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ERRATA

P XXIII, line 10 '1247-1245' should read '1247-1254'
P 17, line 19 'noradrenaline' for 'acetylcholine'
P 77, line 5 '..captopril and bestatin...' should read "...ACE and bestatin-sensitive aminopeptidase...'
P 92, Figure 4.6 '..in the absence of antagonist (□)...' should read '..in the absence of antagonist (△)...'
P 152, line 6 'PPS' should read 'PSS'
P 152, line 13 'PPS' should read 'PSS'
P 155, line 12 'PPS' should read 'PSS'
P 155, line 14 'PPS' should read 'PSS'
P 169, Figure 7.8 '..together with SR 140333 (1nM) (▲) ..together with SR 48968 (1nM) (▼)...' should read '..together with SR 140333 (1nM) (◆) ..together with SR 48968 (1nM) (◆)...'
P 175, line 22 'PPS' should read 'PSS'
P 175, line 25 'PPS' should read 'PSS'
P 175, line 30 'PPS' should read 'PSS'

ADDENDA

P 77, line 16: Add after '..prevent this from happening.'

'It has previously been reported that desensitisation of the NK_1 receptor in cultured enteric neurons and rat striatum is due to internalization of the receptor after exposure to SP, from which recovery ranges from 1 – 8h (Grady *et al.*, 1996; Mantyh *et al.*, 1995). While constructing discrete concentration-response curves using a time between doses of 1h or greater was not feasible in the present study the effect of increasing the interval between doses from 15 to 30min was examined.'

P 78, line 6: Add after '..carried out in our laboratory.'

'While this preliminary work suggests that desensitisation occurs with tachykinins acting at the NK_1 receptors and implies that true maximum responses will not be obtained while constructing concentration-response curves by the cumulative method it is important to note that peptide potency was determined based on the relative position of the curve not the maximum response.'

P 84, 4.2.2 Experimental protocol:
Add at the end of para:

'If antagonists were used these were added to the organ bath at the beginning of the equilibration period and replaced after each washout.'

P 161, Figure 7.2:
Delete '..In this and subsequent figures, a significant difference between log CRCs as indicated by two-way ANOVA followed by Student Newman Keuls is shown by *.'

P 176, line 15: Add after '..to the NK_2 receptor-selective [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) were reduced.'

'It is also interesting to note that whereas the maximum response to [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) in myometrium from pregnant women was decreased in the presence of 1nM SR 48968 and further decreased in the presence of 3nM SR 48968, this was not observed in myometrium obtained from non-pregnant women. This suggests the possibility that differing spare tachykinin NK_2 receptor populations may exist in the non-pregnant and pregnant human uterus.'

P 201, line 1: Add after '..during pregnancy is unclear.'

'A similar unusual phenomenon has also been reported in the guinea-pig airways. Moore *et al.* (2000) observed that the application of SP (0.1-1μM) to nodose neurons isolated from uninflamed airways did not elicit measurable changes in resting electrophysiological properties. In contrast, 80% of nodose neurones isolated 24h after *in vivo* exposure to

aerosolized antigen challenge were depolarised by 100nM SP. The authors suggested that the regulation of tachykinin receptors in these neurons relied on posttranslational mechanisms and have called this phenomenon "unmasking".

P 201, line 4: Add after '..receptor type numbers...'

'..and/or receptor unmasking.'

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THESIS ACCEPTED IN SATISFACTION OF THE
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Sec. Research Graduate School Committee

MODULATION OF MAMMALIAN UTERINE CONTRACTILITY BY TACHYKININS

A THESIS SUBMITTED TO
THE FACULTY OF MEDICINE, NURSING AND HEALTH SCIENCES,
MONASH UNIVERSITY

For the Degree of
DOCTOR OF PHILOSOPHY

By

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SUMMARY OF THESIS

Tachykinin-containing peripheral nerves have been observed in the female reproductive tract of several mammalian species. However, the function of these nerves in reproduction has yet to be elucidated. Tachykinins have been reported to elicit myometrial contractility in the rat by activation of the tachykinin NK_2 receptor. Preliminary reports suggest that tachykinins may also have a uterotonic effect in myometrium from mice and humans. This thesis describes experiments undertaken to determine the tachykinin receptor type(s) involved in mediating myometrial contractility in the non-pregnant and pregnant mouse and human.

Receptor characterization was undertaken by determining the rank order of agonist potency of the mammalian tachykinins SP, NKA and NKB and further confirmed with the use of tachykinin receptor-selective agonists and antagonists. The tachykinin receptor-selective agonists used were the NK_1-selective [$Sar^9Met(O_2)^{11}$]SP, the NK_2-selective [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and the NK_3-selective [$MePhe^7$]NKB. The non-peptide antagonists supplied by Sanofi Recherche included the NK_1-selective SR 140333, the NK_2-selective SR 48968 and the NK_3- selective SR 142801.

The functional experiments described in Chapter 3 established the importance of peptidase inhibitors when examining the uterotonic actions of the mammalian tachykinins in uterine preparations obtained from oestrogen-treated mice. Consequently, subsequent functional experiments examining the effects of the mammalian tachykinins in both the mouse and human uterus were conducted in the presence of the peptidase inhibitors thiorphan, captopril and bestatin.

Experiments described in Chapters 4 and 5 were designed to determine the tachykinin receptor type(s) involved in mediating the uterotonic activity of tachykinins in the oestrogen-treated and pregnant mouse uterus respectively. Results from Chapter 4 indicate

that the tachykinin NK_1 receptor is the predominant receptor type involved in mediating tachykinin-induced contractile activity in the oestrogen-treated mouse uterus, although a component of the response to both NKA and NKB may be mediated by the tachykinin NK_2 receptor. Evidence was also obtained to suggest that neither the release of histamine nor prostaglandins contributed to the uterotonic effects of SP in this preparation. Results from Chapter 5 indicate that the tachykinin NK_2 receptor is the predominant receptor type involved in mediating tachykinin-induced contractions in the pregnant mouse uterus. In addition, peptidase inhibitors were demonstrated to have no effect on the responses to tachykinin peptides in this preparation. Thus, this study has shown that the predominant tachykinin receptor type involved in mediating the uterotonic effects of tachykinins differs in the oestrogen-treated compared to the pregnant mouse uterus. Whether this is due to a change in receptor-effector coupling and/or tachykinin receptor numbers lay outside the scope of the present study. A useful extension of the present study would be to establish whether the lack of effect of peptidase inhibitors in the pregnant mouse uterus was due to a pregnancy-induced regulation of peptidases.

Chapters 6 and 7 describe experiments designed to determine the tachykinin receptor type(s) involved in mediating the contractile activity of tachykinins in the non-pregnant and pregnant human uterus respectively. The results of Chapter 6 indicate that the uterotonic effects of the tachykinins in the non-pregnant human uterus are mediated by activation of an NK_2 receptor. Furthermore, the absence of blockade of the response to the NK_2 receptor-selective agonist [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) by atropine, phentolamine or tetrodotoxin suggests that these peptides are acting on tachykinin receptors that are probably located on the uterine smooth muscle. Peptidase inhibitors did not potentiate the response to the tachykinins in this preparation. The results of Chapter 7 indicate that the uterotonic effects of the tachykinins in the pregnant human uterus are also mediated by activation of the tachykinin NK_2 receptor. In addition, in this preparation responses to the tachykinins were potentiated in the presence of peptidase inhibitors. Interestingly, the NK_2-preferring NKA and the NK_2 receptor-selective agonist [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) were significantly

more potent in the non-pregnant human uterus. This may reflect a pregnancy-related decrease in NK_2 receptor levels and/or pregnancy-induced changes in the activity of peptidases.

In Chapter 8 pilot immunohistochemical experiments were undertaken to demonstrate the presence of tachykinin-containing peripheral nerves in myometrium from non-pregnant and pregnant mice and humans. The results of this chapter demonstrate the sparse distribution of SP-, NKA- and CGRP-immunoreactive (IR) fibres in the oestrogen-treated mouse uterus. SP- and tyrosine hydroxylase-IR fibres were also sparsely distributed in outer myometrium from non-pregnant humans. In contrast, no peptidergic fibres could be visualised in uterine tissue obtained from pregnant mice or humans. Although the absence of tachykinin-IR nerves in both these species during pregnancy is of interest the present pilot study leaves open the question as to whether this was due to a pregnancy-induced sensory degeneration.

Taken together, the results of the present study have established the tachykinin receptor types involved in mediating uterotonic responses to tachykinins in the non-pregnant and pregnant mouse and human uterus. These results indicate that species differences exist in both the tachykinin receptor type involved in mediating uterine contractility and the level of peptidase activity. Based on these observations the rat may provide a better model of pregnancy-induced changes to uterotonic responses to tachykinins of human myometrium than would the mouse.

In conclusion, uterine contractility is a crucial factor determining the emptying of the uterine contents during menstruation, transport of sperm to the fallopian tubes and expulsion of the fetus during parturition. This study has demonstrated that tachykinins are potent agonists eliciting uterotonic effects in both the non-pregnant and pregnant mouse and the human, which could indicate a physiological or pathophysiological role for these peptides in reproductive processes.

ABBREVIATIONS

ACE	angiotensin converting enzyme
Ala	alanine
ANOVA	analysis of variance
Arg	arginine
Asn	asparagine
Asp	aspartic acid
AUC	area under the curve
AVP	arginine vasopressin
°C	degrees Celsius
cDNA	complementary deoxyribonucleic acid
CGRP	calcitonin gene related peptide
CL	confidence limits
CRC	concentration-response curve
CSPANs	capsaicin sensitive primary afferent neurons
Cx	connexins
DAG	diacylglycerol
DAP IV	dipeptidylaminopeptidase IV
d.f.	degrees of freedom
DRG	dorsal root ganglia
ECE	endothelin converting enzyme
EC_{50}	concentration giving 50% of maximum response
E_{max}	maximum response to an agonist
FITC	fluoroscein isothiocyanate
g	gram(s)
Gly	glycine
h	hour(s)
HK-1	hemokinin 1
IC_{50}	concentration which inhibits 50% of binding in the presence of a fixed concentration
Ile	isoleucine
IP_3	inositol trisphosphate
IR	immunoreactive
kg	kilogram(s)
KPSS	high potassium physiological saline solution
Leu	leucine
LUSCS	lower uterine segment caesarean section
Lys	lysine
MCh	methacholine chloride
MePhe	methylphenylalanine
Met	methionine
min	minute(s)
mRNA	messenger ribonucleic acid
MLCK	myosin light chain kinase
n	number of animals/women/preparations

N/A	not applicable
NEP	neprilysin
NK	neurokinin
NK_1	neurokinin 1
NK_2	neurokinin 2
NK_3	neurokinin 3
NK_4	neurokinin 4
NKA	neurokinin A
NKB	neurokinin B
Nle	norleucine
NP	neuropeptide
NPγ	neuropeptide γ
NPK	neuropeptide K
pA_2	negative log of the concentration of antagonist required to give a concentration ratio of two
PBS	phosphate buffered saline
pD_2	negative log of the concentration of agonist producing 50% of the maximal response
PG	paracervical ganglion
Phe	phenylalanine
PIP_2	phosphatidyl inositol bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PPT	preprotachykinin
Pro	proline
PSS	physiological saline solution
Sar	sarcosine
s.c.	subcutaneously
SEM	standard error of the mean
SP	substance P
SR 140333	(1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidine-3-yl]ethyl}-4-phenyl-1-azonia-bicyclo[2.2.2]octane, chloride)
SR 142801	(S)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorpheny)piperidine-3-yl)propyl)-4-phenylpiperidin-4-yl)-N-metylacetamide
SR 48968	((S)-N-methyl-N[4-acetylamino-4 phenylpiperidino)-2-(3,4-dichlorophenyl)butyl] benzamide)
Trp	tryptophan
TTX	tetrodotoxin
Tyr	tyrosine
Val	valine
VR	vanilloid receptor

Additional abbreviations used in Tables are listed in their legends

DECLARATION

The material contained herein has not been presented or the award of any other degree or diploma in any University or other institution. The research conducted has been carried out solely by the candidate, and this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I certify that the writing of this thesis, the results, interpretations, opinions and suggestions are my own work.

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PUBLICATIONS

MANUSCRIPTS

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background of project

The female reproductive tract is innervated by several subgroups of autonomic and sensory nerves. Yet the role of these nerves in reproductive processes, in particular that of the sensory innervation, has received little attention. The sensory nerves contain a variety of putative neurotransmitters, including the tachykinins substance P (SP) and neurokinin A (NKA). It is now known that these neurotransmitters can be released from the peripheral terminals of sensory nerves in an "efferent" fashion to produce effects in surrounding tissue. Previous work in our laboratory has demonstrated that the tachykinins modulate myometrial contractility in the rat uterus. It is therefore probable that the release of tachykinins from sensory nerves could effect coordination of uterine muscular activity, important for facilitating emptying of uterine contents during menstruation, transport of sperm to the fallopian tubes, implantation of the conceptus and expulsion of the fetus during parturition.

To date, the majority of studies investigating the actions of tachykinins on myometrial activity have been conducted in the rat uterus. Less is known about their actions on myometrium from other species. Two early studies reported that SP and the non-mammalian tachykinin eledoisin elicited contractile activity in myometrium from non-pregnant women (Molina and Zappia, 1976; Ottesen *et al.*, 1983). In contrast, tachykinin receptor-selective agonists were reported to be without effect in myometrium from pregnant women (Barr *et al.*, 1991). Pilot experiments conducted in our laboratory have suggested that the tachykinins also mediate uterine contractility in both the non-pregnant and pregnant mouse.

The present study was undertaken to investigate the actions of tachykinins on regulating myometrial contractility in the non-pregnant and pregnant mouse and human. The mouse was of particular interest as gene knockouts for both the tachykinin NK_1 receptor (Ptak *et al.*, 2000; Ripley *et al.*, 2002; Saban *et al.*, 1999; Weng *et al.*, 2001) and the gene that encodes for SP and NKA (Liu *et al.*, 1999; Simons *et al.*, 2001; Zimmer *et al.*, 1998) have been developed in this species. These gene knockout animals are of interest as they have the potential to help better understand uterine dysfunctions.

The following general introduction covers four main themes. The first part (§1.2-1.6) deals with the mammalian uterus and covers anatomy, reproductive cycles, regulation of myometrial contractility, pregnancy and the onset of parturition. Reference to the rat has been made in this section as not only does it share similarities with the mouse but also a majority of studies involving the female reproductive tract have been conducted in this species. The second part (§1.7) deals with the tachykinins and describes their discovery, localization, structure, biosynthesis, release and degradation. The third part (§1.8) deals with the tachykinin receptors and covers nomenclature, structure, second messengers, structure-activity relationships and receptor-selective agonists and antagonists. The fourth part (§1.9) covers what is currently known about tachykinins and their putative roles in the uterus and describes their occurrence, effects on reproductive function and possible sources of their degradation in this organ.

1.2 The uterus

The uterus is a hollow muscular organ whose function is to contain and nourish a developing fetus from implantation to parturition. Broadly speaking the uterus consists of two distinct parts; a body or corpus that is lined by an endometrium and a cervix which, with its shape and musculature, is designed to retain the contents of the uterus during pregnancy (Mossman, 1989). The shape of the uterus varies between species and in mammals can be classified into three basic groups (Mossman, 1989):

- the bicornuate type – where two separate tubes that are joined internally at their cervical ends open into one cervical canal;
- the duplex type – where two tubes open into two cervical canals that are joined externally, as in the mouse and rat;
- the simplex type – where a single unpaired corpus is joined to a single cervical canal, as in the human.

Histologically, the uterine wall consists of three layers; the serosa, the outer covering which is a continuation of the peritoneal epithelium; the myometrium, the middle muscular layer that forms the bulk of the uterine wall and provides the protective and

expulsive functions of the uterus during pregnancy and parturition; and the endometrium, the inner lining of the uterus (Ramsey, 1994).

The composition of the myometrium is itself heterogeneous and is described as consisting of an inner and an outer zone. These two zones have different embryological origins with the inner zone arising from the musculature of the Müllerian ducts while the outer zone develops from the subserous connective tissue (Werth and Grusdew (1898), cited in Daels (1974)). Studies conducted in various species, including humans and rodents, have reported that striking differences exist in not only the spontaneous contractile activity of the different zones but also in their responsiveness to various agonists such as oxytocin, prostanoids, histamine and catecholamines (Daels, 1974; Hartley *et al.*, 1983; Kitazawa *et al.*, 1997; Tuross *et al.*, 1987).

The mouse and rat are two species where the myometrium is seen to consist of two clearly defined layers, an inner layer of circular muscle fibres and an outer layer of longitudinal muscle fibres, with a layer of very vascular loose connective tissue between them (Gude *et al.*, 1982). In contrast, the human myometrium has alternatively been described as comprising either three or four layers. From inner to outer, the three layer model consists of an innermost circular layer, a middle oblique layer where the muscles are arranged in a criss-cross manner and an outermost longitudinal layer which is continuous with the muscle fibres in the uterine ligaments (Burnett, 1962). The four layer model is described as comprising a layer of longitudinal fibres underlying the endometrium, a layer of muscle fibres that run in all directions, a layer of both circular and longitudinal fibres, and an outer layer that is continuous with the myosalpinx and connective tissue of the broad and round ligaments (Finn and Porter, 1975). More recent studies using magnetic resonance imaging have described the human myometrium as consisting of three layers but stated that an intermediate layer between the endometrium and myometrium also exists (Tetlow *et al.*, 1999).

The female reproductive tract receives innervation from three sets of peripheral nerves: sympathetic, parasympathetic and sensory. These nerves contain combinations of the classical neurotransmitters noradrenaline or acetylcholine and neuropeptides such as SP,

neuropeptide-Y, galanin, vasoactive intestinal polypeptide, calcitonin gene-related peptide (CGRP) and others (Papka and Traurig, 1993; Stjernquist and Sjöberg, 1994; Traurig and Papka, 1993). Sympathetic innervation arises from the inferior mesenteric ganglion and lumbosacral sympathetic chain ganglia. Parasympathetic nerves arise from the paracervical ganglion (PG). Sensory nerves arise from dorsal root ganglia (DRG) (Papka and Traurig, 1993) (Figure 1.1A). In general, the uterus, fallopian tubes and ovaries are innervated by the hypogastric nerve with some contribution from the pelvic and vagus nerves, while the cervix and vagina receive their innervation via the pelvic nerve and to a lesser extent the hypogastric nerve (Papka and Traurig, 1993). The nerve fibres supplying the uterus generally accompany the uterine arteries and ramify in the myometrium and endometrium. The innervation of the female reproductive tract is also not uniform. Studies have reported that in the human, mouse and rat the noradrenergic, cholinergic and sensory innervation is denser in the cervix compared to the uterus (Papka and Traurig, 1993; Stjernquist and Sjöberg, 1994; Traurig and Papka, 1993).

Blood supply to the uterus is mainly via the uterine and to a lesser extent the ovarian arteries in the human (Finn and Porter, 1975) (Figure 1.1B) and the uterine branches of the ovarian artery in the rodent (Cook, 1965). Venous return is via the uterine vein (Finn and Porter, 1975).

1.3 Reproductive cycles

The uterus is a dynamic organ, which, unless interrupted by pregnancy, undergoes a repetitive series of cyclic changes that recur throughout reproductive life. Though differences are seen in the reproductive cycles of the human, mouse and rat, in all three species, cycle length is still determined by complex interactions between the hypothalamus, anterior pituitary and ovaries.

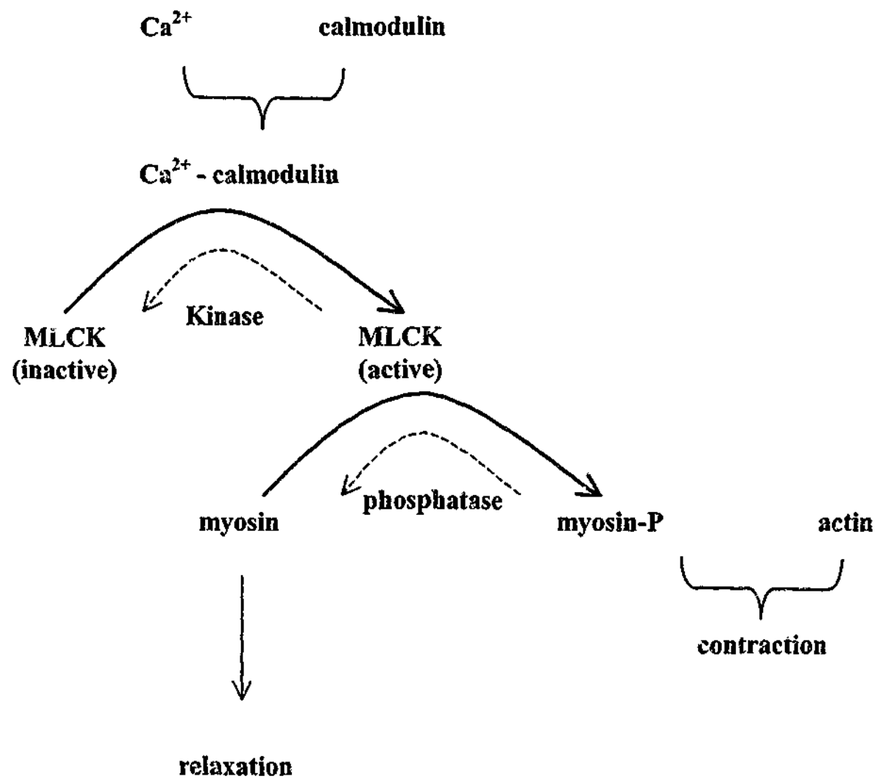
In humans the reproductive cycle lasts approximately 28 days and can be divided into three different phases during which levels of the two ovarian hormones, oestrogen and progesterone, fluctuate markedly (Moffett *et al.*, 1993). The first stage, menstruation, is triggered by low levels of oestrogen and progesterone, caused by degeneration of the

corpus luteum after implantation fails to occur. This is followed by the proliferative phase, during which the follicles secrete oestrogen causing the endometrium to undergo changes such as an increase in thickness and addition of spiral arteries and exocrine glands. A sudden decrease in oestrogen levels triggers ovulation, with the ruptured follicle transforming into a corpus luteum and progesterone starting to be secreted. During this secretory phase, oestrogen levels remain low and progesterone levels increase, preparing the endometrium for possible implantation. If implantation has not taken place, degeneration of the corpus luteum occurs resulting in a decline in progesterone levels and menstruation is once again initiated. This cycle is repeated until a woman reaches menopause. Menopause is the complete cessation of menstruation and on average takes place around 50 years of age. Menopause occurs when oestrogen levels decline and the ovaries become nonfunctional as endocrine organs, causing not only menstruation to cease but also numerous other changes, including atrophy of the uterus (Krouse, 1980).

The oestrous cycle in both the mouse and rat lasts for approximately four to five days and can be divided into four different stages; proestrus, oestrus, metoestrus and dioestrus (Bronson *et al.*, 1966; Long and Evans, 1922; Rugh, 1968). Each stage is characterized by distinct changes in the uterus, with cycle stage able to be readily determined by examination of cell types present in vaginal smears. The first two stages, proestrus and oestrus, are periods of anabolic growth which culminate in ovulation. During this stage levels of oestrogen and progesterone are increasing and a peak is observed during proestrus before rapidly declining during oestrus. Smears from this stage will typically consist of both nucleated and cornified epithelial cells, with the number of cornified cells increasing as oestrus approaches. During metoestrus the uterus undergoes degenerative changes if fertilization has not occurred; throughout this stage oestrogen levels are low while progesterone levels are increasing. A smear obtained at this stage will show cornified epithelial cells and an infiltration of leukocytes. Dioestrus is a period of quiescence. Both oestrogen and progesterone levels are low during this stage and smears typically consist of only leukocytes (Bronson *et al.*, 1966; Butcher *et al.*, 1974; Long and Evans, 1922; Rugh, 1968).

Figure 1.2:

Diagram illustrating regulation of contraction and relaxation in the myometrium (modified from Carsten and Miller, 1987).



It is important to note that during the oestrous cycle, not only does the uterus undergo structural changes but myometrial responses to various pharmacological agents can also be altered (Adam *et al.*, 1984; 1985; Cruz and Rudoiph, 1986; Hartley and Pennefather, 1981; Hartley *et al.*, 1983; Mammen *et al.*, 1987; Wollberg *et al.*, 1992). Therefore, to avoid any complications arising from fluctuating hormone levels, the non-pregnant mice used in the present study have been treated with a single dose of oestrogen 24h prior to experimentation to ensure a similar hormonal status.

