Halls, 2003), leading to the hypothesis that the higher affinity site is positioned in

the ectodomain while the lower affinity site is in the transmembrane domain. The generation of chimeric receptors that contain only the separate LGR8 ecto- and transmembrane domains would allow closer examination of the two hypothesised LGR8 binding sites. Examination of the specific binding of relaxin and INSL3 at LGR8 with an INSL3 radioligand may also reveal further differences in the ability of these peptides to bind to the LGR8 receptor.

Relaxin analogues are not only interacting differently with the LGR8 receptor, the interaction between relaxin analogues and the LGR7 receptor also appears to be an individualised relationship. Further manipulation of the ecto- and transmembrane domains of the LGR7/8 chimeric receptor to include each LGR7 extracellular loop separately revealed that the presence of extracellular loop 2 is important for the interaction between H3RLX and the LGR7 receptor (Sudo *et al.*, 2003). This was not found to be important however in the interaction with H2RLX and PRLX analogues. Signalling through LGR7 also appeared to be agonist-directed as H2RLX, PRLX and RMRLX analogues stimulated a biphasic cAMP response while the response to RatRLX was monophasic (Chapter 6). Although the cAMP response to relaxin is reportedly a biphasic response comprised of a G_S protein- and a PI3K-coupled pathway, this is the first study to show a biphasic cAMP dose-response curve. The ability of relaxin to stimulate the two pathways to produce a cAMP response could also explain the lower efficacy displayed by INSL3 at LGR8 while maintaining the highest potency, as INSL3 may not be able to direct coupling of the receptor to both pathways. Investigation of the cAMP response in the presence of inhibitors to each component is needed to examine the coupling directed by the individual relaxin analogues at LGR7, as well as the difference between relaxin and INSL3 signalling at LGR8.

As the pharmacological differences between the H2RLX and H3RLX analogues become more apparent, the physiological function of these peptides is becoming an intriguing issue. A detailed examination of the expression of relaxin and its receptor in human uterine tissue (Chapter 7) has convincingly shown for the first time the cyclic expression of the relaxin receptor in the endometrial layer of human uterine tissue, with the LGR7 receptor being highly expressed during the early-mid

secretory phase of the menstrual cycle. Although reports have shown differences in relaxin activity (Palejwala *et al.*, 2002) and receptor expression (Ivell *et al.*, 2003; Luna *et al.*, 2004) between the proliferative and secretory phases, solid evidence that the role of relaxin is influenced by the menstrual cycle has been lacking. The localisation and increase in expression of the relaxin receptor in the glandular and luminal epithelial cells indicates a possible role in implantation. These cells are known to maintain an important role in this process, as many of the factors that influence implantation are produced and secreted via the glands during the secretory phase, and the first contact between the embryo and the uterus takes place via the luminal epithelium. Implantation is a complex process involving not only reproductive and growth hormones but also cytokines and adhesion molecules (Lindhard *et al.*, 2002). Cellular adhesion molecules expressed by the luminal epithelium may play a critical role in the initial attachment of the embryo to the endometrial surface (Enders *et al.*, 1969). Reproductive hormones influence the regulation of adhesion molecules, including glycodelin, an endometrial product of the secretory phase. Progesterone is involved in glycodelin production, and its secretion is regulated by relaxin (Stewart *et al.*, 1997). Heparin binding-epidermal growth factor (HB-EGF) is also an important adhesion factor that facilitates blastocyst implantation (Raab *et al.*, 1996). HB-EGF is expressed in the glandular epithelial cells of the endometrium at increasing levels during the implantation window (Leach *et al.*, 1999), and this pattern was reproduced in an endometrial cell line by combined treatment with oestrogen, progesterone and relaxin (Lessey *et al.*, 2002). HB-EGF expression has been linked to the regulation of other key endometrial factors involved in implantation, including leukaemia inhibitory factor (LIF) and integrin $\alpha_v\beta3$, highlighting the complex relationship between implantation factors. Further examination of the relationship between relaxin and various implantation factors could lead to valuable information regarding the specific role relaxin plays in implantation.

The examination of mRNA in the endometrium has revealed local synthesis of H2RLX mRNA while there has been no successful detection of either H1RLX or H3RLX transcripts (Palejwala *et al.*, 2002; Chapter 7). H3RLX mRNA expression was examined in this study and not detected, however as there is currently no human

tissue known to express H3RLX, a positive control for the RT-PCR could not be used and therefore the validity of these results is uncertain. A current hypothesis is that while both relaxin peptides have the potential to function through-out the body, H2RLX is the principle systemic acting relaxin peptide while H3RLX is primarily a centrally acting relaxin peptide. The high affinity displayed by the GPCR135 (Liu *et al.*, 2003b) and GPCR142 (Liu *et al.*, 2003a) receptors for H3RLX, while appearing to show no affinity for H2RLX, confuses this theory slightly as although GPCR135 is abundant in various brain regions with limited expression reported in peripheral tissues, GPCR142 is widely expressed peripherally. A possible explanation is that H3RLX is not the native ligand for GPCR142. Research in rats has shown that expression of Rat3RLX is primarily restricted to the brain (Burazin *et al.*, 2002) and as rats are not capable of expressing a functional GPCR142 receptor (Liu *et al.*, 2003a) while expressing a fully functional Rat3RLX peptide, the relevance of GPCR142 in relaxin gene 3 function is uncertain.

In conclusion, relaxin-like activity at the human relaxin receptor is determined by several factors, including the structural integrity of the peptide and the presence of the relaxin binding motif, however there are other areas important for interaction with the relaxin receptor still to be identified. The interaction between relaxin analogues and a variety of receptors is distinct to each analogue, as shown by the ability with which the various relaxin analogues bind to and activate different receptors. Although the wide variety of receptors that interact with relaxin is confusing in terms of functional relevance, the location and regulation of LGR7 receptor expression in human uterine tissue provides evidence for a role in implantation. Further investigation into the interaction between the human relaxins and the various receptors, as well as their relative expression throughout the body, is needed to provide insight into the specific physiological roles of the individual peptides.

Chapter Nine

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