1.4 Regulation of myometrial contractility

Myometrial contractility is important in both the non-pregnant and pregnant uterus. During the menstrual cycle uterine contractions facilitate emptying of the uterine contents and transport of sperm from the vagina to the fallopian tubes (de Ziegler *et al.*, 2001). During parturition, the ability of the uterus to generate forceful, co-coordinated contractions is important to the successful outcome of the pregnancy.

Contraction of myometrial smooth muscle is due to an interaction between the contractile proteins actin and myosin and is regulated principally by the intracellular calcium concentration and the activity of the calcium dependent enzyme, myosin light chain kinase (MLCK). In brief, an increase in intracellular calcium is required to interact with the calcium binding protein calmodulin to form a calcium-calmodulin complex. This complex activates MLCK, which in turn phosphorylates myosin. Phosphorylated myosin can then interact with actin resulting in muscle contraction (Buxton *et al.*, 2000; Jeyabalan and Caritis, 2002). Other actin-related proteins, troponin, caldesmon and calponin are also phosphorylated and allow for a finer degree of control of the response to a given calcium level (Buxton *et al.*, 2000; Riemer and Heymann, 1998). A decrease in calcium levels causes the calmodulin to dissociate from the MLCK and the MLCK becomes inactive (Figure 1.2). Myosin is then dephosphorylated by phosphatase and relaxation occurs (Carsten and Miller, 1987).

The source of the calcium necessary for uterine contraction is both extracellular and intracellular. Extracellular calcium enters the cell via voltage-gated calcium channels

while intracellular calcium is released from the sarcoplasmic reticulum (Carsten and Miller, 1987; Wray, 1993). The ability of a number of hormones, neurotransmitters and pharmacological agents to regulate uterine activity is governed by their effect on calcium mobilization.

The coordination of rhythmic contractions required during labour is achieved by cell-to-cell coupling through gap junctions. Gap junctions are channels composed of proteins called connexins (Saez *et al.*, 1993) that connect the interior of two cells permitting the passage of small molecules and ions (Garfield and Yallampalli, 1994). Gap junctions have lower resistance than the cell membrane and thus provide pathways for the efficient conduction of action potentials between cells (Garfield and Yallampalli, 1994). The myometrium of both rodents and humans has been found to express various gap junction proteins including Cx43, Cx45 and Cx26, that not only display regional differences but are also under hormonal regulation (Albrecht *et al.*, 1996; Ciray *et al.*, 2000; Orsino *et al.*, 1996; Riemer and Heymann, 1998).

1.5 Pregnancy

The length of gestation varies from species to species and is approximately 38 weeks in the human, 19 days in the mouse and 22 days in the rat (Ramsey, 1975). During this time the uterus undergoes extensive anatomical and physiological changes in order to accommodate the growing fetus. The most noticeable change is an enlargement of the uterus, which in the early stages of pregnancy is accomplished by an increase in both muscle cell size (hypertrophy) and number (hyperplasia) and stretch during the later stages (Theobold, 1973).

A state of quiescence, the absence of coordinated uterine contractions, is normally essential during gestation for the development of the fetus. The processes involved in maintaining uterine quiescence are still poorly understood but factors implicated include the disappearance of noradrenergic and cholinergic nerves from the uterus as pregnancy progresses (Sjöberg, 1968), down-regulation of a number of genes that are essential for parturition such as gap-junction proteins, calcium channels and oxytocin receptors

(Garfield *et al.*, 1998), up-regulation of relaxation mechanisms such as nitric oxide (Garfield *et al.*, 1998) and the up-regulation of enzymes that degrade contractile agents such as oxytocin (Germain *et al.*, 1994; Mitchell and Wong, 1995). Many of these changes are directly attributable to the actions of progesterone and oestrogen, which display species dependent dynamics during pregnancy. In the mouse and rat, oestrogen and progesterone are produced by the corpus luteum (Gross *et al.*, 2000). In both these species, progesterone levels are seen to increase during pregnancy prior to dropping markedly before parturition, while oestrogen levels are initially seen to decline before steadily increasing until parturition (Candenas *et al.*, 2001; Kosaka *et al.*, 1988; Parkening *et al.*, 1978). In the human, oestrogen and progesterone are initially secreted by the ovaries (Challis, 2000) but as pregnancy progresses the secretion of these hormones shifts to the feto-placental unit (Cluett, 2000). Although the levels of both oestrogen and progesterone are seen to increase throughout gestation, there is no pre-partum fall in the level of progesterone in the human, as is observed in the mouse and rat (Batra, 1994; Bernal, 2001).

In mammals, survival of the embryo and fetus during pregnancy is dependent on the presence of a well-established and functional placenta. The placenta is an indispensable structure that attaches the conceptus to the uterus and forms the vascular connections needed for gaseous and nutrient exchange between the maternal and fetal compartments (Sapin *et al.*, 2001). Placental morphology varies from species to species but certain similarities exist which allow for their classification. There are five principal systems of classification, these being origin, shape, internal structure, relation to maternal tissues and composition of the placental membrane (Ramsey, 1975). Based on this classification the human, mouse and rat placentas are similar with the only difference being that the internal structure of the human placenta is villious in contrast to the mouse and rat where it is labyrinthine (Ramsey, 1975).

1.6 Onset of labour

Labour is defined as the onset of regular co-coordinated contractions of the myometrium and progressive dilation and effacement of the cervix that culminates in

expulsion of the fetus(es) (Jeyabalan and Caritis, 2002). In the human, the actual mechanism(s) that cause the uterus to change from a relatively quiescent state during gestation to an active state during labour remain unknown. Numerous hypotheses have been proposed, with the general assumption being that oestrogen, progesterone, oxytocin and prostaglandins play major roles, but no definitive mechanism has been established and further research in this area is needed (for reviews see Bernal, 2001; Schellenberg and Liggens, 1994). This in itself proves to be difficult, with ethical implications limiting experimentation in humans to uterine tissue and fetal membranes obtained during caesarean or vaginal delivery i.e. before or during labour but not at the onset of parturition. Experimentation therefore is mainly carried out using ruminants and rodents. This poses a further problem as extrapolation to humans is often inappropriate due to major differences in reproductive physiology. A good example of this is seen with the sheep in which the mechanism initiating parturition has been elucidated. In this species as pregnancy approaches term, there is a sharp increase in the secretion of fetal hydrocortisone (Liggins, 1989), which results in the increased activity of placental 17-α hydroxylase, an enzyme that promotes the conversion of progesterone to oestrogen (Schellenberg and Liggens, 1994). It is this subsequent change in the oestrogen:progesterone ratio that ultimately causes activation of uterine contractility in the sheep at term. Progesterone withdrawal caused by luteolysis, the dissolution of the corpus luteum, has been established as initiating parturition in both the mouse and rat (Bernal, 2001). In the human, not only is 17-α hydroxylase absent from the placenta but progesterone withdrawal at term is also not seen (Bernal, 2001) and it is doubtful that a single agent, as observed in the sheep, is responsible for initiating parturition. Instead, it is more likely that a combination of the above-mentioned and possibly as yet unidentified factors are acting synergistically to initiate parturition.

1.7 Tachykinins

The tachykinin family are a group of small neuropeptides that occur in many organisms of the animal kingdom ranging from insects to vertebrates. The name tachykinin stems from their quick onset of action on gut smooth muscle (tachy = fast, kinin = to move) compared to that of bradykinin (brady = slow) and defines peptides that share the

common carboxy terminal sequence Phe-X-Gly-Leu-Met-NH_2. To date, nearly 50 tachykinins have been identified and sequenced (Severini *et al.*, 2000). Of these, six are known to naturally occur in mammals, these being substance P (SP), neurokinin A (NKA), neurokinin B (NKB), the N-terminally extended forms of NKA, neuropeptide K (NPK) and neuropeptide γ (NPγ) and the recently described novel tachykinin hemokinin 1 (HK-1) (Bellucci *et al.*, 2002; Camarda *et al.*, 2002; Kurtz *et al.*, 2002; Morteau *et al.*, 2001; Severini *et al.*, 2000; Zhang *et al.*, 2000). Tachykinins are expressed almost exclusively in neurons in both the central and peripheral nervous systems, where, upon release they elicit a wide range of biological actions in various tissues (for reviews see Maggio, 1988; Otsuka and Yoshioka, 1993).

1.7.1 Discovery and isolation

In 1931, Euler and Gaddum discovered a substance in acidic ethanol extracts of equine intestine and brain that caused hypotension when administered *in vivo* in the rabbit, and produced contractions when applied to isolated rabbit duodenum. This substance was given the name preparation P and biological assays determined that it was not one of the compounds already recognized to have a stimulatory effect on the gut (Euler and Gaddum, 1931). Several unsuccessful attempts were made to isolate and purify SP during the 1950's and 1960's (Haefely and Hurlimann, 1962; Pernow, 1953). Finally, nearly 40 years after its discovery, SP was isolated and purified from bovine hypothalamic tissue and its structure determined (Chang and Leeman, 1970; Chang *et al.*, 1971). Shortly after, SP was also isolated from extracts of bovine dorsal root tissue (Otsuka *et al.*, 1972) and equine intestine (Struder *et al.* (1973), cited in Severini *et al.* (2002)) and found to have the same amino acid sequence as determined by Chang and colleagues.

Erspamer (1949), while screening for biogenic amines in posterior salivary gland extracts of the Mediterranean octopi *Eledone Moschata* and *Eledone Aldrovandi* discovered a substance that had properties similar to those of SP (Erspamer, 1949). This substance was first called moschatine but latter renamed eledoisin to avoid confusion with the vegetable alkaloid moschatin extracted from *Achillea moschata*. The non-

mammalian tachykinin eledoisin was present in the salivary glands of the octopus at a much higher concentration than SP in the mammalian brain, thus allowing it to be sequenced before SP. Nevertheless, it still took 1,450kg of octopi in order for eledoisin to be the first tachykinin that was isolated, purified and sequenced (Erspamer, 1981).

In the same year that the structure of eledoisin was established, Erspamer and colleagues discovered a substance in extracts from the skin of the South American amphibian *Physalaemus bigilonigerus* (formerly *fuscumaculatus*) that was found to have eledoisin-like activity (Erspamer and Anastasi, 1962). This substance was named physalaemin and following elucidation of its structure (Anastasi *et al.*, 1964; Erspamer *et al.*, 1964) numerous other related peptides found in the skin, brain and gut of amphibians were identified in quick succession. These included phyllomedusin from *Phyllomedusa bicolor* (Anastasi and Erspamer, 1970), uperolein from *Uperoleia marmorata* and *Uperoleia rugosa* (Anastasi *et al.*, 1975) and kassinin from *Kassina senegalensis* (Anastasi *et al.*, 1977).

For many years it was believed that SP was the only mammalian tachykinin but this changed in 1983 with the discovery of two new mammalian tachykinins. As with SP, both these tachykinins, eventually to be known as NKA and NKB, were isolated and sequenced around the same time by several independent groups. Kimura and colleagues, using the guinea-pig ileum as an assay, reported the presence of two novel peptides in extracts of porcine spinal cord, which they called neurokinin α and neurokinin β (Kimura *et al.*, 1983) to reflect their occurrence in neuronal cells. Using a similar method to search for new neuropeptides, Kangawa and colleagues also discovered two novel peptides in extracts of porcine spinal cord, which they called neuromedin K and neuromedin L (Kangawa *et al.*, 1983; Minamino *et al.*, 1984). Maggio and colleagues used a different approach in that they hypothesized that the mammalian tachykinins had an aliphatic residue at position X in the carboxy-terminal (C-terminal) sequence (see section 1.7.2) and developed an assay to search for this. They discovered a novel tachykinin with a valine in position X in bovine spinal cord extracts and named it substance K to reflect its structural homology with the amphibian tachykinin kassinin (Maggio *et al.* (1983), cited in Maggio, (1988)). It was latter established that neurokinin

Figure 1.3:

Amino acid sequences of some mammalian and non-mammalian tachykinins, common amino acids are indicated in red and the variable amino acid at position 4 from the C-terminus is indicated in blue.

SP	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
NKA	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
NKB	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
NPγ	Asp-Ala-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-Lys-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
NPK	Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu-Lys-Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Gly-Gln-Ile-Ser-His-Lys-Arg-Lys-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
HK-1	Arg-Ser-Arg-Thr-Arg-Gln-Phe-Tyr-Gly-Leu-Met-NH_2
Eledoisin	pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
Physalaemin	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2
Uperolein	pGlu-Pro-Asp-Pro-Asn-Ala-Phe-Tyr-Gly-Leu-Met-NH_2
Kassinin	Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2

α, neuromedin L and substance K were identical, as were neurokinin β and neuromedin K. These peptides were later renamed NKA and NKB respectively (Maggio, 1988).

Three other mammalian tachykinins have also been discovered. NPK is a 36 amino acid residue peptide isolated from the porcine brain (Tatemoto *et al.*, 1985) and NPγ is a 21 amino acid residue peptide isolated from the rabbit intestine (Kage *et al.*, 1988), both being N-terminally extended forms of NKA. NPK and NPγ have been reported as having a modulatory role on the hypothalamo-pituitary-gonadal axis (Debeljuk and Lasaga, 1999). The third, HK-1, is a predicted 11 amino acid peptide generated by the proteolytic processing of a hypothetical 128 amino acid precursor, preprotachykinin C (PPT-C) (refer to section 1.7.4) (Zhang *et al.*, 2000). The expression of PPT-C was first reported in murine hematopoietic cells (Zhang *et al.*, 2000) and more recently in various peripheral tissues and the brain of both humans and rodents (Kurtz *et al.*, 2002). Based on experiments involving synthesized HK-1 this peptide was thought to be implicated in promoting B-cell survival and proliferation but has now been suggested to have other physiological roles beyond its role in B-cell development (Bellucci *et al.*, 2002; Camarda *et al.*, 2002; Kurtz *et al.*, 2002; Morteau *et al.*, 2001; Severini *et al.*, 2000; Zhang *et al.*, 2000).

1.7.2 Structure

Tachykinins all share the conserved C-terminal sequence Phe-X-Gly-Leu-Met-NH_2, where X is either an aromatic (Phe, Tyr) or branched aliphatic (Val, Ile) amino acid as shown in Figure 1.3. Most of the biological activity elicited by tachykinins depends upon the integrity of this conserved, amidated C-terminal sequence (Couture *et al.*, 1979; Erspamer and Melchiorri, 1973; Lee *et al.*, 1982; Pernow, 1983; Sandberg, 1985). Studies have shown that deamidated peptides are virtually inactive, as are N-terminal fragments (Cascieri *et al.*, 1981; Couture *et al.*, 1979; Piercey *et al.*, 1982; Severini *et al.*, 2000). Conversely, partial C-terminal sequences of tachykinins have been reported as being either equipotent or more potent than the parent peptide in some systems (Bury and Mashford, 1976; Maggio, 1988; Osakada *et al.*, 1986; Regoli *et al.*, 1984; Watson,

1984). In general, a minimum of six residues from the C-terminus for SP and at least seven for both NKA and NKB are required for these peptides to be active (Bury and Mashford, 1976; Osakada *et al.*, 1986). One view is that the common C-terminal sequence is important for binding and signal transduction while the variant N-terminal region confers selectivity of different tachykinins for the various receptors (Ingi *et al.*, 1991; Yokota *et al.*, 1992).

1.7.3 Localisation

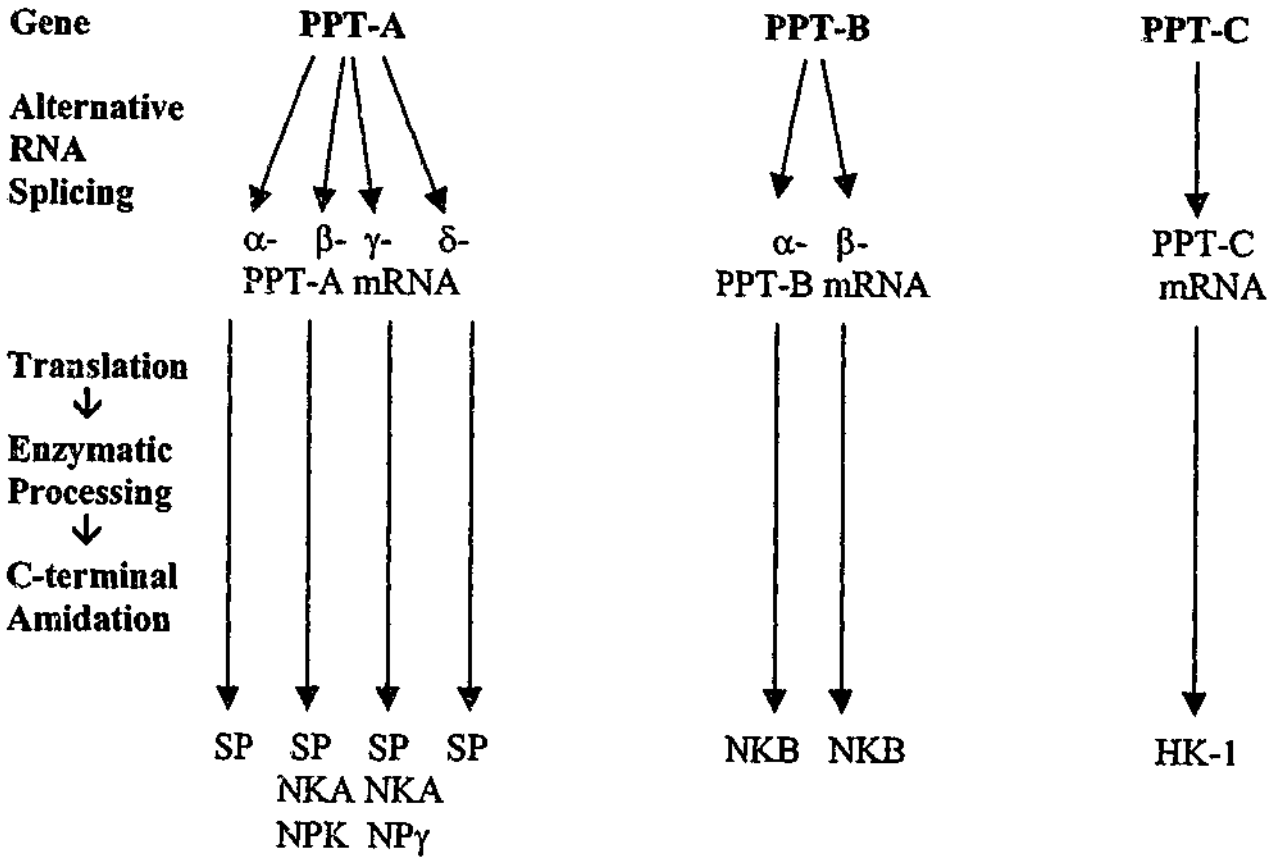
Tachykinins are expressed almost exclusively in neurons in the central, peripheral and enteric nervous systems (Maggi, 2000a; Otsuka and Yoshioka, 1993) with the exception of HK-1 which is the only known mammalian tachykinin to be produced primarily outside neuronal tissues (Zhang *et al.*, 2000). In the peripheral nervous system the most wide spread source of neuronal tachykinins are the peripheral terminals of a distinct subpopulation of afferent neurons, characterized by their sensitivity to capsaicin, the pungent ingredient of a wide variety of red peppers of the genus *Capsicum* (Maggi, 1995b). In addition to expressing SP and NKA, these capsaicin-sensitive primary afferent neurons (CSPANs) have been shown to contain a number of other peptides including CGRP, galanin, vasoactive intestinal peptide, somatostatin and glutamate which can be co-stored with the tachykinins (De Biasi and Rustioni, 1988; Franco-Cereceda *et al.*, 1987; Hanesch *et al.*, 1995; Holzer, 1988; Isaacs *et al.*, 1995; Maggi and Meli, 1988; Majewski *et al.*, 1996; Schmidt *et al.*, 1991; Zhu and Dey, 1992).

It was previously thought that NKB was only expressed in the central nervous system (Moussaoui *et al.*, 1992). However, recent reports have described its occurrence, or that of its precursor gene, in peripheral tissues including the uterus and placenta (Cintado *et al.*, 2001; Page *et al.*, 2000; Pinto *et al.*, 2001).

SP-like immunoreactivity has also been reported in various non-neuronal cells in the periphery such as eosinophils (Aliakbari *et al.*, 1987; Weinstock *et al.*, 1988), chromaffin cells in the adrenal gland (Bucsics *et al.*, 1981; Kuramoto *et al.*, 1985), enterochromaffin cells (Heitz *et al.*, 1976; Sundler *et al.*, 1977), macrophages (Pascual

Figure 1.4:

Biosynthesis of mammalian tachykinins



and Bost, 1990), Leydig cells (Chiwakata *et al.*, 1991) and Merkel cells in pig skin (Weihe *et al.*, 1991). However, it has not been confirmed whether tachykinin synthesis occurs in these non-neuronal cells (Maggi, 1995b).

1.7.4 Biosynthesis

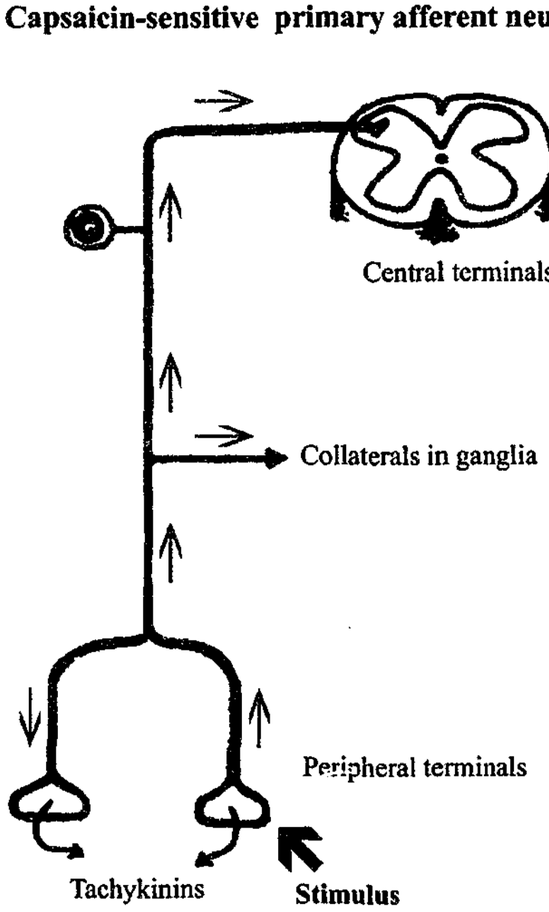
Tachykinins are synthesized ribosomally as larger protein precursors in the neuronal cell body and shipped to the terminals where proteolytic cleaving converts them to their active form (Maggio, 1988).

The mammalian tachykinins SP, NKA, the N-terminally extended forms of NKA and NKB are derived from two similar genes termed preprotachykinin A and preprotachykinin B (PPT-A and PPT-B) (Kotani *et al.*, 1986; Maggio, 1988; Nakanishi, 1987) (see Figure 1.4). The PPT-A gene consists of seven exons, with sequences in exon 3 and exon 6 encoding SP and NKA respectively. Alternative splicing of the PPT-A gene yields four different mRNAs designated α-, β-, γ- and δ-PPT mRNA (Harmar *et al.*, 1990; Khan and Collins, 1994; Kotani *et al.*, 1986; Nawa *et al.*, 1984). The biological significance of this alternative splicing of the gene is unclear. α-PPT lacks exon 6 so only produces SP after post-translational processing (Kotani *et al.*, 1986; Nawa *et al.*, 1984). β-PPT is encoded by all seven exons and produces SP, NKA and NPK (Kotani *et al.*, 1986; Nawa *et al.*, 1984; Tatemoto *et al.*, 1985). γ-PPT lacks exon 4 and produces SP, NKA and NP-γ (Kage *et al.*, 1988; Kotani *et al.*, 1986; Nawa *et al.*, 1984). δ-PPT lacks exon 4 and 6 and produces SP (Harmar *et al.*, 1990; Khan and Collins, 1994). Expression of the four mRNAs is both species and tissue dependant (Nakanishi, 1987).

The PPT-B gene consists of nine exons with sequences in exon 5 encoding NKB. Alternative splicing of the PPT-B gene gives rise to α- and β-PPTB mRNA, both of which produce NKB (Kotani *et al.*, 1986; Nawa *et al.*, 1984).

More recently, a putative new member of the PPT family, PPT-C, has been reported to be expressed in murine hematopoietic cells and various human and rat tissues.

Figure 1.5:
Schematic diagram of the functional anatomy of the capsaicin-sensitive primary afferent neuron. Environmental stimulus induces the release of neuropeptides from the peripheral terminals (direct activation, TTX-resistant). Sensory impulses (indicated by red arrows) are then conducted to other peripheral branches of the same neuron (axon reflex, TTX-sensitive), to collaterals in ganglia and to the central endings of the nerves.
(modified from Maggi, 1995b)



Proteolytic cleaving of PPT-C could yield the novel tachykinin HK-1, however the mature peptide has yet to be isolated (Kurtz *et al.*, 2002; Zhang *et al.*, 2000).

1.7.5 Release

It is now recognised that capsaicin-sensitive sensory neurons serve a dual "sensory-efferent" function in that not only do they relay information concerning changes in external environment to the central nervous system but they also have the ability to release, upon adequate stimulation, neuropeptides from their peripheral terminals which can then produce effects in surrounding tissues (Lembeck and Holzer, 1979; Maggi and Meli, 1988; Szolcsanyi, 1983). In peripheral tissues, release of neuropeptides from CSPANs can be mediated by direct activation of the nerve terminal (tetrodotoxin (TTX)-resistant release) as well as by an axon-reflex arrangement (TTX-sensitive release) (Maggi, 1995b) (Figure 1.5).

The concept of the axon-reflex arrangement was first established early last century when it was demonstrated that vasodilation could be initiated by "antidromic conduction" i.e. after mechanical, chemical, thermal or electrical stimulation, sensory impulses are transmitted to other peripheral branches of the same neuron causing release of mediators (Bayliss, 1901; Langley, 1923; Lewis and Marvin, 1927). Release of peptides via the axon-reflex arrangement has been shown to occur in numerous peripheral organs including the uterus and cervix, with Pinter and Szolcsanyi (1995) demonstrating that antidromic stimulation of lumbosacral dorsal roots in the rat leads to plasma extravasation in these tissues and that this was highly reduced after systemic capsaicin treatment, indicating release of transmitters from CSPANs.

The idea that direct activation of the CSPAN by an environmental stimulus can also lead to the release of transmitters is more recent and is based on the observation that responses bought about by stimulants such as capsaicin, histamine, prostanoids, bradykinin and hydrogen ions are resistant to agents which block axonal conduction, such as TTX (Bileviciute *et al.*, 1997; Franco-Cereceda *et al.*, 1987; Geppetti, 1993; Geppetti *et al.*, 1991a; 1991b; Hingtgen and Vasko, 1994; Holzer, 1988; Lembeck and

Holzer, 1979; Maggi *et al.*, 1987; Pinter and Szolcsanyi, 1995; Southall *et al.*, 2002; Szolcsanyi, 1983; White and Helme, 1985).

Much of this information concerning release of neuropeptides from CSPANs has arisen from the use of capsaicin as a tool in sensory neuroscience (reviewed in Holzer, 1991). As mentioned previously capsaicin is the pungent ingredient in a wide variety of peppers from the genus *Capsicum* (Maggi, 1995b) that shows remarkable selectivity for a distinct subset of primary sensory neurons (Holzer, 1991). Capsaicin acts at vanilloid receptors (VRs) expressed along the entire length of these sensory neurons (Szallasi and Blumberg, 1999) and can produce both acute excitatory effects and long-term neurotoxic effects. Initial exposure to capsaicin causes a stimulation of the sensory terminals followed by a subsequent period of desensitization to capsaicin and other stimuli of sensory neurons (Maggi and Meli, 1988). The administration of capsaicin to newborn rats however, causes an irreversible degeneration of primary afferent fibres. (Holzer, 1991) allowing researchers to investigate the functional implications of these nerve fibres.

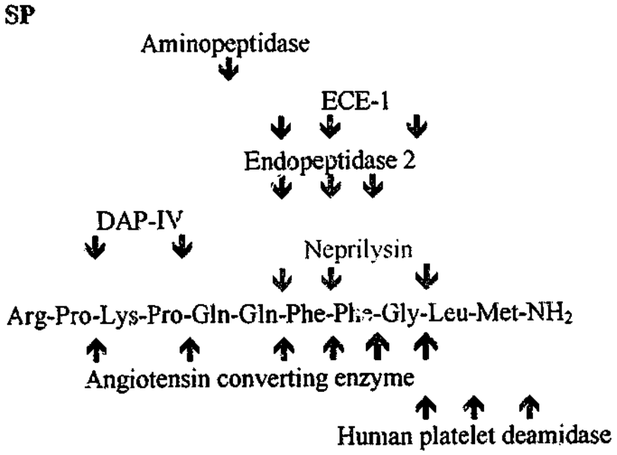
1.7.6 Tachykinin degradation

One of the requisites in accepting that a given substance is a neurotransmitter is that there has to exist a mechanism for its inactivation. Unlike classical neurotransmitters, such as acetylcholine, no re-uptake mechanism exists for tachykinins and the main mechanism for terminating their actions is via peptidase degradation.

In determining whether a given peptidase is involved in the degradation of endogenously released peptides a number of criteria need to be satisfied, namely that the peptidase is present at the target site, that it cleaves the given peptide and that inhibitors of the peptidase enhance the physiological response and the recovery of the peptide. Based on these criteria a number of cell-surface peptidases have been implicated in the degradation of tachykinins including neprilysin, angiotensin converting enzyme (ACE), bestatin-sensitive aminopeptidase, endopeptidase 2, endothelin-converting enzyme (ECE)-1 and human platelet deamidase. These peptidases are anchored to the plasma

Figure 1.6:

Reported sites of enzymatic cleavage of mammalian tachykinins (Ahmad *et al.*, 1992; Cascieri *et al.*, 1984; Heymann and Mentlein, 1978; Hooper *et al.*, 1985; Hooper and Turner, 1985; Jackman *et al.*, 1990; Johnson *et al.*, 1999; Matsas *et al.*, 1984; Michael-Titus *et al.*, 2002; Nau *et al.*, 1986; Skidgel *et al.*, 1984; Stephenson and Kenny, 1988; Wang *et al.*, 1994a).



membrane of cells and have an active site that projects into the extracellular fluid, thus making them ideally placed to degrade tachykinins at the cell surface in the vicinity of their receptors (Grady *et al.*, 1997). All are able to cleave the mammalian tachykinins at numerous sites as indicated in Figure 1.6.

Of these peptidases, neprilysin is believed to be the main enzyme responsible for tachykinin degradation. Neprilysin, previously known as neutral endopeptidase, neutral metalloendopeptidase, EC 3.4.24.11, enkephalinase, CALLA, CD 10 and gp 100 was first discovered in the brush-border membranes of the rabbit kidney (Kerr and Kenny, 1974). Subsequently, it has been found in several tissues, including the brain, gastrointestinal tract, airways and other smooth muscles and is able to be expressed by multiple cell types including neurons, epithelial, endothelial and smooth muscle cells (see Grady *et al.*, 1997; Roques *et al.*, 1993). Neprilysin is able to degrade several peptides, including the tachykinins (Matsas *et al.*, 1983), which, to date, have been reported as being the best substrate for this enzyme (Katayama *et al.*, 1991; Matsas *et al.*, 1984). Neprilysin cleaves peptides at bonds involving the α amino group of hydrophobic residues, such as Leu, Ile, Phe, Val or Tyr (Kerr and Kenny, 1974; Matsas *et al.*, 1983) with the Gly-Leu bond in tachykinins being particularly susceptible (Hooper *et al.*, 1985; Hooper and Turner, 1985; Matsas *et al.*, 1983; 1984). Given that the C-terminal sequence is crucial for activation by the tachykinins of their receptors, degradation at the Gly-Leu bond terminates their biological activity.

Several neprilysin inhibitors are available including thiorphan (Almenoff and Orlowski, 1983; Roques *et al.*, 1993), phosphoramidon (Schwartz *et al.*, 1985) and SCH 39370 (Roques *et al.*, 1993; Sybertz *et al.*, 1989). Only thiorphan will be used in the present study. Thiorphan was the first described, synthetic, potent inhibitor of neprilysin, inhibiting activity of this enzyme by interacting with a hydrophobic pocket (S_1) on the active site of the enzyme, preventing the attachment of substrates (Almenoff and Orlowski, 1983; Roques *et al.*, 1993; Schwartz *et al.*, 1985). Thiorphan has been shown to potentiate the biological actions of tachykinins in numerous systems including human, rabbit and guinea-pig airways (Devillier *et al.*, 1988; Djokic *et al.*, 1989; Frossard and Barnes, 1991; Kondo *et al.*, 1990; Maggi *et al.*, 1990c; Shore and Drazen,

1989), guinea-pig bladder (Maggi *et al.*, 1990a), hamster oral mucosa (Gao and Rubinstein, 1995), human iris (Anderson *et al.*, 1990) and human lymphocytes (Covas *et al.*, 1997).

ACE, also called EC 3.4.15.1, dipeptidyl carboxypeptidase and peptidyl dipeptidase A, is another peptidase that is reported to be important in the degradation of peptides. Although angiotensin I and bradykinin are the preferred substrates for this peptidase (Erdos and Skidgel, 1985), SP is still highly susceptible with ACE hydrolysing SP at the Pro^2-Lys^3, Pro^4-Gln^5, Phe^7-Phe^8 and Phe^8-Gly^9 bonds. The Phe^8-Gly^9 bond is reported to be the initial site of cleavage, which generates the tripeptide amide (Cascieri *et al.*, 1984). This is followed by the successive release of dipeptides (Yokosawa *et al.*, 1983; 1985). The release of C-terminal tripeptides and dipeptides has been reported to occur in a four to one ratio (Skidgel *et al.*, 1984). ACE is unable to cleave either NKA or NKB (Hooper *et al.*, 1985; Hooper and Turner, 1985). ACE activity can be inhibited by captopril (Couture and Regoli, 1981), a molecule based on the active component of venom from a Brazilian snake, which binds to a zinc atom in the active site of the enzyme (for recent review see Opie and Kowolik, 1995). Captopril has previously been shown to be an effective inhibitor of the inactivation of SP by purified ACE in various assays (Cascieri *et al.*, 1984; Couture and Regoli, 1981; Skidgel *et al.*, 1984; Yokosawa *et al.*, 1985).

In addition, NKA, NKB (Hooper and Turner, 1985; Nau *et al.*, 1986) and the SP metabolite SP[5-11] (Wang *et al.*, 1991), but not SP (Palmieri *et al.*, 1985; Ward *et al.*, 1990; Xu *et al.*, 1995), are also substrates for several aminopeptidases. These peptidases can be inhibited by bestatin (Tieku and Hooper, 1992) an inhibitor that contains hydroxyl and carbonyl groups that bind to a zinc atom in the active site of the enzymes (Rich *et al.*, 1984). Hooper and colleagues have shown that though substantial inhibition of NKA and NKB hydrolysis by pig brain striatal synaptic membranes was achieved by incubation with phosphoramidon (1μM), complete inhibition only occurred with phosphoramidon (1μM) in combination with bestatin (0.1mM) (Hooper *et al.*, 1985; Hooper and Turner, 1985; Rich *et al.*, 1984).

SP has also been reported as being a substrate for human platelet deamidase (Jackman *et al.*, 1990), dipeptidylaminopeptidase IV (DAP IV) (Ahmad *et al.*, 1992), endopeptidase 2 (Stephenson and Kenny, 1988) and ECE-1 (Johnson *et al.*, 1999).

It is of interest to note that although peptidase degradation is seen as a mechanism of terminating the actions of peptides, in some cases certain biological actions of the released tachykinins can be maintained or even enhanced by the production of the resulting peptide fragments (Bury and Mashford, 1976; Chipkin *et al.*, 1979; Hall *et al.*, 1989; Igwe *et al.*, 1990; Khan *et al.*, 1998; Konecka *et al.*, 1981; Lei *et al.*, 1991; Piotrowski *et al.*, 1987; Warner *et al.*, 2001). In particular, breakdown at the N-terminal end of the tachykinin peptides is often without effect on their potency (refer to section 1.7.2).

1.8 Tachykinin receptors

1.8.1 Nomenclature and classification

Prior to the discovery of NKA and NKB, it was proposed that two tachykinin receptors existed. This proposal was based on the observations that tachykinins displayed two distinct patterns of activity in various smooth muscle preparations. Either, they were roughly equipotent with an order of agonist potency of physalaemin ≥ SP ~ kassinin ~ eledoisin, or eledoisin was substantially more potent than SP and physalaemin with an order of agonist potency of eledoisin ≥ kassinin ≥ physalaemin ~ SP (Sandberg and Iversen, 1982). The two postulated receptors were therefore termed SP-P and SP-E with physalaemin and eledoisin being the key discriminants (Lee *et al.*, 1982).

Following the discovery of NKA and NKB, Buck *et al.* (1984) proposed a third type of tachykinin receptor. This was based on their observation that in gastrointestinal and urinary bladder smooth muscle there existed a tachykinin binding site that had a pharmacological profile that was distinct from SP-E and SP-P. This receptor was designated SP-K to reflect its preference for NKA. Another suggestion at that the time

also included SP for SP-preferring, SK for NKA-preferring and NK for NKB preferring receptors (Quirion, 1985).

The nomenclature currently used is that put forward during the 1986 meeting "Substance P and Neurokinins" held in Montreal. An agreement was reached such that the receptors were termed neurokinin $(NK)_1$, NK_2 and NK_3, with an order of agonist potency of SP>NKA>NKB at NK_1 receptors; NKA>NKB>SP at NK_2 receptors and NKB>NKA>SP at NK_3 receptors (Henry, 1986). Molecular cloning has since confirmed the existence of three tachykinin receptor types (Buell *et al.*, 1992; Gerard and Gerard, 1991; Hershey *et al.*, 1991; Ingi *et al.*, 1991; Masu *et al.*, 1987; Shigemoto *et al.*, 1990; Yokota *et al.*, 1989).

To date, some authors are not satisfied with this nomenclature arguing that, firstly, NK was not originally meant as an abbreviation for neurokinin as SP is also expressed in non-neuronal cell types and, secondly, that the terms tachykinin receptors and neurokinin receptors have been used interchangeably in the literature (Maggi, 2000b). This was discussed at both the "10th Meeting of the European Neuropeptide Club" (Innsbruck, Austria, 2000) and "Tachykinins 2000" (La Grand Motte, France, 2000), where it was decided that the Montreal nomenclature should be adhered to (Maggi, 2001).

There have been proposals that the tachykinin receptor classification should be extended to include NK_4, a novel receptor expressed in some human tissues that shares 81% sequence homology with the human NK_3 receptor (Donaldson *et al.*, 1996; Xie *et al.*, 1992). The existence of this putative receptor is still contentious with neither Sarau *et al.* (2000) nor Page and Bell (2002) able to demonstrate its presence or expression in numerous species including the human. Page and Bell (2002) went as far as to speculate that the human NK_4 receptor is in fact the guinea-pig NK_3 receptor and that Xie and colleagues somehow managed to cross contaminate their isolated human cDNA with that of the guinea-pig.

1.8.2 Receptor structure

The three tachykinin receptor types belong to the "super family" of rhodopsin-like G-protein coupled receptors (Maggi, 1995a). However, unlike many other genes for this family the tachykinin receptor genes differ in that they contain introns, which are believed to define several important functional units of this receptor family (Takahashi *et al.*, 1992). The tachykinin receptors all have a similar structure consisting of an extracellular amine terminal and an intracellular carboxy terminal bridged by seven domains spanning the cell membrane (Strader *et al.*, 1994) as shown in Figure 1.7. Sequence homology between the three receptors varies from species to species, with human tachykinin receptors having approximately 40% sequence homology (Gerard *et al.*, 1993) while the rat has 54-66% (Shigemoto *et al.*, 1990). Between species, sequence homology tends to be greatest for the NK_1 receptor though homology for the NK_2 and NK_3 receptors is still high (Khawaja and Rogers, 1996) (for further discussion refer to section 1.8.7).

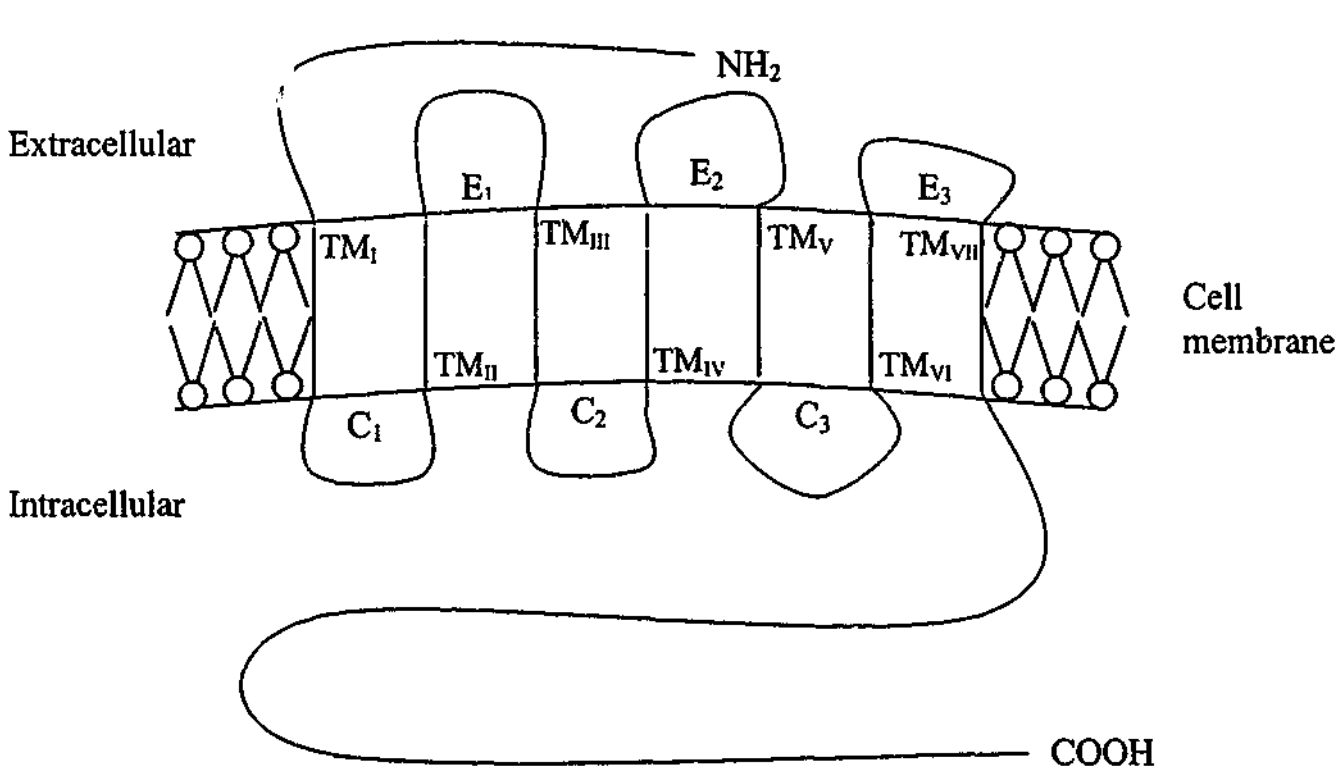
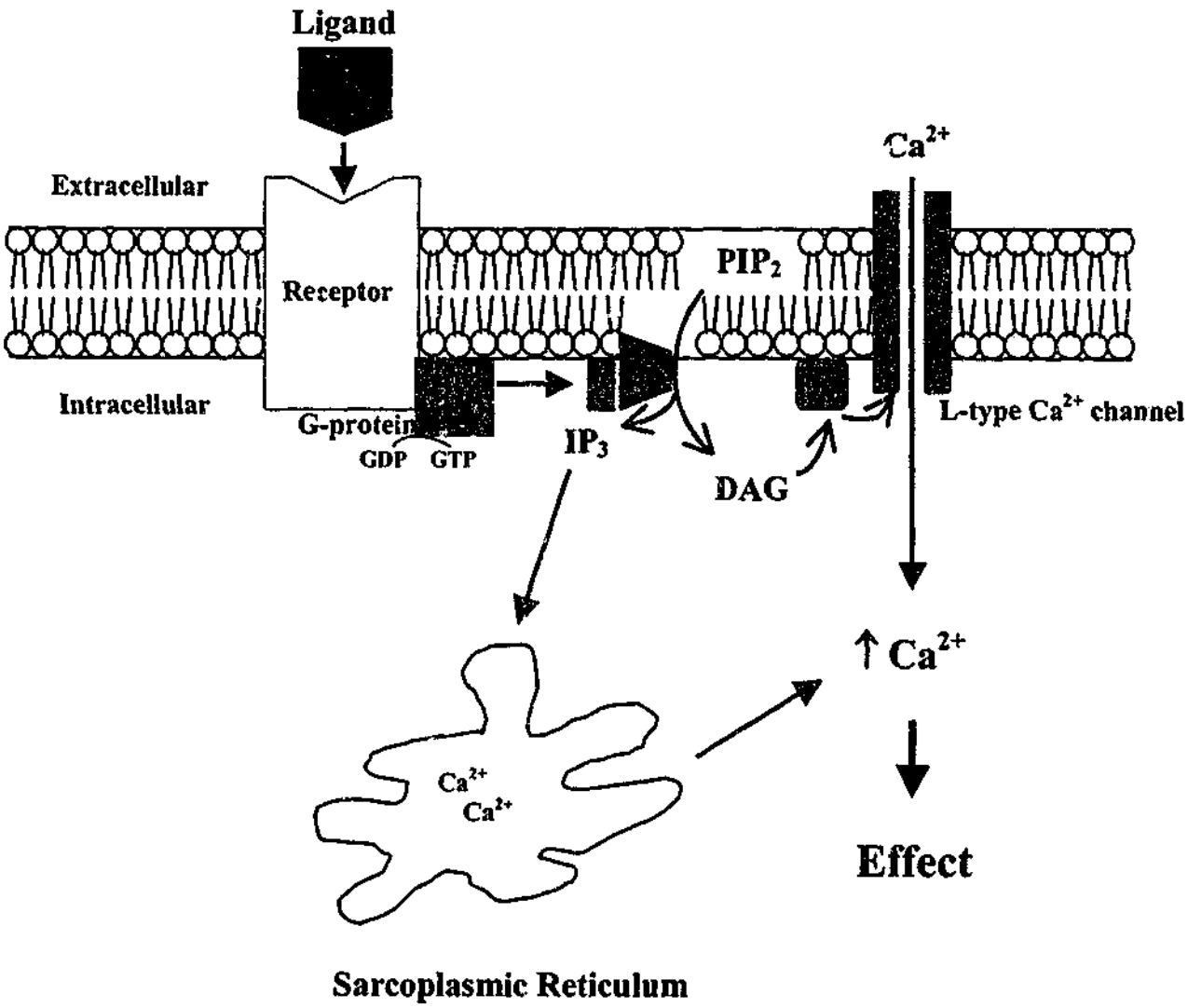


Figure 1.7: Schematic diagram of the primary structure of tachykinin receptors. $E_1 - E_3$ designate the extracellular loops, C_1-C_3 designate the cytoplasmic loops and TM_I-TM_{VII} denote the seven transmembrane segments (modified from Khawaja and Rogers, 1996).

Figure 1.8:

Schematic diagram showing the second messenger system for tachykinin peptides (modified from Khawaja and Rogers, 1996).



The presence of a short and long isoform of the NK_1 receptor has been reported in various tissues in the human (Fong *et al.*, 1992a; Goode *et al.*, 2000; Richardson *et al.*, 2003) and rat (Mantyh *et al.*, 1996; Raddatz *et al.*, 1995). The two isoforms differ in the length of their cytoplasmic carboxy terminal tails with the short isoform lacking the C-terminus phosphorylation sites and a putative palmitoylation site (Fong *et al.*, 1992a). As the C-terminus is believed to play a key role in G protein-coupled receptor desensitization it is suggested that the truncated isoform of the NK_1 receptor would have impaired desensitization (Richardson *et al.*, 2003). However, the precise role of the C-terminus in NK_1 receptor desensitization remains unclear.

Numerous studies have been undertaken to determine the binding sites of tachykinins, with evidence suggesting that the natural tachykinins bind to sites that are not distinct but overlap (Strader *et al.*, 1994). All three receptors possess conserved amino acid sequences on the amino terminal and first intracellular loop that are thought to be important for interacting with the common C-terminal of natural tachykinins (Watling and Krause, 1993). A conserved sequence on the third extracellular loop also confers high affinity binding of the tachykinins (Fong *et al.*, 1992b). Differences are seen though in the binding sites of the preferred ligand for each receptor type.

1.8.3 Second messengers

As with other members of the rhodopsin-like G-protein "super family", phospholipase C (PLC) is believed to be a principal intracellular effector for tachykinin receptors (Khawaja and Rogers, 1996). Briefly, G-proteins are composed of three distinct subunits termed α, β, and γ which are joined together at rest. Binding of a ligand causes the α subunit of the G-protein to disassociate and activate the intracellular enzyme PLC. PLC hydrolyses phosphatidyl inositol bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 acts on specific receptors on the sarcoplasmic reticulum to cause release of calcium from intracellular stores, while DAG also causes increases in intracellular calcium by acting on protein kinase C (PKC) to open L-type calcium channels in the plasma membrane. This increase in calcium then leads to a biological response taking place (see Figure 1.8).

Although PLC is thought to be the main intracellular effector, tachykinin receptor activation can also be coupled to both adenyl cyclase activation (Narumi and Maki, 1978; Yamashita *et al.*, 1983) and to adenyl cyclase inhibition (Laniyonu *et al.*, 1988). Tachykinins also play a major role in the induction of endothelium-dependent vasodilation, whereby activation of NK_1 receptors localized on endothelial cells leads to nitric oxide (NO) generation (for review see Maggi, 1995a). This indicates that, depending on the cell type, tachykinins can elicit their effects by activating multiple effectors.

1.8.4 Structure activity relationships

All natural tachykinins can act as full agonists at the three known tachykinin receptors (Maggi *et al.*, 1993b). Though the tachykinins share a similar spectrum of biological activities they can be distinguished by their relative potencies across various bioassays. The major determinant of this is the nature of the amino acid at position 4 from the C-terminus. If the amino acid is aromatic (Phe, Tyr), such as in physalaemin then selectivity for the NK_1 receptor is increased. Conversely, if the amino acid is aliphatic (Val, Ile), such as in eledoisin, NKA and NKB then selectivity is increased for the NK_2/NK_3 receptors (Severini *et al.*, 2000) (refer to Figure 1.3).

Other residues also important in influencing receptor selectivity include the residues that occupy positions 6 and 7 from the C-terminus (Munekata *et al.*, 1987; Severini *et al.*, 2000) and the presence of a Pro residue in the N-terminal sequence (Cascieri *et al.*, 1992). A basic or neutral residue at position 7 from the C-terminus, such as is present in SP, increases affinity for the NK_1 receptor while acidic residues, such as in NKA and NKB, increase affinity for the NK_2 and NK_3 receptors (Osakada *et al.*, 1986; Regoli *et al.*, 1984; Severini *et al.*, 2000). The residue at position 6 from the C-terminus is important for differentiating NKA and NKB from each other (Munekata *et al.* (1988), cited in Comis and Burcher (1999); Munekata, 1991) and if the peptide has an acidic residue at both positions 6 and 7 from the C-terminus than preference for the NK_3 over the NK_2 receptor is shown (Severini *et al.*, 2000). The presence of a Phe residue in position 6 from the C-terminus in NKA is also important for both affinity and potency

of this peptide (Warner *et al.*, 2001). SP and some of the non-mammalian tachykinins have a Pro residue in the N-terminal moiety of the peptide sequence, usually in either position 8 or 10 from the C-terminus (Severini *et al.*, 2000). It has been suggested that tachykinins with a Pro at position 8 from the C-terminus, such as SP, physalaemin and uperolein, have reduced affinity for NK_2 and NK_3 receptors (Cascieri *et al.*, 1992).

1.8.5 Receptor-selective agonists

The ability of the mammalian tachykinins SP, NKA and NKB to act at all three tachykinin receptors (Maggi *et al.*, 1993b) has made receptor characterization problematic, thus prompting the need for receptor-selective agonists. Several tachykinin receptor-selective agonists have been developed for the three tachykinin receptors and all are based on alterations of the amino acid sequence of the parent peptide, particularly at the common C-terminal (Regoli *et al.*, 1988). In the present study the respective NK_1, NK_2 and NK_3 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP (Drapeau *et al.*, 1987), [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) (Chassaing *et al.*, 1991) and [$MePhe^7$]NKB (Drapeau *et al.*, 1987) were used.

The development of [$Sar^9Met(O_2)^{11}$]SP was instigated as a result of the discovery by Drapeau and colleagues that replacing Gly^8 with Sar improved the affinity of NKB(4-10) and [$MePhe^7$]NKB in the dog carotid artery (an NK_1 receptor assay) (Drapeau *et al.*, 1987). The potency and selectivity of [Sar^9]SP for NK_1 receptors was then further improved by oxidizing Met^{11} to $Met(O_2)$. The resulting [$Sar^9Met(O_2)^{11}$]SP was shown to be a potent agonist on the dog carotid artery but inactive on both the rabbit pulmonary artery (NK_2 receptor assay) and rat portal vein (NK_3 receptor assay) (Drapeau *et al.*, 1987).

[$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) was developed as the result of two different observations. Drapeau and colleagues had reported that the reduction of NKA by three residues from the N-terminal end combined with the replacement of Met^{10} with Nle, which resulted in [Nle^{10}]NKA(4-10), conferred selectivity for the NK_2 over NK_1 receptor (Drapeau *et al.*, 1987). Meanwhile, Chassaing and colleagues had noticed that

scyliorhinin I, a non-mammalian tachykinin isolated from the dogfish, which has a Lys in position 5, was as potent as NKA at NK_2 receptors (Lavielle *et al.*, 1990). Binding studies showed that [Lys^5]NKA(4-10) was indeed highly selective for NK_2 receptors but this selectivity was not seen in functional experiments (Lavielle *et al.*, 1990). However, further studies showed that N-methylation of Leu^9 increased the potency of this compound. When these two structural changes were incorporated together the resultant [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) showed a high selectivity for NK_2 receptors in both binding and functional studies (Chassaing *et al.*, 1991) while displaying reduced activity in the guinea-pig ileum (NK_1 receptor assay) and rat portal vein (NK_3 receptor assay) (Chassaing *et al.*, 1991).

[$MePhe^7$]NKB was developed after it was shown that selectivity and affinity for NK_3 receptors in the rat portal vein was markedly increased when Val^7 was replaced with MePhe (Drapeau *et al.*, 1987). [$MePhe^7$]NKB shows high selectivity for NK_3 receptors while displaying reduced activity in the dog carotid artery (NK_1 receptor assay) and rabbit pulmonary artery (NK_2 receptor assay) (Regoli *et al.*, 1987).

It is suggested that synthetic analogues may be less susceptible to enzymatic degradation than the natural tachykinins. In keeping with this suggestion [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) has been reported as being a metabolically stable agonist (Fisher and Pennefather, 1997; Fisher *et al.*, 1993). In contrast, the response to [$Sar^9Met(O_2)^{11}$]SP in the isolated rat uterus was potentiated in the presence of the neprilysin inhibitor SCH 39370 (3μM) and captopril (10μM) (Fisher and Pennefather, 1997). The response to [$MePhe^7$]NKB in the isolated guinea-pig oesophagus was also seen to be significantly enhanced in the combined presence of thiorphan (1μM), captopril (1μM) and the aminopeptidase inhibitor amastatin (20μM) (Kerr *et al.*, 1997). These findings indicate that both [$Sar^9Met(O_2)^{11}$]SP and [$MePhe^7$]NKB may be susceptible to peptidase degradation.

1.8.6 Tachykinin antagonists

1.8.6.1 Discovery and development

Knowledge of tachykinin receptor types and elucidation of their physiological roles has further increased with the advent of tachykinin receptor-selective antagonists. The search for tachykinin antagonists began in the early 70's and by the early 80's, what were later to be called "first generation" antagonists, had been developed. These were peptide analogues based upon the amino acid sequence of SP where D-amino acids were substituted for L-amino acids on the SP backbone. The first of these was [D-Pro^2, D-Trp^{7,9}]SP (Leander *et al.*, 1981), and though it was reported to antagonise SP-induced responses in numerous preparations (Bjorkroth *et al.*, 1982; Holmdahl *et al.*, 1981) it was also shown to have neurotoxic side effects (Hokfelt *et al.*, 1981). This led to the development of other, less toxic compounds such as [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]SP(1-11), [D-Pro^2, D-Trp^{7,9,10}]SP(4-11) and spantide or [D-Arg^1, D-Trp^{7,9}, Leu^{11}]SP (1-11) (Akerman *et al.*, 1982; Bjorkroth *et al.*, 1982; Folkers *et al.*, 1984; Rosell *et al.*, 1983). As well as the aforementioned neurotoxicity (Hokfelt *et al.*, 1981; Post and Paulsson, 1985; Rodriguez *et al.*, 1983), it was later revealed that the first generation antagonists suffered from many other shortcomings including poor selectivity (Buck and Shatzer, 1988), partial agonist activity (Folkers *et al.*, 1984), local anaesthetic activity (Post *et al.*, 1985) and antagonism of peptides unrelated to the tachykinins (Fabregat and Rozengurt, 1990; Jensen *et al.*, 1988; Yachnis *et al.*, 1984).

Over the next few years, "second generation" antagonists were developed. These were still peptide in nature but were more potent and selective compared to the first generation antagonists, and took into account the recent classification of tachykinin NK_1 and NK_2 receptors. Examples include the NK_1 receptor-selective antagonists L 668,169, spantide II, GR 82,334 and FK 888 and the NK_2 receptor-selective antagonists L 659,877, MEN 10,207 and R 396 (Maggi *et al.*, 1993b). Although second generation antagonists had overcome many of the problems associated with first generation antagonists they still had drawbacks, including the fact that their peptide nature made them susceptible to enzymatic degradation and that their high molecular weight made them expensive to synthesize.

"Third generation" antagonists are non-peptide compounds. The first one developed was the Pfizer compound CP 96,345 (Snider *et al.*, 1991). CP 96,345 is a potent NK_1 receptor-selective antagonist (McLean *et al.*, 1991) but was subsequently found not only to bind to L-type calcium channels (Schmidt *et al.*, 1992) but also to exhibit local anaesthetic activity (Wang *et al.*, 1994b). Newer tachykinin antagonists have since been synthesized that display fewer non-selective actions and shortcomings than their predecessors. These include the Sanofi Recherche compounds SR 140333, SR 48968 and SR 142801, the respective NK_1, NK_2 and NK_3 receptor-selective antagonists used in the present study, that are discussed in detail below.

1.8.6.2 SR 140333

SR 140333 (1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl}-4-phenyl-1-azonia-bicyclo[2.2.2]octane, chloride) is a non-peptide NK_1 antagonist, the structure of which is shown below (Emonds-Alt *et al.*, 1993b). SR 140333 has advantages over other non-peptide NK_1 receptor antagonists in that it appears not to display species dependency nor does it discriminate between the classical and septide-like NK_1 receptor agonists or interact with Ca^{2+} channels (Emonds-Alt *et al.*, 1993b; Jensen *et al.*, 1994).

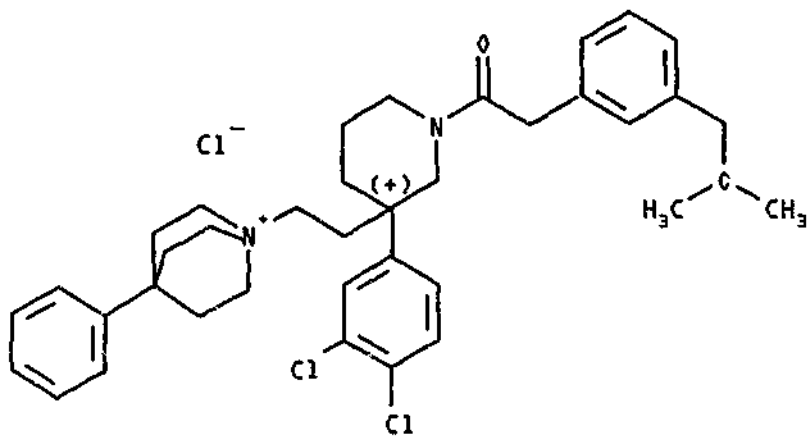


Figure 1.9: Structure of SR 140333

In binding studies, SR 140333 was reported to be a potent competitive inhibitor of the binding of SP to NK_1 receptors from various species including humans. It was further found that SR 140333 did not significantly inhibit the binding of either NKA to rat NK_2 receptors or [MePhe^7]NKB to rat NK_3 receptors; it also lacked activity in binding assays for several other receptors (Emonds-Alt *et al.*, 1993b).

In vitro studies have shown SR 140333 to be a potent NK_1 receptor antagonist with pA_2 values of 9.7 versus [Sar^{9}Met$(O_2)^{11}$]SP and 9.9 versus septide in the guinea-pig ileum (NK_1 receptor assay). However, the antagonism exerted by SR 140333 toward NK_1 receptors in the guinea-pig ileum was apparently non-competitive (Croci *et al.*, 1995a; Emonds-Alt *et al.*, 1993b). SR 140333, at concentrations up to 1μM had no effect on [β-Ala^8]NKA-induced contractions of rabbit pulmonary artery (NK_2 receptor assay) or [MePhe^7]NKB-induced contractions of rat portal vein (NK_3 receptor assay) (Emonds-Alt *et al.*, 1993b). SR 140333 displayed no partial agonist activity in any of these assays.

SR 140333 has also been reported as being a potent and long lasting selective antagonist of NK_1 receptors in the central nervous system. Given intravenously, SR 140333 dose-dependently inhibited [Sar^{9}Met$(O_2)^{11}$]SP-induced hypotension in the anaesthetised dog and antagonised [Sar^{9}Met$(O_2)^{11}$]SP-induced but not [Nle^{10}]NKA(4-10)-induced bronchoconstriction in the anaesthetised guinea-pig (Emonds-Alt *et al.*, 1993b). Given intraperitoneally, SR 140333 antagonised scratching responses in mice induced by intracerebroventricular SP and septide, and turning behaviour elicited by intrastriatal SP and septide, but interestingly had no effect on scratching and turning responses elicited by [Sar^{9}Met$(O_2)^{11}$]SP (Jung *et al.*, 1994). SR 140333 also reduced SP-, [Sar^{9}Met$(O_2)^{11}$]SP- and septide-induced salivation in rats (Jung *et al.*, 1994).

1.8.6.3 SR 48968

SR 48968 ((S)-N-methyl-N[4-acetylamino-4 phenylpiperidino)-2-(3,4-dichlorophenyl) butyl] benzamide), the structure of which is shown below, was the first non-peptide NK_2 receptor antagonist developed (Advenier *et al.*, 1992a).

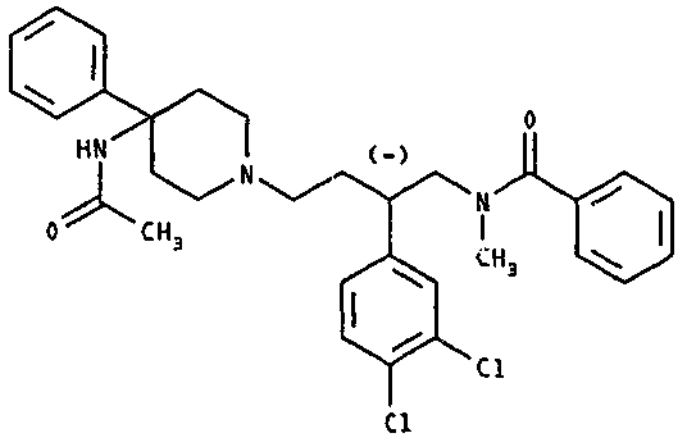


Figure 1.10: Structure of SR 48968

Binding studies have shown that SR 48968 bound in a reversible and concentration-dependent manner to a single class of high affinity binding sites (Emonds-Alt *et al.*, 1992b). SR 48968 competitively inhibited NKA from binding to NK_2 receptors in the rat duodenum and hamster urinary bladder (Boyle *et al.*, 1993) but did not significantly inhibit binding of SP from either rat cortex membranes or human IM9 cells (NK_1 receptor binding assays), or eledoisin and senktide from rat cortex membranes (NK_3 receptor assays) (Advenier *et al.*, 1992a; 1992c; Boyle *et al.*, 1993; Emonds-Alt *et al.*, 1992a; 1992b). However, appreciable affinity for NK_3 receptors was reported in both the guinea-pig (Boyle *et al.*, 1993; Petitet *et al.*, 1993) and human (Chung *et al.*, 1994), indicating a species-dependent lack of receptor selectivity.

In vitro studies demonstrated that SR 48968 behaved as a competitive and potent antagonist on most preparations studied. pA_2 values were obtained in different preparations from rabbits (9.8-10.5), guinea-pigs (9.5-10.5), rats (9.0-9.6), hamsters (7.5-8.6) and humans (9.2-9.6) (Advenier *et al.*, 1992a; 1992b; 1992c; Boyle, 1993; Emonds-Alt *et al.*, 1992b; Maggi *et al.*, 1993a). However, SR 48968 non-competitively

antagonised the response to [β-Ala^8]NKA(4-10) and NKA in the rat oesophagus (Croci *et al.*, 1995a). SR 48968 was inactive at concentrations up to 3μM in the guinea-pig ileum (NK_1 receptor assay) and rat portal vein (NK_3 receptor assay) (Emonds-Alt *et al.*, 1992b; Maggi *et al.*, 1993b). In these preparations SR 48968 was inactive as a stimulant or relaxant (Advenier *et al.*, 1992a; 1992b; 1992c). In addition SR 48968 was without effect on responses to bradykinin, angiotensin, bombesin, acetylcholine, histamine, potassium chloride and prostaglandin $F_{2\alpha}$ (Advenier *et al.*, 1992a; 1992b; 1992c).

As seen with the binding studies a species-dependent effect of SR 48968 at NK_3 receptors was also reported with functional experiments. SR 48968 (0.1-10μM) was without significant activity at NK_3 receptors in the rat portal vein (Advenier *et al.*, 1992a) but non-competitively antagonised the response to senktide at NK_3 receptors in the guinea-pig ileum (Boyle *et al.*, 1993). In addition, SR 48968 inhibited [Pro^7]-NKB- and senktide-increased inositol phospholipid turnover in cells transfected with human NK_3 receptor cDNA and at NK_3 receptors in guinea-pig ileum slices respectively (Petitet *et al.*, 1993).

SR 48968 also has a limited ability to discriminate between species variants of the NK_2 receptor, with results from functional and binding experiments indicating a slightly higher affinity for putative NK_{2A} receptors than NK_{2B} receptors (see section 1.8.7) (Emonds-Alt *et al.*, 1993c; Maggi *et al.*, 1993a).

SR 48968 is highly active *in vivo*, potently antagonizing NK_2 receptor-mediated effects in the periphery and central nervous systems when administered intravenously, intraperitoneally, intraduodenally, subcutaneously and orally (Advenier *et al.*, 1992c; Emonds-Alt *et al.*, 1993a; Emonds-Alt *et al.*, 1992b; Lau *et al.*, 1993).

The nature of the interaction of SR 48968 with NK_2 receptors is not altogether clear. Firstly, in some preparations the longer the period of contact with the tissue the higher the affinity (Advenier *et al.*, 1992c). Secondly, the blockade is slowly reversible *in vitro* (Advenier *et al.*, 1992c; Maggi *et al.*, 1993b), with the exception of NK_2 receptors in the

guinea-pig isolated gall bladder and proximal colon where it was irreversible (Patacchini *et al.*, 1994) and was found to be long lasting *in vivo* (Emonds-Alt *et al.*, 1992b; Maggi *et al.*, 1993b).

1.8.6.4 SR 142801

SR 142801 (S)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylpiperidin-4-yl)-N-methylacetamide, the structure of which is shown below, was the first highly potent non-peptide antagonist for NK_3 receptor (Emonds-Alt *et al.*, 1995).

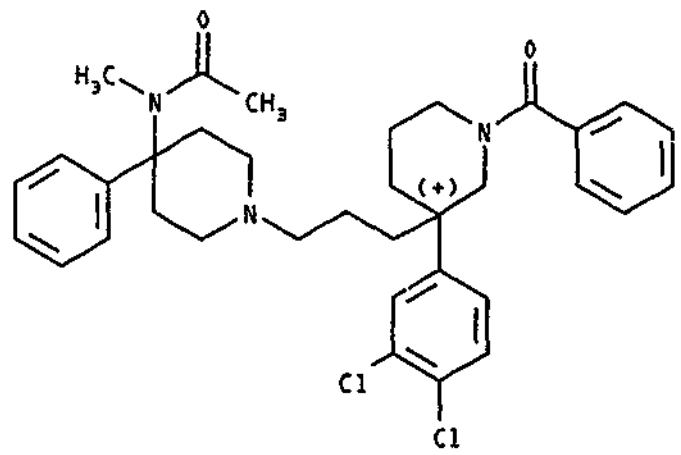


Figure 1.11: Structure of SR 142801

Binding studies have shown that SR 142801 potently inhibited the binding of [MePhe^7]NKB to NK_3 receptors from various species including humans, gerbils, guinea-pigs and rats, though was markedly less potent in the rat than the other species. SR 142801 only slightly inhibited the binding of SP and NKA to NK_1 and NK_2 receptors respectively, and lacked activity at other receptors except for calcium (verapamil site) and sodium (site 2) channels and opiate (binding of naloxone) receptors where IC_{50} values were in the range of 0.1 to 1μM (Beaujouan *et al.*, 1997; Emonds-Alt *et al.*, 1995).

Selectivity for NK_3 receptors has also been reported *in vitro*. SR 142801 inhibited both [MePhe^7]NKB- and senktide-stimulated inositol monophosphate formation in Chinese hamster ovary cells transfected with the human NK_3 receptor (Oury-Donat *et al.*, 1995) and inhibited NK_3 mediated acetylcholine release in the guinea-pig ileum (Emonds-Alt *et al.*, 1995). SR 142801 produced an apparently competitive antagonism of [MePhe^7]NKB-induced contractions of the guinea-pig ileum, which assuming competitive antagonism yielded an apparent pA_2 of 9.2. SR 142801 did not significantly antagonise [Sar^{9}Met$(O_2)^{11}$]SP-induced endothelium-dependent relaxation of the rabbit pulmonary artery or [βAla^8]NKA(4-10)-induced contraction of the denuded rabbit pulmonary artery, demonstrating a lack of effect upon NK_1 and NK_2 receptors respectively (Emonds-Alt *et al.*, 1995). As with the binding studies, apparent species dependency was also seen, with SR 142801 competitively antagonising [MePhe^7]NKB-induced contractions of the guinea-pig ileum while exhibiting a low pA_2 in the rat portal vein (Croci *et al.*, 1995b; Emonds-Alt *et al.*, 1995). *In vitro*, SR 142801 has a slow onset of action, 140 minutes at low concentrations and 60-80 minutes at higher concentrations, which is thought to be due either to a slow interaction with the receptor protein or slow penetration of the receptor compartment (Nguyen-Le *et al.*, 1996).

When administered *in vivo*, SR 142801 dose-dependently inhibited senktide-induced turning behavior in gerbils when administered either orally or intraperitoneally (Emonds-Alt *et al.*, 1995) and failed to inhibit either [Sar^{9}Met$(O_2)^{11}$]SP-induced hypotension in dogs or [Nle^{10}]NKA(4-10)-induced bronchoconstriction in anaesthetised guinea-pigs when administered either intravenously or intraperitoneally (Emonds-Alt *et al.*, 1995).

1.8.7 Species Heterogeneity

Species-related heterogeneity has been observed for all three tachykinin receptors and stems mostly from work conducted with receptor-selective antagonists. Much of this research has been on the tachykinin NK_1 receptor and the differing affinities seen for the two NK_1 receptor-selective antagonists CP 96345 and RP 67580 amongst various

species. CP 96345 was demonstrated to possess a 100-fold higher affinity for NK_1 receptors expressed in bovine, guinea-pig, rabbit, hamster and human tissues than NK_1 receptors expressed in rat, mouse or chicken tissues (Garret *et al.*, 1991; Gitter *et al.*, 1991; Snider *et al.*, 1991). The opposite pattern of affinities was seen with RP 67580 (Maggi *et al.*, 1993b).

Point mutations studies have since confirmed the existence of NK_1 receptor heterogeneity with Fong *et al.* (1992c) showing that the variable potencies of RP 67580 and CP 96345 on human and rat NK_1 receptors was due to species-dependent variations at positions 116 and 290 of the NK_1 receptor protein. Replacing residues 116 and 290 (Val and Ile) of the human NK_1 receptor with the corresponding residues (Leu and Ser) from the rat NK_1 receptor conferred full sensitivity to RP 67580 while decreasing affinity for CP 96345 while the exchange of residues from the human to the rat NK_1 receptor produced the opposite effect. Fong *et al.* (1992c) noted that the exchange of these residues did not affect the binding of SP indicating that the agonist and antagonist binding sites differed.

The existence of a "septide-sensitive" NK_1 receptor subtype has also been proposed based on the observation that septide was equipotent with SP in contracting the guinea-pig ileum yet was much less effective than SP at displacing SP sulfone in binding studies conducted on guinea-pig ileum membranes (Petitet *et al.*, 1992). The results of two recent studies suggest that septide is not acting at a different receptor to SP, rather that it is binding to a different site on the NK_1 receptor. Wijkhuisen *et al.* (1999) showed that two simultaneous point mutations in the 193-197 region of human NK_1 receptors expressed in COS-7 cells abolished the high functional activities of septide but did not affect SP. Furthermore, Torrens *et al.* (2000) demonstrated that in rat CHO cells transfected with NK_1 receptor cDNA and human astrocytoma U373MG and mouse cortical astrocyte cell lines, where only NK_1 receptors are expressed, NKA binding sites were different from SP and that septide, NKA and NKB had a much higher affinity for the NKA binding sites.

Similar species heterogeneity is also seen with the tachykinin NK_2 receptor. MEN 10207 (NK_2 receptor-selective antagonist) was reported to have a higher affinity for

NK_2 receptors in the endothelium-denuded rabbit pulmonary artery than in the isolated hamster trachea, while the opposite was seen with two other NK_2 receptor-selective antagonists, R 396 and L 659877 (Maggi *et al.*, 1990b; Patacchini *et al.*, 1991). Maggi *et al.* (1993a; 1993b) proposed that NK_2 receptors fell into two categories designated NK_{2A} and NK_{2B}. Rabbit, guinea-pig, bovine and human NK_2 receptors were designated as 2A whil[illegible] and hamster were 2B. Examination of the primary sequence of NK_2 receptors f[illegible] various species revealed that NK_2 receptors from the rat, hamster and mouse w[illegible]deed more similar to one another than to human and bovine NK_2 receptors (Maggi, 1994).

Intraspecies variations of NK_2 receptors have also been proposed. Nimmo *et al.* (1992) reported that the rat urinary bladder contained two different NK_2 receptor populations, with epithelial NK_2 receptors showing a high affinity for MEN 10207 while those from the smooth muscle demonstrated a much lower affinity for the same antagonist. The expression of mRNA for two types of NK_2 receptors in both the human and rat uterus and other rat tissues has also been reported (Candenas *et al.*, 2002). These receptors have provisionally been termed $NK_{2\alpha}$ and $NK_{2\beta}$, (Candenas *et al.*, 2002).

Species heterogeneity of the NK_3 receptor was firstly shown when SR 48968 was reported to have a higher affinity for guinea-pig and human than rat NK_3 receptors (Chung *et al.*, 1994; Petitet *et al.*, 1993). This was later confirmed with SR 142801 also showing a higher affinity for human, gerbil and guinea-pig NK_3 receptors than rat in both binding and functional experiments (Beaujouan *et al.*, 1997; Emonds-Alt *et al.*, 1995; Nguyen-Le *et al.*, 1996; Patacchini *et al.*, 1995).

1.9 Tachykinins and the uterus

1.9.1 Occurrence and distribution

The first report of tachykinin–immunoreactive (IR) nerves in the female genital tract was by Alm *et al.* (1978) who observed the presence of SP-IR nerves beneath the surface epithelium, around blood vessels and following smooth muscle fibres in the

guinea-pig uterus. Later studies conducted by Huang *et al.* (1984) and Heinrich *et al.* (1986) also demonstrated the presence of SP-IR fibres in the guinea-pig uterus. Alm and Lundberg (1988) subsequently showed that these SP-IR fibres coexisted with NKA. Tachykinin-IR nerves have since been reported in the uterus and cervix of numerous species including the mouse (Huang *et al.*, 1984), cat (Huang *et al.*, 1984), horse (Bae *et al.*, 2001), pig (Czaja *et al.*, 1996), cow (Lakomy *et al.*, 1995) and human (Butler-Manuel *et al.*, 2002; Franco-Cereceda *et al.*, 1987; Fried *et al.*, 1990; Heinrich *et al.*, 1986; Reinecke *et al.*, 1989; Samuelson *et al.*, 1985). In these species a similar pattern of distribution is usually observed in that the tachykinin-IR fibres are quite sparse and are seen to be associated with the smooth muscle, blood vessels and endometrial glands.

To date, the majority of work on tachykinin-immunoreactivity in the female reproductive tract has been conducted using the rat uterus. In this species the presence of both SP- and NKA-immunoreactivity has been demonstrated in CSPANs supplying the endometrium, myometrium and vasculature of the uterus, as well as being present in the ovaries, cervix and paracervical ganglia (Papka *et al.*, 1985; 1987; Papka and Traurig, 1993; Shew *et al.*, 1991; Springall *et al.*, 1984; Traurig *et al.*, 1984; 1991; Traurig and Papka, 1993).

It is still not clear whether the CSPANs or their tachykinin-IR content are affected by hormonal changes or pregnancy as is observed with noradrenergic and cholinergic nerves (Sjöberg, 1968). In the guinea-pig, Alm and Lundberg (1988) reported the disappearance of neuropeptide-IR from the uterus at full term pregnancy. In contrast, Mione *et al.* (1990) have reported that there was no difference in the pattern of SP-IR nerve fibres observed in uterine artery preparations obtained from virgin and late-pregnant guinea-pigs. In the rat, Traurig *et al.* (1984) observed no change in the SP-IR content of the uterus during the oestrous cycle, after ovariectomy or during pregnancy, while Amira *et al.* (1995) reported an increase in the uterine content of SP-immunoreactivity during gestation.

In addition to tachykinins being present in the primary afferent neurons of the female reproductive tract SP has also been demonstrated in the human placenta, where it was

observed in decidual cells (Sastry *et al.*, 1981) and amniotic fluid from mid-trimester onwards (Sanfilippo *et al.*, 1992). The presence of SP in neuroendocrine cells present on the epithelial surface of the urogenital tract was also reported (Skrabanek and Powell, 1983), although no SP-IR neuroendocrine cells were able to be demonstrated in the porcine urogenital tract (Czaja *et al.*, 1996). More recently, Page *et al.* (2000) have reported the presence of NKB in the outer syncytiotrophoblasts of the human placenta. This finding is of interest as NKB had previously been undetectable in peripheral tissues (Moussaoui *et al.*, 1992) other than the enteric nerves of the rat ileum (Yunker *et al.*, 1999). Molecular studies have since also reported the expression of the PPT-B gene, which encodes for NKB, in the both the rat and human uterus, however, in both these species its precise cellular localization was not determined (Cintado *et al.*, 2001; Patak *et al.*, 2003; Pinto *et al.*, 2001).

1.9.2 Actions

Tachykinins have been implicated in various processes of reproductive function, however, a detailed discussion of their actions other than that on myometrial smooth muscle is beyond the scope of this thesis. Briefly, available evidence suggests that in the female reproductive tract neuropeptides are released from peripheral nerve endings following sensory nerve activation and participate in the regulation of blood flow, glandular secretions and myometrial contractility (Traurig and Papka, 1993). There have been several studies describing the effects of tachykinins on blood flow, in which SP was shown to relax human intramyometrial and uterine arteries. This suggests a possible role for this peptide in the regulation of uteroplacental blood flow during pregnancy (Bodelsson and Stjernquist, 1992; Hansen *et al.*, 1988; Skrabanek *et al.*, 1980). SP has also been reported to be involved in neuro-immune interactions resulting in stress-induced abortion in rodents (Arck *et al.*, 1995; Cocchiara *et al.*, 1995; 1997; Markert *et al.*, 1997) and cervical ripening in rats (Collins *et al.*, 2002). In addition to releasing neuropeptides from their peripheral terminals, sensory neurons also relay information to the spinal cord and brainstem initiating visceral reflexes important for homeostasis and reproduction (Traurig and Papka, 1993).

The female reproductive tract is one of the few peripheral tissues in which NKB is expressed. In a study by Page *et al.* (2000) NKB levels in the maternal circulation were found to be grossly elevated during pre-eclampsia and pregnancy-induced hypertension prompting the authors to propose a role for this peptide in the pathogenesis of pre-eclampsia (Page and Lowry, 2000; 2001; Page *et al.*, 2001).

The focus of the present study is to investigate the actions of tachykinins on uterine smooth muscle. In previous studies the effects of tachykinins on the myometrium have most extensively been investigated in the rat, where they have been reported to induce contractions of the longitudinal myometrium. In both oestrogen-primed and untreated rats, the relative order of agonist potency was NKA>SP≈NKB indicating activation of primarily NK_2 receptors (Fisher and Pennefather, 1997; 1999; Fisher *et al.*, 1993; Magraner *et al.*, 1998; Moodley *et al.*, 1999; Pennefather *et al.*, 1993). NK_1 and NK_3 receptors have also been reported as contributing to myometrial contractility though to a lesser degree than the NK_2 receptors (Barr *et al.*, 1991; Candenas *et al.*, 2001; Cintado *et al.*, 2001; Fisher and Pennefather, 1999; Magraner *et al.*, 1998).

Evidence suggests that some of the effects of tachykinins on the uterus can be regulated by hormonal status. In the rat, Pinto and colleagues observed that hormonal treatment and pregnancy affected the expression and function of both NK_1 and NK_3 receptors and that a correlation existed between the magnitude of the contractile response elicited by tachykinin peptides and the level of the corresponding receptor mRNA detected (Candenas *et al.*, 2001; Pinto *et al.*, 1999). Expression and function of NK_2 receptors were not seen to vary with hormonal status or pregnancy (Candenas *et al.*, 2001; Pinto *et al.*, 1999) and this finding is consistent with the observations of Moodley *et al.* (1999) who reported that hormonal changes during the rat oestrous cycle had little influence on uterine contractions mediated by the NK_2 receptor. In contrast, Hamlin *et al.* (2000) observed that uterotonic responses to the tachykinins mediated by not only the NK_1 and NK_3 but also the NK_2 receptors were all dependent upon the hormonal status of the rat.

There have been relatively few studies of the effects of tachykinins on the human uterus. Two early investigations indicated that both eledoisin and SP were able to elicit contractions in uterine tissue obtained from non-pregnant women (Molina and Zappia, 1976; Ottesen *et al.*, 1983) while the respective NK_1, NK_2 and NK_3 receptor-selective agonists SP methyl ester, [β-Ala^8]NKA(4-10) and senktide were reported to be without effect in tissues obtained from pregnant women (Barr *et al.*, 1991).

The effect of tachykinins on myometrial contractility has also been studied in both the guinea-pig and mouse. Though cDNA encoding a guinea-pig SP receptor has been isolated from the guinea-pig uterus (Gorbulev *et al.*, 1992), tachykinins were seen to have a minimal effect in uterine tissue obtained from oestrogen-treated, progesterone-treated and pregnant guinea-pigs (Fisher, 1997). In the oestrogen-primed mouse uterus, tachykinins were reported to have a stimulant effect mediated primarily through an NK_1 receptor (Fleming *et al.*, 1998). Preliminary experiments indicated that during late-term pregnancy, in this species, as in the rat, uterotonic activity is mediated through an NK_2 receptor (Fleming *et al.*, 1998).

1.9.3 Degradation

As discussed in section 1.7.6, enzymatic degradation represents the major mechanism by which the actions of tachykinins are terminated. Several enzymes that have been reported to hydrolyse the mammalian tachykinins have been found in the female reproductive tract of various species. Neprilysin, the main enzyme believed to be responsible for tachykinin degradation, has been detected in human placenta, ovaries, endometrium, serum, maternal plasma, amniotic fluid, fetal membranes, cerebrospinal fluid and blood leukocytes (Casey *et al.*, 1991; Germain *et al.*, 1994; Imai *et al.*, 1992; 1994; Iwamoto *et al.*, 1991; Johnson *et al.*, 1984; Kuno *et al.*, 1997; Li *et al.*, 1995; Malfroy *et al.*, 1988; Spillantini *et al.*, 1990; Watanabe *et al.*, 1987). Neprilysin has also been detected in both the rat (Ottlecz *et al.*, 1991; Pinto *et al.*, 1999), ovine (Riley *et al.*, 1995) and human uterus (Salamonsen *et al.*, 1999) where it was shown to be expressed in the myometrium and endometrium. In addition, the levels of neprilysin in uterine

tissues have been reported to be up-regulated by pregnancy in both the rat and the human uterus (Casey *et al.*, 1991; Ottlecz *et al.*, 1991).

ACE has been detected in human and rat placenta (Johnson *et al.*, 1984; Sim and Seng, 1984; Taira *et al.*, 1985; Yagami *et al.*, 1994), human amniotic fluid (Yasui *et al.*, 1984) and rat and bovine uterus (Cushman and Cheung, 1971; Schauser *et al.*, 2001). The presence of aminopeptidase N has been demonstrated in human ovaries, placenta and endometrium (Imai *et al.*, 1994; Imai *et al.*, 1992; Mizutani *et al.*, 1993) and porcine allantoic and amniotic fluids (Basha *et al.*, 1978). DAP IV has been detected in human ovaries, endometrium and placenta (Imai *et al.*, 1992; 1994; Mizutani *et al.*, 1993) and the mouse uterus (Ohta *et al.*, 1992).

The susceptibility of the tachykinins to enzymatic degradation together with the reported presence of peptidases in the female reproductive tract therefore suggests the possible need for peptidase inhibitors when examining the effects of tachykinins on the uterus. Indeed, a study by Fisher *et al.* (1993) revealed an 18-fold increase in the contractile effects of NKA on the uterus of the oestrogen-primed rat in the presence of the neprilysin inhibitor phosphoramidon. In another study by the same authors responses to SP, NKA and NKB were potentiated in the presence of the neprilysin inhibitor SCH 39370 (Fisher and Pennefather, 1997). Magraner *et al.* (1998) also reported the potentiation of SP, NKA and NKB and various receptor-selective analogues by phosphoramidon in the rat uterus. The uterotonic effect of NKA in the pregnant rat uterus was also seen to be significantly increased in the presence of phosphoramidon (Shintani *et al.*, 2000). However, though both ACE and aminopeptidases are found in the uterus, in the rat neither captopril nor amastatin had any significant effect on uterine responsiveness to the tachykinins in this species (Fisher and Pennefather, 1997; Pennefather *et al.*, 1993). These data indicate that in the uterus, as in other tissues such as airways, certain peptidases may play a more important role in peptide inactivation than others.

1.10 Aims of thesis

Tachykinins have the potential to play an important physiological role as modulators of myometrial contractility; however there have been few pharmacological investigations into the uterotonic actions of these peptides in species other than the rat. Thus, the main aims of the present study were:

1. to determine the susceptibility of tachykinins to various peptidases in the mouse and human uterus;

2. to characterize the tachykinin receptor types involved in mediating myometrial contractility in the mouse and human uterus by determining the order of agonist potency for the mammalian tachykinins and through the use of tachykinin receptor-selective agonists and antagonists;

3. to attempt to demonstrate the presence of tachykinin-containing nerves in myometrium from mice and humans using immunohistochemical methods;

4. to investigate the effect of pregnancy on the phenomena associated with the above stated aims.

A more comprehensive account of each of the specific aims of this thesis is presented in the relevant chapters.

CHAPTER 2

GENERAL METHODS

Ethical approval was obtained from the Monash University Standing Committee on Animal Experimentation (Phar 1998/04 and Phar 1999/02), the Monash University Standing Committee on Ethics in Research on Humans and the Royal Women's Hospital Ethics Committee (Project 99/46) prior to commencement of experiments.

2.1 Species

2.1.1 Mice

Female, virgin (18 – 25g) and pregnant (35 – 55g) Balb C mice obtained from the Monash University Central Animal House were housed in the Departmental Animal House. Mice resided six or less to a cage in a rodent stock room that contained both male and female rats and mice. Environmental conditions were set at 22°C, 50 – 60% humidity with a 12h day / 12h night cycle. Mice were allowed access to Gr2 cubes and water *ad libitum*.

2.1.1.1 Oestrogen-treated

Twenty-four hours prior to experimentation non-pregnant mice were administered a single subcutaneous injection of oestradiol-cypionate (200μg/kg) in peanut oil (2ml/kg; i.e. a mouse weighing 20g would be injected with 0.04ml). The injection was dispensed using a 0.25ml glass tuberculin syringe and disposable 26 gauge needles. Animals were killed by placing them into a chamber and exposing them to a mixture of 80% CO_2 and 20% O_2 until unconscious, quickly followed by decapitation with a guillotine. Before uterine horns were removed vaginal smears were taken to confirm the presence of cornified epithelial cells indicating an oestrous-like state. Vaginal smears were obtained by inserting the tip of a Pasteur pipette containing distilled water into the vagina; the contents of the pipette were flushed into the vagina and drawn back into the pipette. The contents were then transferred to a glass slide and allowed to air dry. Once dry, the slides were fixed with 100% methanol, allowed to dry and then placed into Giemsa stain. Slides were then visualized using a standard laboratory microscope (x400 magnification) and cell types noted.

2.1.1.2 Pregnant

Timed pregnant mice were used on day 17 of the average 19 day gestation period. Animals were killed by placing them into a chamber and exposing them to a mixture of 80% CO_2 and 20% O_2 until unconscious, quickly followed by decapitation with a guillotine. Both uterine horns were opened along the mesometrial border to expose the fetuses, which were removed and decapitated.

2.1.2 Humans

All tissue samples were obtained from the Royal Women's Hospital. Patients were considered for participation if they were undergoing either a total abdominal or vaginal hysterectomy or a lower uterine segment caesarean section (LUSCS). Those deemed suitable for the study were approached by a research nurse, verbally briefed about the project and allowed to read an information sheet detailing the purpose of the study, why they were considered as suitable participants and the benefits and risks that were associated with participating. If participation was agreed to, patients signed a consent form in the presence of the research nurse and another witness (see Appendix 1 for copies of the participant information sheet and consent forms). Exclusion criteria included a positive HIV or hepatitis status, a known history of drug abuse, oncological patients, women in labour, where there was the possibility of complications arising during the procedure and women who were not able to comprehend either the verbal briefing or participant information sheet (i.e. were not able to speak English).

2.1.2.1 Non-pregnant

Table 2.1 lists the patient details of non-pregnant women who had participated in this study. Information collected included the age of the patient, indication for hysterectomy, anaesthetic details, parity and gravida, date of last pregnancy (if applicable), menopausal status and any medication that was currently being taken. Please note that for the sake of clarity, this is an abridged version, full details are listed in Appendix 2.

2.1.2.2 Pregnant

Table 2.2 lists the patient details of pregnant women who had participated in this study. Information collected included the age of the patient, the stage of gestation, parity and gravida, indication for LUSCS, anaesthetic details and any medication that was currently being taken. Please note that this is an abridged version and full details are listed in Appendix 3.

Table 2.1: Details of non-pregnant women undergoing either a total abdominal or vaginal hysterectomy who participated in the present study.

Patient	Age	Indication	Parity/Gravida	Last pregnancy (years ago)	Anaesthetic
1	39	fibroids	P_1G_2	5	G
2	57	complex ovarian cyst	P_1G_1	29	G
3	49	dysmenorrhoea, menorrhagia	P_1G_1	15	G
4	49	fibroids	P_5G_5	15	G
5	38	dysmenorrhoea, menorrhagia, fibroids	P_2G_2	17	G
6	38	dysmenorrhoea, menorrhagia	P_5G_5	5	G
7	49	fibroids	P_0G_0	N/A	G
8	57	fibroids	P_3G_3	26	G
9	55	dysmenorrhoea, menorrhagia, fibroids	P_4G_5	20	G
10	41	dysmenorrhoea, menorrhagia	P_0G_0	N/A	G
11	46	menorrhagia	P_2G_2	17	G
12	65	post-menopausal bleeding	P_4G_6	31	G
13	41	dysmenorrhoea, menorrhagia	P_0G_2	12	G
14	33	familial uterine cancer – prophylactic removal	P_3G_3	7	G
15	44	fibroids, menorrhagia	P_3G_5	12	G
16	53	fibroids	P_3G_4	20	G
17	58	ovarian cyst	P_2G_2	27	G
18	49	menorrhagia, fibroids	P_2G_2	25	G
19	46	fibroids	P_2G_2	24	G
20	49	dysmenorrhoea, menorrhagia, fibroids	P_3G_3	18	G
21	43	dysmenorrhoea, menorrhagia	P_2G_3	17	G
22	42	fibroids	P_2G_2	8	G
23	47	dysmenorrhoea, menorrhagia, fibroids	P_1G_1	25	G
24	42	fibroids	P_2G_2	25	G
25	43	menorrhagia	P_2G_2	20	G
26	42	dysmenorrhoea, menorrhagia	P_1G_3	4	G
27	41	fibroids	P_3G_3	10	G
28	38	menorrhagia	P_2G_2	5	G
29	43	fibroids, menorrhagia	P_2G_2	9	G
30	47	dysmenorrhoea, menorrhagia, fibroids	P_3G_5	7	G
31	31	dysmenorrhoea, menorrhagia	P_2G_7	2	G
32	39	dysmenorrhoea	P_2G_2	13	G
33	47	fibroids, menorrhagia	P_4G_4	22	G
34	51	fibroids	P_2G_4	29	G
35	40	menorrhagia	P_3G_3	16	G
36	51	menorrhagia	P_5G_{10}	18	G
37	46	fibroids, endometriosis	P_1G_1	20	G
38	46	menorrhagia	P_2G_3	13	G

Parity = number of live births, Gravida = number of pregnancies; i.e. P_2G_2 = two children from two pregnancies.
N/A – not applicable
Anaesthetic: G – general

Table 2.2: Details of pregnant women undergoing a lower uterine caesarean section who participated in the present study.

Patient	Age	Gestation (weeks)	Indication	Parity/Gravida	Anaesthetic
1	31	39	2nd LUSCS	P_2G_3	S
2	36	39	3rd LUSCS	P_3G_5	S
3	33	38	diabetic, 2nd LUSCS	P_1G_2	S
4	30	40	4th LUSCS	P_2G_4	G
5	27	37	2nd LUSCS	P_1G_2	S
6	29	39	1st LUSCS	P_1G_4	S
7	29	38	1st LUSCS	P_0G_2	S
8	36	38	gestational diabetic, 2nd LUSCS	P_2G_7	S
9	29	38	2nd LUSCS	P_2G_4	S
10	32	38	2nd LUSCS	P_1G_2	S
11	28	38	2nd LUSCS	P_1G_9	S
12	30	37	2nd LUSCS	P_3G_{10}	S
13	32	39	breech	P_0G_2	S
14	37	38	breech, 2nd LUSCS	P_1G_2	S
15	28	36	placenta praevia	P_0G_1	G
16	31	38	2nd LUSCS	P_2G_4	S
17	33	38	2nd LUSCS	P_1G_2	S
18	23	38	breech	P_0G_1	S
19	35	39	2nd LUSCS	P_4G_7	S
20	30	38	2nd LUSCS	P_2G_4	S
21	31	39	2nd LUSCS	P_1G_2	S
22	29	38	2nd LUSCS	P_1G_4	S
23	31	39	2nd LUSCS	P_1G_2	S
24	34	41	pelvic injury	P_5G_7	G
25	37	39	2nd LUSCS	P_2G_5	S
26	33	39	3rd LUSCS	P_2G_4	S
27	38	38	7th LUSCS	P_6G_{10}	S
28	36	39	4th LUSCS	P_3G_5	S/E
31	26	39	3rd LUSCS	P_2G_3	S
32	36	38	3rd LUSCS high blood pressure	P_2G_3	S
33	38	38	4th LUSCS	P_3G_5	S
34	35	37	twins	P_0G_2	S
35	36	38	breech	P_1G_2	S
36	38	39	2nd LUSCS	P_1G_7	S
37	32	39	1st LUSCS	P_2G_3	S
38	33	39	baby changing positions	P_0G_1	S
39	35	39	3rd LUSCS	P_2G_4	S
40	21	39	breech	P_1G_3	S
41	34	38	2nd LUSCS	P_1G_2	S
42	30	38	3rd LUSCS	P_2G_3	S
43	32	38	placenta praevia	P_1G_4	S
44	33	39	2nd LUSCS	P_1G_4	S
45	35	33	twins	P_1G_3	S
46	23	41	cephalopelvic disproportion	P_0G_1	S
47	34	38	placenta praevia	P_0G_2	S
48	30	38	gestational diabetic, 2nd LUSCS	P_1G_2	S
49	29	36	LuCTR in 2nd twin	P_0G_1	S
50	38	39	breech	P_3G_4	S
51	35	38	3rd LUSCS	P_5G_7	S
52	32	39	1st LUSCS	P_1G_3	S
53	26	40	breech	P_0G_1	S
54	26	39	breech	P_0G_3	S
55	28	38	3rd LUSCS	P_2G_4	S

Parity = number of live births, Gravida = number of pregnancies; i.e. P_1G_2 = one child from two pregnancies (does not include present pregnancy)
Anaesthetic: S – spinal, E – epidural, G - general

2.2 Preparations

2.2.1 Physiological salt solution

Experiments using both mouse and human uterine tissue were carried out in a modified Krebs-Henseleit solution (PSS) of the following composition (mM: NaCl, 118.0; KCl, 4.7; $MgSO_4.7H_2O$, 1.1; KH_2PO_4, 1.18; $NaHCO_3$, 25.0; glucose, 11.66; $CaCl_2.2H_2O$, 1.9). At the conclusion of each experiment tissues were exposed to a high potassium (40mM) modified Krebs-Henseleit solution (KPSS) (mM: NaCl, 82.7; KCl, 40.0; $CaCl_2.2H_20$, 2.5; $MgSO_4.7H_20$, 0.5; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 11.7)

2.2.2 Mouse myometrium

All uterine tissue obtained for contractility studies remained intact, i.e. endometrial tissue was not removed nor were the layers separated. All tissue weights were recorded at the conclusion of the experiment.

2.2.2.1 Tissue from oestrogen-treated animals

Both uterine horns were removed, cleared of surrounding fat and connective tissue and placed in a petri dish containing PSS. Each horn was opened along the mesometrial border and transected medially allowing for four preparations per animal. Average size of a preparation was 5mm by 10mm with a mean tissue weight of 29.2 ± 0.4mg (n=430).

2.2.2.2 Tissue from pregnant animals

After removal of the fetuses both uterine horns were excised and placed in a petri dish containing PSS. Placental and fetal membranes and sites of implantation were removed and typically, six preparations were obtained from the uterine horn that contained the most fetuses, with the remainder two preparations being obtained from the other horn. Average size of a preparation was 5mm by 10mm with a mean tissue weight of 30.4 ± 0.8mg (n=196).

2.2.3 Human myometrium

2.2.3.1 Myometrium obtained from non-pregnant women

After the surgeon removed the uterus from the body, a segment of myometrium was obtained from approximately the same location as specimens obtained at caesarean section (see below, 2.2.3.2). The sample was placed in a petri dish containing PSS. The inner and outer myometrial layers were identified and strips of myometrium were then obtained from the outer layer. Average size of a preparation was 3mm by 3mm by 10mm with a mean tissue weight of 101.2 ± 2.9mg (n=98).

2.2.3.2 Myometrium obtained from pregnant women

After delivering the fetus(es) and placenta(e), a segment of myometrium, not always of full thickness, would be removed from the upper edge of the original incision by the surgeon. The sample was placed in a petri dish containing PSS and the outer and inner myometrial layers were identified and strips of myometrium were obtained from the outer layer. Average size of a preparation was 3mm by 3mm by 10mm with a mean tissue weight of 115.2 ± 2.9mg (n=169).

2.3 Experimental set-up and protocol

2.3.1 Contractility studies

Myometrial preparations were tied to tissue holders so as to record contractile force produced by the longitudinally-oriented smooth muscle fibres. Stainless steel wire tissue holders were used with all mouse uterine preparations. Plastic tissue holders incorporating platinum stimulating electrodes were used with all human uterine preparations. Tissues were tied to holders using either cotton thread or silk suture. Preparations were placed into siliconised, 5ml isolated organ baths containing PSS warmed to 37°C and continuously bubbled with carbogen gas (95% O_2, 5% CO_2) to maintain a pH of 7.4. The upper end of each preparation was attached with either cotton

thread or silk suture to a Grass FT03 force transducer that was connected to a MacLab data acquisition system.

Initial resting force varied between the species. Preparations from oestrogen-treated and pregnant mice were set up under an initial force of 1g and 2g respectively. Preparations from non-pregnant and pregnant humans were set up under an initial force of 15-20g to approximate intrauterine pressure (Story *et al.*, 1988). Tissues were allowed to equilibrate for 1h (preparations from oestrogen-treated mice or pregnant humans) or 2h (preparations from pregnant mice and non-pregnant humans). During the equilibration period the bathing solution was replaced every 20min. Following the equilibration period discrete concentration-response curves were constructed. Each agonist concentration remained in contact with the tissue for 5min, was washed out and a higher concentration of agonist was added 15min later (initial concentrations increasing in log units until a response was observed and then increasing in 0.5 log increments). Only one concentration-response curve was generated on each preparation.

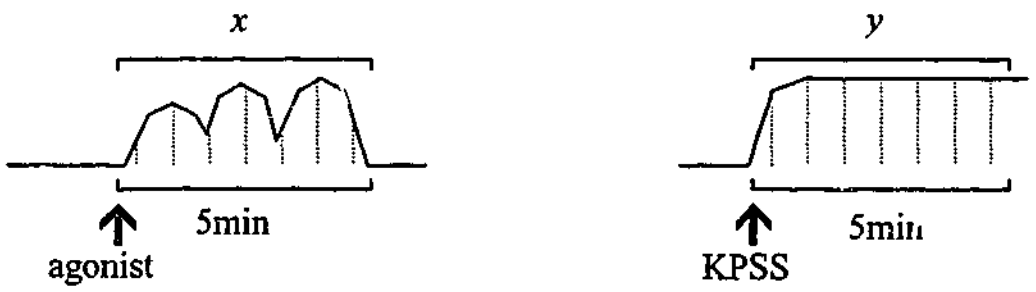
If enzyme inhibitors or antagonists were used these were added to the organ bath at the beginning of the equilibration period (or after 1h if the preparation had a 2h equilibration period) and replaced after each washout.

Fifteen minutes after the completion of the concentration-response curve the preparation was exposed to KPSS warmed to 37°C (see 2.2.1) that had been bubbled with carbogen. This was added directly to each organ bath for 5min and maximal responses for each preparation were obtained. Tissues were then removed from the organ baths, blotted and weighed.

2.4 Measurement of responses

Responses to all agonists were measured as area under the force-time curve (grammes.seconds) for the duration of agonist exposure (5min). Responses were then expressed as a percentage of the corresponding response to KPSS and presented as mean ± standard error of the mean (SEM) (see Figure 2.1).

Figure 2.1: Measurement of myometrial responses to agonists.



The area under the force time curve is measured for the 5min that the agonist is in contact with the tissue. This response is then expressed as a percentage of the tissue's response to KPSS; i.e. $\frac{x}{y}$ x 100%.

2.5 Immunohistochemistry

2.5.1 Tissue Preparation

Full details of immunohistochemical experiments are given in Chapter 8. In brief, uterine horns or cervices from oestrogen-treated and pregnant mice, and outer myometrial tissue from non-pregnant and pregnant women, were fixed in phosphate-buffered saline (mM: NaCl, 136.90; KCl, 2.68; KH_2PO_4, 1.46; Na_2HPO_4, 8.10) (PBS) containing 4% paraformaldehyde for 2h. Tissues were then washed in PBS containing 7% sucrose and 0.01% sodium azide four times for 10min each time, then left in this solution and refrigerated at 4°C for between 48-72h. Tissues were then embedded in "Tissue Tek" (OCT embedding compound) in vinyl specimen moulds (cryomold) and snap-frozen with liquid nitrogen and stored in a -70°C freezer until use. 14μm sections of tissue were cut using a cryostat (Riechert-Jung CM 1800, -20°C) and thawed onto gelatin-coated slides.

2.5.2 Immunohistochemistry

Slides were incubated with rabbit polyclonal antibodies for substance P (Serotec), neurokinin A (Phoenix) and calcitonin gene-related peptide (Serotec) and a mouse monoclonal antibody for tyrosine hydroxylase (Boehringer Mannheim) for 18-20h at room temperature. Antibodies were diluted (substance P, 1:1000; neurokinin A, 1:1000; calcitonin gene-related peptide, 1:2000; tyrosine hydroxylase, 1:100) in an antibody-diluting medium containing 0.1% w/v sodium azide, 0.01% w/v bovine serum albumin, 0.1% w/v lysine and 0.1% v/v Triton in phosphate-buffered solution. Tissue sections were then washed with PBS four times for 10min each time before a 1h incubation with a secondary antibody at room temperature. Fluoroscein isothiocyanate (FITC-)-conjugated anti-rabbit immunoglobulin (1:250 or 1:500, Vector) was used for tissues that had been incubated with substance P, neurokinin A or calcitonin gene-related peptide, and Texas Red-conjugated anti-mouse immunoglobulin (1:500, Vector) for tissues that had been incubated with tyrosine hydroxylase. Tissues were then washed in PBS four times every 10min, mounted in "Vectashield" (Vector), coverslipped and examined under an Olympus fluorescence photomicroscope.

2.6 Statistical analysis of data

All data are expressed as mean ± standard error of the mean (SEM) for n experiments. Mean log concentration-response curves (CRCs) were constructed by pooling data from individual log CRCs. If the mean log CRCs reached a maximum, mean pD_2 values were determined using non-linear regression analysis in the GraphPad Prism program (version 3.0) by pooling pD_2 values for individual CRCs. Comparison of mean pD_2 values was by either Student's unpaired t-test or one-way ANOVA followed by Student Newman Keuls. Agonist potency ratios were determined using COMPAR (Orre *et al.*, 1996). Briefly, when there was significant regression of response with increasing agonist concentrations, least squares regression lines were fitted to the linear portions of the log CRCs (typically 15-85% of the maximum response of the reference agonist) and analysis of variance was undertaken to determine deviation from parallelism and coincidence as outlined in Geigy Scientific Tables (Lentner, 1982). When lines were

parallel to the reference agonist, estimates of potency ratio, together with 95% confidence limits, were determined for each agonist. A significant difference in apparent potency of the agonist was indicated when the 95% confidence limits of the potency ratio did not include one. Mean log CRCs were also compared using two-way ANOVA followed by Student Newman Keuls. Comparison of mean KPSS responses was either by Student's unpaired t-test or one-way ANOVA followed by Student Newman Keuls. Mean E_{max} values were determined by pooling the maximum agonist response observed for individual CRCs at the concentrations tested. Comparison of mean E_{max} values was either by Student's unpaired t-test or one-way ANOVA followed by Student Newman Keuls.

Other statistical tests used are described in the relevant chapters.

In all cases, statistical significance was accepted if $p<0.05$.

2.7 Drugs and chemicals

The drugs used in this study were: arginine vasopressin (Sigma); atropine sulphate (Sigma); bestatin HCl (N-[2S,3R)-3-Amino-2-hydroxy-4-phenylbutyryl]-L-leucine hydrochloride) (Sigma); captopril (D-3-mercapto-2-methyl propanoyl-L-proline) (Sigma); eledoisin (Auspep); histamine (2-[-4-imidazolyl]ethylamine dihydrochloride (Sigma); indomethacin (1-(p-Chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid) (Sigma); [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) (RBI); mepyramine maleate (N-(4-Methoxyphenyl)methyl-N',N'-di-methyl-N-(2-pyridinyl)-1,2-ethanediamine (Sigma); methacholine chloride ((2-Acetoxypropyl)trimethylammonium chloride) (Sigma); neurokinin A (Auspep); neurokinin B (Auspep); [*N*-$MePhe^7$]NKB (Auspep); oestradiol-17β cypionate (Sigma); oxytocin (Sigma); phentolamine HCl (Ciba-geigy); ranitidine HCL (Sigma); [$Sar^9Met(O_2)^{11}$]SP (Auspep); SR 140333 ((1-{2-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl) piperidin-3-yl]ethyl}-4-phenyl-1-azonia-bicyclo[2.2.2]octane, chloride); SR 48968 ((S)-N-methyl-N[4-acetylamino-4-phenylpiperidino-2-(3,4-dichlorophenyl)butyl]benzamide) and SR 142801 ((S)-(*N*)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylpiperidine-4-yl)-*N*-

methylacetamide) (all generous gifts from Sanofi Recherche); substance P (Auspep); [Glu^5]substance P (Auspep); [Glu^{5,6}]substance P (Auspep); [Glu^6]substance P (Auspep); DL-Thiorphan (Sigma); tetrodotoxin (Sigma).

Arginine vasopressin, atropine, captopril, histamine, mepyramine, methacholine, oxytocin, phentolamine, ranitidine and thiorphan were dissolved in distilled water. Neurokinin B and [MePhe^7]NKB were dissolved in 0.1M ammonia. Indomethacin was dissolved in a 0.1M sodium carbonate solution. SR 140333, SR 48968 and SR 142801 were dissolved in absolute ethanol. Nifedipine was dissolved in 80% ethanol. Tetrodotoxin was dissolved in citrate buffer. All remaining compounds were dissolved in dilute hydrochloric acid (0.01M). Stock solutions of bestatin (10mM), captopril (10mM), carbachol (10mM), histamine (1mM), indomethacin (10mM), isoprenaline (1mM), mepyramine (10mM), methacholine (10mM), ranitidine (10mM), SR 140333 (1mM), SR 48968 (1mM) and SR 142801(1mM) were stored at 4°C. Standard solutions (1mM) of all peptides, thiorphan and tetrodotoxin were aliquoted into eppendorf tubes and stored at -20°C

Absolute ethanol was used in vehicle controls described in Chapter 4.

Salts used in physiological solutions were all standard analytical reagents from either UNIVAR or AnalaR.

CHAPTER 3

PILOT INVESTIGATIONS ON THE UTERUS OF THE OESTROGEN-PRIMED MOUSE

Summary

1. The aims were (1) to examine the contractile effects of SP, NKA and the respective NK_1, NK_2 and NK_3 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP, [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and [$MePhe^7$]NKB on myometrium obtained from oestrogen-treated mice and (2) to determine the importance of peptidases in constraining the response to these peptides in this tissue preparation.

2. Mice were oestrogen-treated (200μg/kg, s.c.) 24h prior to use. Four longitudinal preparations were obtained from each animal. No significant differences were seen in the mean pD_2 values for MCh in the four preparations.

3. A reduction in successive responses to repeated applications of high concentrations of SP (100nM), NKA (100nM) and MCh (10μM) was observed. This reduction occurred irrespectively of whether the agonist contact time was 5min (SP) or 2min (SP, NKA and MCh) and the interval between doses 15 or 30min, and in the case of SP achieved statistical significance with the 2min contact time / 15min interval combination.

4. In the absence of peptidase inhibitors SP and NKA were equipotent and the NK_1 receptor-selective [$Sar^9Met(O_2)^{11}$]SP produced a modest contractile response. The NK_2 and NK_3 receptor-selective agonists [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and [$MePhe^7$]NKB were inactive.

5. The responses to SP, NKA and [$Sar^9Met(O_2)^{11}$]SP but not [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) or [$MePhe^7$]NKB were significantly potentiated in the presence of peptidase inhibitors. Peptidase inhibitors used included thiorphan (3μM), captopril (10μM) and bestatin (10μM). The peptidase inhibitors did not potentiate the responses to either MCh or KPSS in these tissues.

6. The results of this chapter indicate that the contractile effects of the tachykinins in myometrium from oestrogen-primed mice are predominantly mediated by the tachykinin NK_1 receptor and that in this tissue the peptidases neprilysin and to a lesser extent, captopril and bestatin, play a role in modulating the responses to these peptides. Experimental conditions to be adopted for further functional experiments conducted using mouse myometrium were also established. These conditions have included the use of a randomization procedure to allocate preparations to treatments, the use of an agonist contact time of 5min with 15min between concentrations when constructing log concentration-response curves and the inclusion of a "peptidase inhibitor cocktail" consisting of thiorphan (3μM), captopril (10μM) and bestatin (10μM) to prevent peptide degradation.

3.1 Introduction

The tachykinins represent a family of neurotransmitters including substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) (Maggio, 1988). They act on three distinct receptors termed NK_1, NK_2 and NK_3, which are preferentially, though not exclusively activated by SP, NKA and NKB respectively (Maggi, 1995a). In the female reproductive tract, SP- and NKA-immunoreactivity has been detected in a population of capsaicin-sensitive sensory neurons, the presence of which has been demonstrated in numerous species including the human (Butler-Manuel *et al.*, 2002; Franco-Cereceda *et al.*, 1987; Fried *et al.*, 1990; Heinrich *et al.*, 1986; Reinecke *et al.*, 1989; Samuelson *et al.*, 1985), rat (Huang *et al.*, 1984; Papka and Traurig, 1993; Springall *et al.*, 1984; Traurig and Papka, 1993), guinea-pig (Alm and Lundberg, 1988; Huang *et al.*, 1984), mouse (Huang *et al.*, 1984), cow (Lakomy *et al.*, 1995), pig (Czaja *et al.*, 1996) and horse (Bae *et al.*, 2001). The release of tachykinins from the peripheral terminals of these sensory neurons can exert a local effect on tissue function (Lembeck and Holzer, 1979; Maggi and Meli, 1988).

The presence of NKB, which previously was considered to be restricted to the central nervous system, has recently been reported in the human placenta (Page *et al.*, 2000). Molecular studies have since also reported the expression of the preprotachykinin B gene, which encodes for NKB, in both the rat and human uterus (Cintado *et al.*, 2001; Patak *et al.*, 2003; Pinto *et al.*, 2001).

Tachykinins have been implicated in numerous processes important for reproduction including the regulation of blood flow and myometrial contractility (Traurig and Papka, 1993), cervical ripening (Collins *et al.*, 2002) and possible participation in stress-induced abortions in the both the mouse and human (Arck *et al.*, 1995; Joachim *et al.*, 2001; Markert *et al.*, 1997; Marx *et al.*, 1999).

The effects of tachykinins in regulating myometrial contractility have been studied extensively in the rat (Barr *et al.*, 1991; Candenas *et al.*, 2001; Fisher and Pennefather, 1997; 1998; 1999; Fisher *et al.*, 1993; Hamlin *et al.*, 2000; Magraner *et al.*, 1998; Moodley *et al.*, 1999; Shintani *et al.*, 2000). In this species it has been established that

although NK_1 and NK_3 receptors are expressed in the uterus of non-pregnant animals, the contractile activity of the tachykinins is predominantly mediated by the NK_2 receptor. Furthermore, their activity is enhanced in the presence of peptidase inhibitors, chiefly those that inhibit neprilysin (Fisher *et al.*, 1993; Magraner *et al.*, 1998). To date, very few studies have examined the actions of tachykinins on the mouse uterus (Fleming *et al.*, 1998). This is surprising as the mouse is the animal most frequently used for producing gene knockouts, and accordingly has the potential of determining the physiological role that tachykinins play in reproductive processes.

The aim of the experiments described in this chapter is to examine the effects of SP, NKA and the respective NK_1, NK_2 and NK_3 tachykinin receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP, [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and [$MePhe^7$]NKB in the absence and presence of peptidase inhibitors on the smooth muscle of the oestrogen-treated mouse uterus. These pilot studies were undertaken to determine some of the characteristics of the mouse myometrium and to establish the experimental conditions to be adopted in more extensive studies using mouse myometrium described in the following two chapters.

3.2 Materials and Methods

3.2.1 Animals and tissue preparations

Full details of animals and preparations are given in section 2.1.1 and 2.2.2. Briefly, virgin, female Balb C mice weighing between 20–25g were treated with oestradiol-17β-cypionate (200μg/kg), s.c., 24h prior to use. Four uterine preparations were obtained from each animal and set up for recording of force from the longitudinal smooth muscle layer.

3.2.2 Experimental protocol

Refer to Chapter 2 (section 2.3). In brief, following the equilibration period discrete concentration-response curves were constructed. Each agonist concentration remained in contact with the tissue for 5min, was washed out and a higher concentration of agonist was added 15min later (initial concentrations increasing in log units until a response was observed and then increasing in 0.5 log increments). Only one concentration-response curve was generated on each preparation. Preparations used to construct log concentration-response curves to SP, NKA, [$Sar^{9}Met(O_2)^{11}$]SP, [$Lys^{5}MeLeu^{9}Nle^{10}$]NKA(4-10) and [$MePhe^{7}$]NKB were also exposed to methacholine (MCh) (10μM) prior to the addition of KPSS to the bath.

In an additional series of experiments, following the equilibration period tissues were exposed to six repeated applications of SP (100nM), NKA (100nM) or MCh (10μM). The agonists remained in contact with the tissue for either 2 (SP, NKA and MCh) or 5min (SP) were washed out and next dose added either 15 or 30min later. Only one agonist was tested on each preparation. Following the six repeated applications of the agonist the tissues were exposed to KPSS.

Table 3.1: Overview of experiments undertaken on uterine preparations obtained from oestrogen-treated mice. Note that, with the exception of Series 1, preparations of upper and lower segments of left and right uterine horns are allocated as preparation 1, 2, 3 or 4 using a randomized procedure.

	Preparation 1		**Preparation 2**		**Preparation 3**		**Preparation 4**	
Series	**Agonist**	**Treatment**	**Agonist**	**Treatment**	**Agonist**	**Treatment**	**Agonist**	**Treatment**
1: Response of upper and lower uterine horns to MCh (n=4)	MCh	left upper uterine horn	MCh	left lower uterine horn	MCh	right upper uterine horn	MCh	right lower uterine horn
2: Repeated doses of SP (100nM) in the presence of thiorphan (3μM) and captopril (10μM) varying drug contact time (2 or 5min) and time between doses (15 or 30min) (n=4)	SP 2min	15min	SP 2min	30min	SP 5min	15min	SP 5min	30min
3: Repeated doses of NKA (100nM) in the presence of thiorphan (3μM) and bestatin (10μM) and MCh (10μM) varying time between doses (15 or 30min) (n=4)	NKA 2min	15min	NKA 2min	30min	MCh 2min	15min	MCh 2min	30min
4: SP in the absence and presence of peptidase inhibitors (n=8)	SP	-	SP	T	SP	T & C		
5: NKA in the absence and presence of peptidase inhibitors (n=10)	NKA	-	NKA	T	NKA	T & B	NKA	T, C & B
6: Sar^9 and Lys^5 in the absence and presence of peptidase inhibitors (n=6-8)	Sar^9	-	Sar^9	T, C & B	Lys^5	-	Lys^5	T, C & B
7: $MePhe^7$ in the absence and presence of peptidase inhibitors (n=6)	$MePhe^7$	-	$MePhe^7$	T, C & B				

Sar^9 = [$Sar^9Met(O_2)^{11}$]SP, Lys^5 = [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10), $MePhe^7$ = [$MePhe^7$]NKB
T - thiorphan (3μM), C - captopril (10μM), B - bestatin (10μM)
n = number of animals
- = no treatment

3.2.3 Measurement of results and statistical analyses

Responses to all agonists were measured as area under the force-time curve for the duration of agonist exposure as described in section 2.4. Responses were then expressed as a percentage of the response to the KPSS and presented as mean ± SEM.

Statistical analysis was undertaken as described in section 2.6.

E_{max} is defined as the maximum response to an agonist observed over the concentration range used; note that might not in all cases be the greatest response that an agonist could elicit if higher concentrations had been tested.

3.2.4 Drugs

The drugs used included all those previously described in section 2.7, which included details of stock solutions, vehicles used and vehicle controls as appropriate.

3.3 Results

Uterine tissue was obtained from 44 oestrogen-treated mice (200μg/kg; s.c) weighing between 18-25g. Histological examination of vaginal smears confirmed cornification of the vaginal epithelium. The mean weight of the tissue preparations was 29.7 ± 0.7mg (n = 156 preparations). The mean response to KPSS was 17.8 ± 0.5g.s/mg tissue (n = 156 preparations). The mean response to MCh (10μM) was 46.0 ± 2.1% of the response to KPSS (n = 104 preparations). Spontaneous activity was observed in nearly all tissue preparations and ranged from 1 – 25 contractions over any 15min period, this would gradually decrease over the duration of the equilibration period. Figure 3.1 shows a representative trace of responses to increasing concentrations of SP in the absence and presence of peptidase inhibitors.

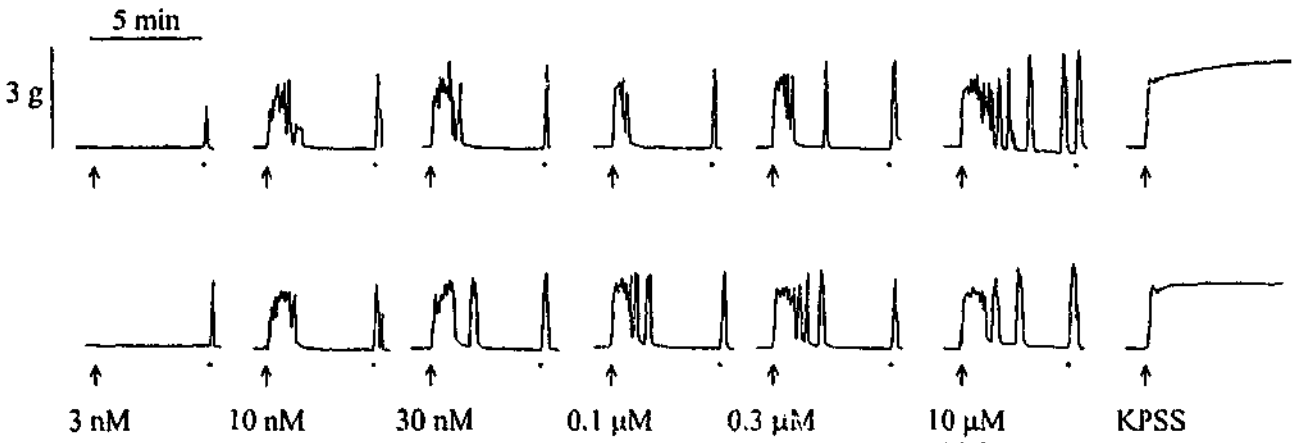


Figure 3.1: Representative trace showing response of longitudinal myometrium from an oestrogen-treated mouse to SP in the absence (upper panel) and presence of thiorphan (3μM) and captopril (10μM) (lower panel) together with responses to MCh (10μM) and KPSS. The arrows indicate agonist addition, the dots represent washes.

3.3.1 Response to methacholine

Two-way ANOVA showed significant differences in the response to MCh of preparations obtained from the upper right uterine horn compared to those obtained from the upper left, lower right and lower left ($P<0.05$; d.f. = 104) (Figure 3.2), however

no significant differences were seen in either mean E_{max} or pD_2 values for the four preparations, the mean response to KPSS was also similar in all four preparations (one-way ANOVAs, P>0.05) (Table 3.2).

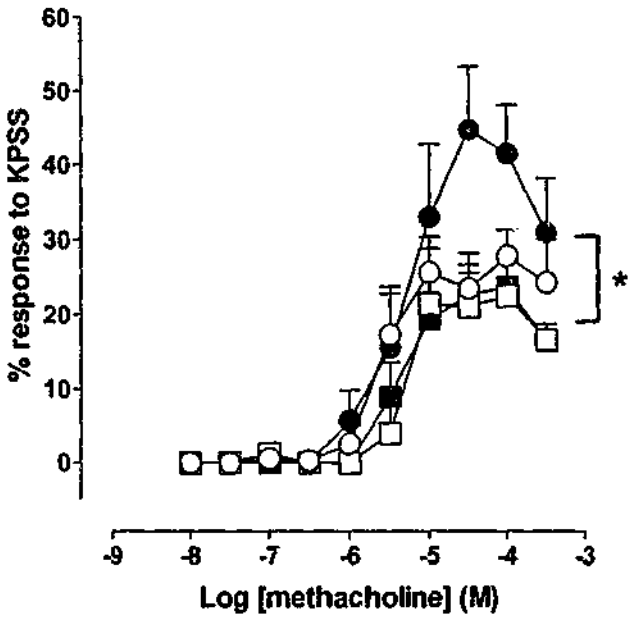


Figure 3.2: Log concentration-response curves (CRCs) to MCh on upper left (○), upper right (●), lower left (□) and lower right (■) uterine preparations from oestrogen-treated mice. Each point is the mean ± SEM, n=4 animals. In this and subsequent figures, a significant difference between log CRCs as indicated by two-way ANOVA followed by Student Newman Keuls is shown by *.

* indicates significant difference from preparation obtained from the upper right uterine horn, two-way ANOVA, P<0.05; d.f. = 104

Table 3.2: Effect of MCh and KPSS on upper and lower uterine preparations from oestrogen-treated mice (n=4).

Uterine horn position	**Response to KPSS (g.s/mg tissue ± SEM)**	**E_{max} (% of KPSS ± SEM)**	**Potency (pD_2 ± SEM)**
Upper left	12.9 ± 1.3	29.7 ± 4.5	5.58 ± 0.19
Upper right	13.6 ± 1.8	45.5 ± 8.4	5.35 ± 0.17
Lower left	11.6 ± 1.6	27.4 ± 5.4	5.21 ± 0.10
Lower right	17.1 ± 2.7	25.95 ± 4.4	5.42 ± 0.10

3.3.2 Response to repeated doses of substance P, neurokinin A and methacholine: the effect of varying drug contact time and time between doses.

3.3.2.1 Substance P

A reduction in successive responses to repeated doses of SP (100nM) was seen when the agonist was present for either 2 or 5min with an interval of either 15 or 30min between doses (Figure 3.3 and 3.4).

A significant reduction was seen in successive responses when the agonist contact time was 2min with 15min between doses (one-way ANOVA, $P<0.05$; d.f = 17), which was not seen when the time between doses was increased to 30min (see Figure 3.3). Subsequent analysis indicated a significant linear trend ($P<0.05$) in both cases.

No significant difference between doses was found by one-way ANOVA ($P>0.05$) for when the agonist contact time was 5min with either 15 or 30min between doses, however subsequent analysis indicated a significant linear trend ($P<0.05$) when the interval between doses was 15min but not 30min (see Figure 3.4).

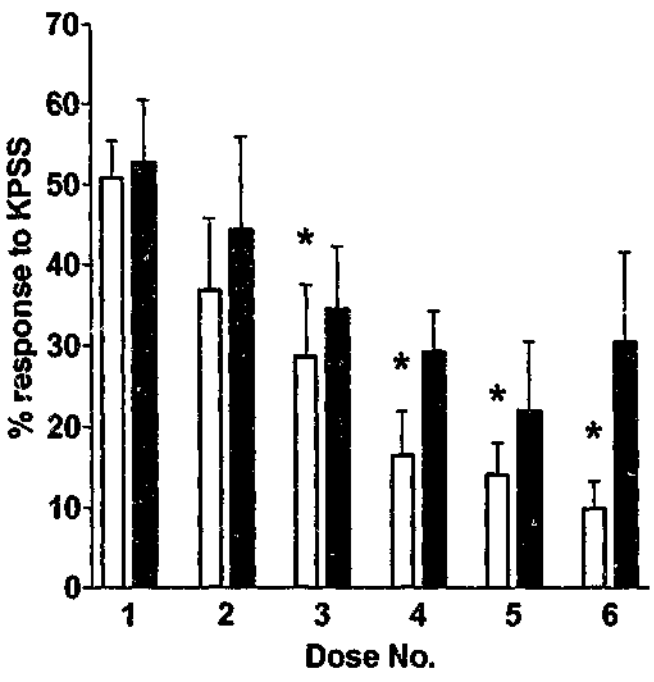


Figure 3.3: Response of uterine preparations from oestrogen-treated mice to repeat doses of substance P (100nM) (2min contact time) in the presence of thiorphan (3μM) and captopril (10μM) with either 15min (open bars) or 30min (closed bars) intervals between doses. Each bar represents the mean ± SEM, n=4 animals.

* indicates significant difference from dose 1, one-way ANOVA, $P<0.05$, d.f. = 17

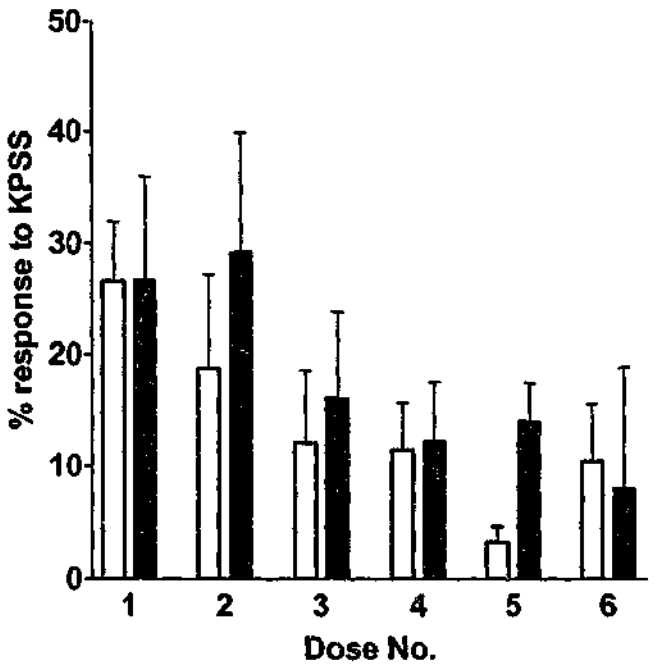


Figure 3.4: Response of uterine preparations from oestrogen-treated mice to repeat doses of SP (100nM) (5min contact time) in the presence of thiorphan (3μM) and captopril (10μM) with either 15min (open bars) or 30min (closed bars) intervals between doses. Each bar represents the mean ± SEM, n=4 animals.

3.3.2.2 Neurokinin A and methacholine

A reduction in successive responses to repeated doses of NKA (100nM) and MCh (10μM) was seen for both agonists when the agonist contact time was 2min with an interval of either 15 or 30min between doses (Figure 3.5). This reduction was not significant for either NKA or MCh (one-way ANOVA, $P>0.05$), however subsequent analysis indicated a significant linear trend for both NKA and MCh when the interval between doses was 15min but not 30min ($P<0.05$).

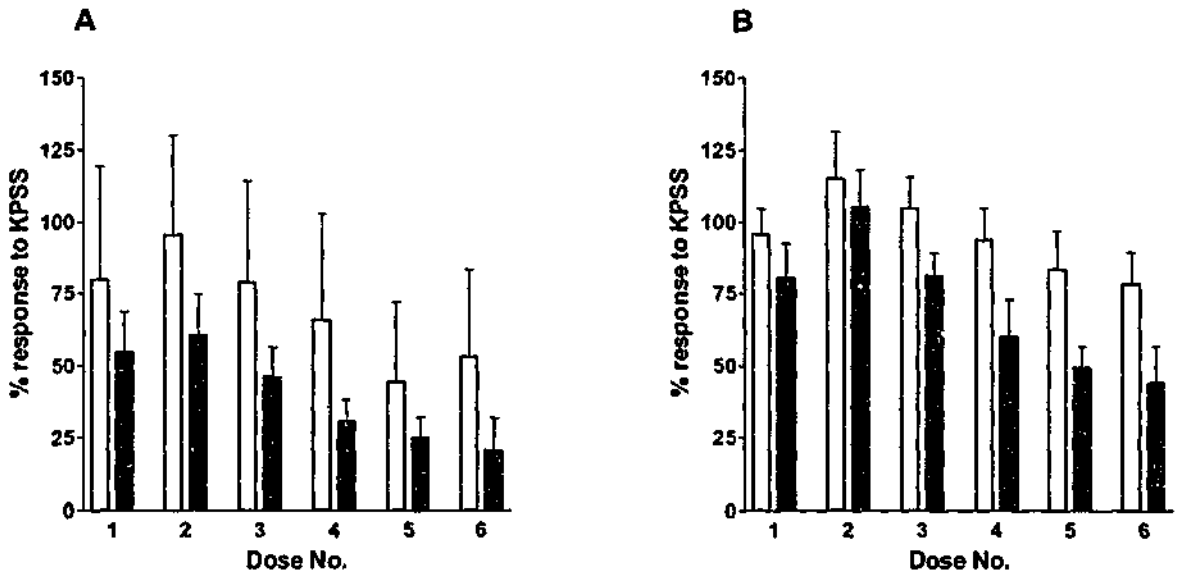


Figure 3.5: Response of uterine preparations from oestrogen-treated mice to repeat doses of (A) NKA (100nM) in the presence of thiorphan (3μM) and bestatin (10μM) and (B) MCh (10μM) with either 15min (open bars) or 30min (closed bars) between doses. Agonist contact time is 2min. Each bar represents the mean ± SEM, n=4 animals.

3.3.3 Influence of peptidase inhibitors on responses to tachykinin peptides, methacholine and KPSS in the mouse uterus

3.3.3.1 Substance P

Responses to SP were unaffected by thiorphan (3μM) alone (potency ratio = 5.8, 95% C.L. = 0.7, 123.2; d.f. = 60). Two-way ANOVA indicated a significant difference in the response to SP in the presence of both thiorphan (3μM) and captopril (10μM) ($P<0.05$; d.f. = 168), which corresponded to a 17.3 fold leftward shift (95% C.L. = 2.5, 366.1; d.f. = 61) (Figure 3.6).

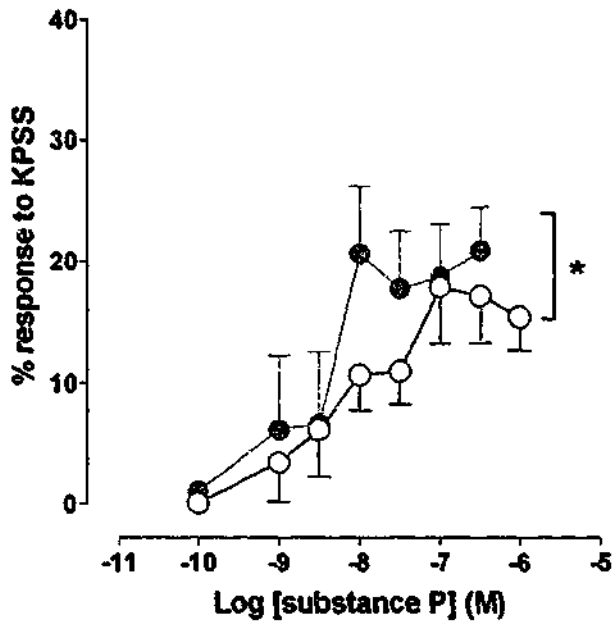


Figure 3.6: Log CRCs to SP on uterine preparations from oestrogen-treated mice in the absence of peptidase inhibitors (○) and in the presence of thiorphan (3μM) (●) and thiorphan (3μM) and captopril (10μM) ( ). Note that log CRCs to SP reached a ceiling at concentrations over 0.1μM. Each point is the mean ± SEM, n=8 animals.

* indicates significant difference from control, two-way ANOVA, $P<0.05$; d.f. = 168

3.3.3.2 Neurokinin A

Two-way ANOVA indicated a significant difference in the response to NKA in the presence of all three peptidase inhibitor combinations tested ($P<0.05$; d.f. = 288). This corresponded to 2.4, 5.0 and 5.0 fold leftward shifts in the presence of thiorphan (3μM) (95% C.L. = 1.1, 6.6; d.f. = 76), thiorphan (3μM) and bestatin (10μM) (95% C.L. = 1.5, 35.1; 70 d.f) and thiorphan (3μM), captopril (10μM) and bestatin (10μM) (95% C.L. = 1.8, 23.5; d.f. = 67) respectively (Figure 3.7).

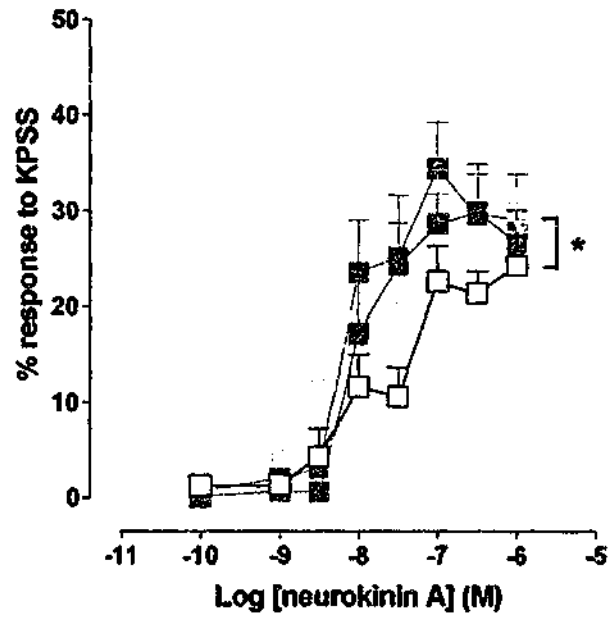


Figure 3.7: Log CRCs to NKA on uterine preparations from oestrogen-treated mice in the absence of peptidase inhibitors (□) and in the presence of thiorphan (3μM) (■), thiorphan (3μM) and bestatin (10μM) (▪) and thiorphan (3μM), captopril (10μM) and bestatin (10μM) (■). Note that log CRCs to NKA reached a clear ceiling. Each point is the mean ± SEM, n=10 animals.

* indicates significant difference from control, two-way ANOVA, $P<0.05$; d.f. = 288

3.3.3.3 [Sar^{9}Met(O$_2$)^{11}]SP

Two-way ANOVA indicated a significant difference in the response to [Sar^{9}Met(O$_2$)^{11}]SP in the presence of thiorphan (3μM) captopril (10μM) and bestatin (10μM) ($P<0.05$; d.f. = 98) (Figure 3.8). It was not possible to obtain a potency ratio for [Sar^{9}Met(O$_2$)^{11}]SP in the absence of peptidase inhibitors as there were insufficient points on the linear part of the curve, however, it was found that the maximum response to [Sar^{9}Met(O$_2$)^{11}]SP was enhanced in the presence of peptidase inhibitors although this did not quite achieve statistical significance (Student's unpaired t-test, $P<0.08$).

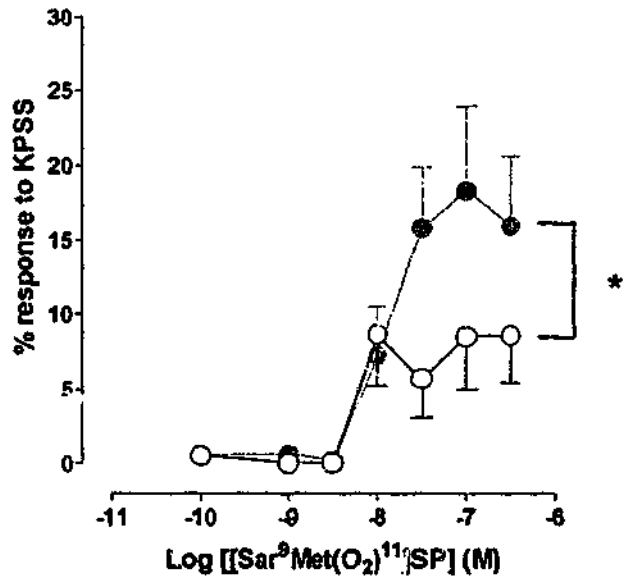


Figure 3.8: Log CRCs to [Sar^{9}Met(O$_2$)^{11}]SP on uterine preparations from oestrogen-treated mice in the absence of peptidase inhibitors (○) and in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM) (●). Note that the log CRC to [Sar^{9}Met(O$_2$)^{11}]SP in the presence of peptidase inhibitors reached a clear ceiling at concentrations over 0.1μM. Each point is the mean ± SEM, n=8 animals.

* indicates significant difference from control, two-way ANOVA, P<0.05; d.f. = 98

3.3.3.4 [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10)

Responses to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) were unaffected by thiorphan (3μM) captopril (10μM) and bestatin (10μM) (two-way ANOVA, P<0.05; d.f. = 123) (Figure 3.9).

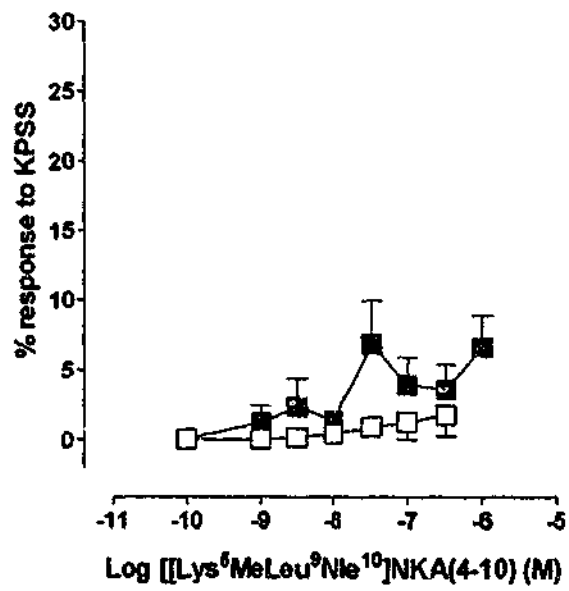
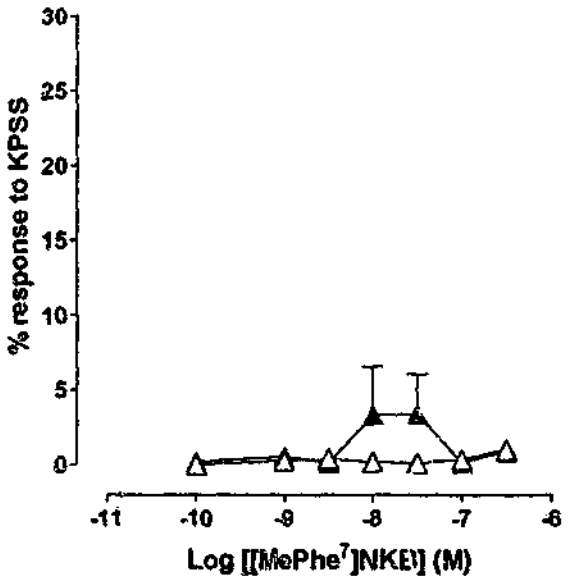


Figure 3.9: Log CRCs to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) on uterine preparations from oestrogen-treated mice in the absence of peptidase inhibitors (□) and in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM) (■). Each point is the mean ± SEM, n=6 animals.

3.3.3.5 [MePhe^7]NKB

Responses to [MePhe^7]NKB were unaffected by thiorphan (3μM) captopril (10μM) and bestatin (10μM) (two-way ANOVA, P>0.05; d.f. = 70) (Figure 3.10).

Figure 3.10: Log CRCs to [MePhe^7]NKB on uterine preparations from oestrogen-treated mice in the absence of peptidase inhibitors (△) and in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM) (▲). Each point is the mean ± SEM, n=6 animals.



3.3.3.6 Methacholine and KPSS

None of the peptidase inhibitor combinations used in this study; thiorphan (3μM); thiorphan (3μM) and captopril (10μM); thiorphan (3μM) and bestatin (10μM) or thiorphan (3μM), captopril (10μM) and bestatin (10μM) significantly affected the responses to MCh or KPSS (one-way ANOVAs, P>0.05) (Figure 3.11).

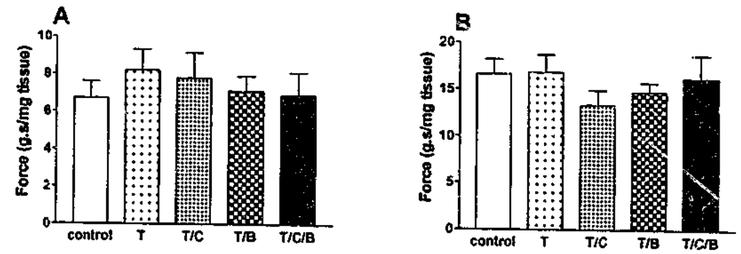


Figure 3.11: Responses to (A) MCh (10μM) and (B) KPSS in the absence of peptidase inhibitors (control; n=38) and in the presence of thiorphan (3μM) (T; n=18), thiorphan (3μM) and captopril (10μM) (T/C; n=8), thiorphan (3μM) and bestatin (10μM) (T/B; n=10) and thiorphan (3μM), captopril (10μM) and bestatin (10μM) (T/C/B; n=30). These data have been pooled from the responses to MCh and KPSS applied at the completion of log CRCs shown in Figures 3.6-3.10. Each bar is the mean ± SEM, n=number of preparations.

No significant differences in mean responses to KPSS were observed between treatment groups for each series of experiments (one-way ANOVA or Student's unpaired t-test, $P>0.05$; Table 3.3). Responses to MCh were similar in all series of experiments with the exception of when [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) was used as an agonist and a significantly lower response to MCh was seen in tissues when peptidase inhibitors were not present (Student's unpaired t-test, $P<0.05$). The maximal responses to peptides (E_{max} % KPSS) did not differ significantly between treatment groups for each series of experiments with the exception of [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) where a significantly lower E_{max} was seen in the absence of peptidase inhibitors (Student's unpaired t-test, $P<0.05$; Table 3.3). Maximal responses to peptides were either similar to or significantly lower than the mean response to MCh (Student's unpaired t-test, $P<0.05$).

In the absence of peptidase inhibitors SP and NKA had similar potencies. Of the receptor-selective agonists only [Sar^{9}Met(O$_2$)^{11}]SP acted as an agonist but a pD_2 value could not be obtained in the absence of peptidase inhibitors. A direct comparison of the potency of SP, NKA and [Sar^{9}Met(O$_2$)^{11}]SP in the presence of peptidase inhibitors was not made as differing combinations of peptidase inhibitors had been used in this pilot investigation.

Table 3.3: Effects of peptides, MCh (10μM) and KPSS in the absence and presence of peptidase inhibitors on myometrium from oestrogen-primed mice.

N	Agonist	Peptidase inhibitor	Response to KPSS (gs/mg tissue ± SEM)	Response to MCh (% KPSS ± SEM)	Peptide E_{max} (% KPSS ± SEM)	Peptide potency (pD_2 ± SEM)
8	SP		19.2 ± 2.6	60.2 ± 10.9	23.2 ± 4.1	7.58 ± 0.39
8	SP	T	17.6 ± 3.8	61.6 ± 9.8	28.6 ± 4.6	7.97 ± 0.70
8	SP	T & C	13.3 ± 1.6	57.1 ± 6.5	34.6 ± 5.0	7.94 ± 0.74
10	NKA		18.3 ± 2.1	49.2 ± 5.2	26.7 ± 3.5	7.64 ± 0.13
10	NKA	T	16.4 ± 1.0	45.1 ± 4.1	33.2 ± 3.7	7.77 ± 0.14
10	NKA	T & B	14.7 ± 1.0	53.9 ± 5.0	36.8 ± 3.6	7.84 ± 0.23
10	NKA	T, C & B	15.7 ± 1.4	46.3 ± 4.7	37.1 ± 5.0	8.02 ± 0.10
8	[Sar^{9}Met(O$_2$)^{11}]SP		19.3 ± 2.7	32.1 ± 5.7	10.6 ± 3.6	N/A
8	[Sar^{9}Met(O$_2$)^{11}]SP	T, C & B	19.2 ± 3.0	48.0 ± 5.9	22.2 ± 5.1	8.22 ± 0.28
6	[Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10)		13.0 ± 2.8	15.9 ± 2.0[a]	2.6 ± 1.4[b]	N/A
6	[Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10)	T, C & B	15.8 ± 1.8	48.3 ± 5.9	9.4 ± 2.0	N/A
6	[MePhe^7]NKB		13.4 ± 2.8	22.7 ± 5.5	1.2 ± 0.6	N/A
6	[MePhe^7]NKB	T, C & B	14.2 ± 2.2	40.3 ± 10.2	4.6 ± 3.1	N/A

N – number of preparations
T – thiorphan (3μM), C – captopril (10μM), B – bestatin (10μM)
a – significantly different from the corresponding value obtained in the presence of peptidase inhibitors
b – significantly different from the corresponding value obtained in the presence of peptidase inhibitors
N/A – not applicable (could not be estimated as log CRC did not reach a clear maximum

3.4 Discussion

This pilot study was undertaken as a preliminary investigation of the effects of tachykinins on myometrium from the oestrogen-primed mouse. Due to the susceptibility of tachykinins to peptidase-mediated degradation it was important to ensure that inactivation of these peptides in this tissue was minimized before undertaking the more extensive studies reported in chapters 4 and 5. Both SP and NKA produced contractions of the longitudinal myometrium and these responses were usually potentiated in the presence of peptidase inhibitors. The NK_1 receptor-selective agonist [$Sar^9Met(O_2)^{11}$]SP produced a modest contractile effect and this effect was also potentiated in the presence of peptidase inhibitors. The NK_2 and NK_3 receptor-selective agonists [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and [$MePhe^7$]NKB were without effect in this tissue in either the absence or presence of peptidase inhibitors.

The effects of tachykinins in regulating myometrial activity have been studied extensively in the rat (Barr *et al.*, 1991; Candenas *et al.*, 2001; Fisher and Pennefather, 1997; 1998; 1999; Fisher *et al.*, 1993; Hamlin *et al.*, 2000; Magraner *et al.*, 1998; Moodley *et al.*, 1999; Shintani *et al.*, 2000). In the uterus of the oestrogen-primed rat, mammalian tachykinins were reported to enhance myometrial activity with an order of potency of NKA>SP≥NKB, indicating activation of NK_2 receptors (Fisher and Pennefather, 1997; Magraner *et al.*, 1998; Pennefather *et al.*, 1993). In the present pilot study, experiments were undertaken only with the mammalian tachykinins SP and NKA. In the absence of peptidase inhibitors these two peptides were approximately equipotent and therefore no assumption could be made regarding the nature of the tachykinin receptor(s) mediating these contractions, given that at high concentrations the mammalian tachykinins can activate all three receptor types (Maggi, 1995a).

Additional experiments were then undertaken using the respective NK_1, NK_2 and NK_3 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP (Drapeau *et al.*, 1987), [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) (Chassaing *et al.*, 1991) and [$MePhe^7$]NKB (Drapeau *et al.*, 1987). In the oestrogen-primed mouse uterus [$Sar^9Met(O_2)^{11}$]SP enhanced myometrial activity. In contrast, neither [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) nor [$MePhe^7$]NKB were effective in producing myometrial contractions. These results

suggest that in the oestrogen-primed mouse uterus activation of the NK_1 receptor is involved in the contractile responses elicited by tachykinins in this tissue. This contrasts with corresponding studies using rat myometrium where [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) enhanced myometrial contractions but the NK_1 and NK_3 receptor-selective agonists were ineffective (Fisher and Pennefather, 1997; Fisher *et al.*, 1993; Moodley *et al.*, 1999).

In the present study the responses to both the mammalian tachykinins SP and NKA were potentiated in the presence of peptidase inhibitors. This is consistent with the extensive literature documenting the susceptibility of tachykinins to degradation by various peptidases. Neprilysin is considered the most effective of the peptidases cleaving not only the mammalian tachykinins (Matsas *et al.*, 1983; 1985) but also several of the receptor-selective analogues (Medeiros and Turner, 1995). The presence of neprilysin has been detected in the rat uterus (Ottlecz *et al.*, 1991; Pinto *et al.*, 1999) and accordingly studies have shown that in this tissue the response to the mammalian tachykinins are potentiated in the presence of the neprilysin inhibitors phosphoramidon and SCH 39370 (Fisher and Pennefather, 1997; Fisher *et al.*, 1993). There has been one report of phosphoramidon, at the same concentration as used by Pennefather and colleagues, having no effect on the response to NKA in the non-pregnant rat uterus (Shintani *et al.*, 2000). The inconsistency in results between these two studies is likely to be due to differences in methodology, with Pennefather and colleagues measuring responses to the agonists using area under the force-time curve while Shintani *et al.* (2000) measured peak height. The expression of neprilysin in the rat uterus has also been reported to be under hormonal regulation (Pinto *et al.*, 1999) and while rats used in the study by Fisher *et al.* (1993) had been oestrogen-primed, those used by Shintani *et al.* (2000) were not, nor were the cycle stages monitored. To date there is no literature indicating whether neprilysin is also present in the mouse uterus but the potentiation of NKA in the presence of thiorphan alone suggests that this is likely to be the case.

Interestingly, thiorphan alone was not shown to potentiate the response to SP in the oestrogen-primed mouse uterus. This finding is inconsistent with the results of other investigators who have found that SP is hydrolysed by purified neprilysin (Matsas *et al.*,

1984). It also contrasts with that observed in the oestrogen-primed rat uterus where SCH 39370 was reported to potentiate the response to SP (Fisher and Pennefather, 1997). In this pilot study the effect of captopril alone on the response to SP was not investigated. However, as the response to SP was potentiated in the combined presence of thiorphan and captopril and not thiorphan alone it is likely that captopril potentiates the response to SP in the oestrogen-primed mouse uterus. This is consistent with SP being a substrate for ACE (Cascieri *et al.*, 1984; Skidgel *et al.*, 1984; Yokosawa *et al.*, 1983). Though ACE has been detected in the rat uterus (Cushman and Cheung, 1971), captopril was without effect on the response to SP in this tissue (Fisher and Pennefather, 1997). It has previously been reported that SP is equally susceptible to neprilysin (K_m = 25.4μM; Nau *et al.*, 1986) as for ACE (K_m = 25μM; Skidgel *et al.*, 1984) but that neprilysin is able to breakdown SP considerably faster than ACE. As the K_{cat}/K_m value is significantly lower for ACE then that for neprilysin it has been suggested that ACE cannot successfully compete for available SP (Nau *et al.*, 1986) unless the concentration of ACE is considerably in excess of that of neprilysin (Matsas *et al.*, 1985). To date there is no literature on the concentrations of neprilysin and ACE in the uterus of the oestrogen-primed mouse uterus but it would be of interest to confirm whether ACE is present in a much greater concentration than neprilysin in this tissue.

It has previously been suggested that SP degradation occurs in two steps (Teichberg and Blumberg, 1980), with SP firstly being cleaved by a post-proline cleaving enzyme giving an N-terminal tetrapeptide and a C-terminal heptapeptide, the latter which then undergoes further degradation. Present literature indicates that only ACE (Yokosawa *et al.*, 1983; 1985) and dipeptidylaminopeptidase IV (DAP IV) (Ahmad *et al.*, 1992; Heymann and Mentlein, 1978; Wang *et al.*, 1991) degrade SP at the Pro^4-Gln^5 bond. Though the presence of DAP IV has been reported in the mouse uterus (Ohta *et al.*, 1992), it was not considered necessary to include a peptidase inhibitor for this enzyme when examining the response of SP in this study as the K_m value is approximately 2.0mM (Kato *et al.*, 1978) and therefore would not represent a major mechanism of inactivation of SP. In addition, the resulting heptapeptide after DAP IV hydrolysis is also reported to be equipotent with SP (Teichberg and Blumberg, 1980).

In the oestrogen-primed mouse uterus responses to NKA were potentiated in the presence of thiorphan alone and further potentiated in the presence of thiorphan together with bestatin. There was no additional potentiation of the response to NKA in the presence of thiorphan and bestatin together with captopril. This is consistent with NKA being a substrate for both neprilysin and bestatin-sensitive aminopeptidases but not ACE and that complete inhibition of NKA hydrolysis only occurs in the combined presence of phosphoramidon together with bestatin (Hooper *et al.*, 1985; Hooper and Turner, 1985). In the oestrogen-primed rat uterus SCH 39370 was reported to potentiate the response to NKA but no further potentiation was seen in the combined presence of SCH 39370 and the aminopeptidase inhibitor amastatin (Fisher and Pennefather, 1997). The authors suggest that as the aminopeptidases only hydrolyse the N-terminal of NKA, the resulting NKA(4-10) is sufficient for tachykinin NK_2 receptor activation as has been found by other investigators (Regoli *et al.*, 1990; Rovero *et al.*, 1989).

It has previously been suggested that the synthetic tachykinin agonists are less susceptible to enzymatic degradation than the natural tachykinins (Brokaw and White, 1994). However, in the present study the response to [$Sar^9Met(O_2)^{11}$]SP was significantly potentiated in the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM) indicating that this peptide is still susceptible to peptidase degradation, although it has been suggested that the Sar^9 substitution possibly protects SP from the actions of some endopeptidases (Regoli *et al.*, 1988). A similar effect was also observed in the oestrogen-primed rat uterus where the response to [$Sar^9Met(O_2)^{11}$]SP was potentiated four-fold in the presence of both SCH 39370 and captopril (Fisher and Pennefather, 1997).

Neither [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) nor [$MePhe^7$]NKB were effective in producing contractions in the oestrogen-primed mouse uterus. A previous study reported that in the guinea-pig isolated oesophagus the response to [$MePhe^7$]NKB was potentiated in the presence of the peptidase inhibitors captopril, thiorphan and amastatin indicating that it is susceptible to peptidase degradation (Kerr *et al.*, 1997). [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) has previously been reported as being metabolically stable (Fisher and Pennefather, 1997; Fisher *et al.*, 1993). The lack of activity of [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and

[MePhe^7]NKB in the mouse uterus does not appear to be due to peptidase degradation as no potentiation of the response to either peptide was seen in the presence of the peptidase inhibitors.

Taken together, the results of the present study suggest that neprilysin and to a lesser extent captopril and bestatin may play a key role in inactivating tachykinins in the mouse uterus and emphasize the need to include peptidase inhibitors when conducting functional studies of tachykinin receptor subtypes as the degradation of these peptides could lead to inaccurate potency estimates.

In a separate series of experiments the responses to repeated applications of high concentrations of SP (100nM) and NKA (100nM) were examined. These experiments were undertaken as it was noticed that during the construction of log concentration-response curves to SP and NKA that belling of the curve occurred with the higher concentrations of peptides. This is suggestive that desensitization was occurring and it was therefore prudent to check whether decreasing the time that the peptide was in contact with the tissue or increasing the interval between doses would prevent this from happening. It was found that successive responses to six repeated applications of SP or NKA were reduced irrespective of whether the agonist contact time was 5 or 2min and the interval between doses 15 or 30min. This is consistent with the literature indicating that cellular responses to SP that are mediated by the NK_1 receptor are rapidly desensitized (Grady *et al.*, 1997). Other studies have suggested that this desensitisation may not occur with responses mediated through the NK_2 or NK_3 receptors. In the mouse distal colon it was found that after desensitization to SP, the contractile activity evoked by SP and the NK_1 receptor-preferring physalaemin was totally abolished yet responses evoked by the NK_2/NK_3 receptor-preferring eledoisin and kassinin were barely affected (Fontaine and Lebrun, 1989). Though NKA is NK_2 receptor preferring, the inactivity of the NK_2 receptor-selective [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) in the mouse myometrium indicates that it is unlikely that NKA was mediating responses through an NK_2 receptor in this tissue. Thus the observed desensitization seen with NKA is consistent with the response being mediated predominantly by an NK_1 receptor. Some degree of desensitization occurred when SP was present for either 2 or 5min but it achieved

statistical significance with the shorter contact time, hence it was decided that the agonist contact time in subsequent experiments would remain as 5min. While the extent of desensitization was not as great when the time between doses was increased from 15 to 30min, desensitization still occurred with 30min and it was decided to adhere to the shorter time interval in order to be consistent with previous work carried out in our laboratory.

In conclusion, the results of the present pilot study indicate the importance of the tachykinin NK_1 receptor in mediating tachykinin-induced contractions in myometrial preparations from oestrogen-primed mice. The uterotonic effects of SP, NKA and $[Sar^9Met(O_2)^{11}]SP$ also appear to be influenced by inhibition of neprilysin, ACE and bestatin-sensitive aminopeptidases, thus emphasizing the need to include peptidase inhibitors when conducting functional studies looking at the effects of tachykinins in this tissue. Both these findings are consistent with previous work conducted in our laboratory on the effects of tachykinins on the oestrogen-primed mouse myometrium (Fleming *et al.*, 1998). Further studies are needed to confirm the relative importance of the individual tachykinin receptors in contributing to the contractile activity of tachykinins in this tissue. These studies include examination of the effects of the mammalian tachykinins SP, NKA and NKB in the presence of the same combination of peptidase inhibitors so that an order of agonist potency can be determined, and the use of the respective tachykinin NK_1, NK_2 and NK_3 receptor-selective antagonists SR 140333, SR 48968 and SR 142801 to determine their effects on the responses to the tachykinin agonists. These findings are described in the following chapter.

CHAPTER 4

CHARACTERIZATION OF THE UTEROTONIC EFFECTS OF TACHYKININ PEPTIDES ON THE OESTROGEN-PRIMED MOUSE UTERUS

Summary

1. The aims were (1) to characterize the receptor type(s) mediating the uterotonic activity of the tachykinins in uterus from oestrogen-primed mice, (2) to examine whether hydrolysis of glutamine in positions 5 and 6 of the SP sequence alters the potency of SP and (3) to examine whether either histamine or prostaglandin contributed to the response to SP in this preparation.

2. In the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM) the mammalian tachykinins SP, NKA and NKB acted as full agonists with an order of potency of SP≥NKA>NKB.

3. The NK_1 receptor-selective agonist [$Sar^9Met(O_2)^{11}$]SP elicited concentration-related contractions. In contrast the NK_2 receptor-selective agonist [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) was relatively inactive. The potencies of the SP analogues in which Glu replaced Gln^5 and/or Gln^6 were similar to that of SP indicating that hydrolysis of glutamine at positions 5 and/or 6 did not significantly alter the potency of SP.

4. The NK_1 receptor-selective antagonist SR 140333 (10nM) alone or combined with the NK_2 receptor-selective antagonist SR 48968 (10nM) significantly attenuated responses to SP, NKA, NKB and [$Sar^9Met(O_2)^{11}$]SP. SR 48968 (10nM) was without effect on the response to SP and [$Sar^9Met(O_2)^{11}$]SP but significantly attenuated the response to NKA and NKB. The NK_3 receptor-selective antagonist SR 142801 (0.3μM) slightly potentiated the response to SP and slightly attenuated the response to NKB.

5. Responses to SP were unaffected by the prostaglandin synthesis inhibitor indomethacin (10μM) either alone or combined with the histamine H_1 receptor antagonist mepyramine (0.1μM), or the histamine H_2 receptor antagonist ranitidine (10μM).

6. The results of this chapter indicate that the contractile effects of the tachykinins in uterus from oestrogen-primed mice are predominantly mediated by the NK_1 receptor. The response to NKA and NKB may also in part be mediated by the NK_2 receptor. No evidence was obtained to indicate that release of either histamine or prostaglandins contributed to the uterotonic effects of SP in this preparation.

4.1 Introduction

In the previous chapter it was established that the mammalian tachykinins SP and NKA and the NK_1 receptor-selective agonist [$Sar^9Met(O_2)^{11}$]SP elicit contractile activity in uterus from oestrogen-treated mice. In addition, it was also reported that the inclusion of peptidase inhibitors is necessary when testing the effects of tachykinin peptides in this tissue.

Tachykinins mediate their actions through three distinct receptors termed NK_1, NK_2 and NK_3, with an order of agonist potency of SP>NKA>NKB at NK_1 receptors; NKA>NKB>SP at NK_2 receptors and NKB>NKA>SP at NK_3 receptors (Henry, 1986). Although at low concentrations the mammalian tachykinins show preference for their respective receptors, at high concentrations they can act at all three receptors (Maggi, 1995a). The ability of the endogenous tachykinins to interact with all three receptor types has made receptor characterization difficult. Consequently, the availability of highly selective agonists and antagonists acting at the tachykinin receptor types provides an additional opportunity to characterize the relative contribution of the three tachykinin receptors to tachykinin-induced responses.

Preliminary observations in our laboratory examining the effects of the mammalian tachykinins and tachykinin receptor-selective agonists and antagonists on uterus from oestrogen-treated mice indicated the need for further studies to elucidate the nature of the tachykinin receptor types contributing to the uterotonic activity of these peptides in this tissue (Fleming, 1998). In the preliminary study in the presence of thiorphan (10μM; for the endogenous tachykinins only) the rank order of agonist potency was SP≥NKA>[$Lys^5MeLeu^9Nle^{10}$]NKA(4-10)>NKB=[$Sar^9Met(O_2)^{11}$]SP>>>[$MePhe^7$]NKB which is consistent with activation of an NK_1 receptor, although the high potency of [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) also suggests activation of NK_2 receptors. However, while the NK_1 receptor-selective antagonist SR 140333 (10nM) antagonised responses to SP and [$Sar^9Met(O_2)^{11}$]SP, the NK_2 receptor-selective antagonist SR 48968 (10nM) did not antagonise responses to either NKA or [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10), which suggested activation of only NK_1 receptors (Fleming, 1998).

The aims of the experiments described in this chapter were three-fold. The first aim was to complete the characterization of the tachykinin receptor type(s) mediating contractile activity in myometrium from oestrogen-treated mice by determining an order of agonist potency for the mammalian tachykinins in the presence of peptidase inhibitors and by using receptor-selective agonists. Tachykinin receptor types present in this preparation would be further confirmed by the use of the potent and selective NK_1, NK_2 and NK_3 non-peptide tachykinin antagonists SR 140333, SR 48968 and SR 142801 from Sanofi Recherche. Analysis of SP purity by Auspep, the suppliers of this peptide, has revealed that over time hydrolysis of glutamine in position 5 and 6 may occur thus potentially affecting the potency of this peptide. The second aim was to examine whether hydrolysis of glutamine in positions 5 and/or 6 of the SP sequence alters its potency. The third aim was to investigate whether the response to SP in the uterus of the oestrogen-treated mouse was due in part to the release of either histamine or prostaglandins as has been reported to occur in other tissues and cells such as the guinea-pig lung (Gerard, 1987) and human skin mast cell (Columbo *et al.*, 1996).

4.2 Materials and Methods

4.2.1 Animals and tissue preparations

Full details of animals and preparations are given in section 2.1.1 and 2.2.2. Briefly, virgin, female Balb C mice weighing between 20 –25g were treated with oestradiol-17β-cypionate (200μg/kg), s.c., 24h prior to use. Four uterine preparations were obtained from each animal and set up for recording of force from the longitudinal smooth muscle layer.

4.2.2 Experimental protocol

Refer to Chapter 2 (section 2.3). In brief, following the equilibration period discrete concentration-response curves were constructed. Each agonist concentration remained in contact with the tissue for 5min, was washed out and a higher concentration of agonist was added 15min later (initial concentrations increasing in log units until a response was observed and then increasing in 0.5 log increments). Only one concentration-response curve was generated on each preparation. All preparations were also exposed to methacholine (MCh) (10μM) prior to the addition of KPSS to the bath.

4.2.3 Measurement of results and statistical analyses

Responses to all agonists were measured as area under the force-time curve for the duration of agonist exposure as described in section 2.4. Responses were then expressed as a percentage of the response to the KPSS and presented as mean ± SEM.

Statistical analysis was undertaken as described in section 2.6.

E_{max} is defined as the maximum response to an agonist observed over the concentration range used; note that might not in all cases be the greatest response that an agonist may have been able to elicit if higher concentrations had been tested.

Table 4.1: Overview of experiments undertaken on uterine preparations obtained from oestrogen-treated mice. Note that preparations of upper and lower segments of left and right uterine horns are allocated as preparation 1, 2, 3 or 4 using a randomized procedure.

Series	Preparation 1		Preparation 2		Preparation 3		Preparation 4	
	Agonist	Treatment	Agonist	Treatment	Agonist	Treatment	Agonist	Treatment
1: SP in the absence and presence of SR 140333 and SR 48968 (n=10)	SP*	0.001% ethanol	SP*	SR 140333 (10nM)	SP*	SR 48968 (10nM)	SP*	SR 140333 (10nM) & SR 48968 (10nM)
2: NKA in the absence and presence of SR 140333 and SR 48968 (n=9)	NKA*	0.001% ethanol	NKA*	SR 140333 (10nM)	NKA*	SR 48968 (10nM)	NKA*	SR 140333 (10nM) & SR 48968 (10nM)
3: NKB in the absence and presence of SR 140333 and SR 48968 (n=9)	NKB*	0.001% ethanol	NKB*	SR 140333 (10nM)	NKB*	SR 48968 (10nM)	NKB*	SR 140333 (10nM) & SR 48968 (10nM)
4: SP and NKB in the absence and presence of SR 142801 (n=10)	SP*	-	SP*	SR 142801 (0.3 μM)	NKB*	-	NKB*	SR 142801 (0.3 μM)
5: Sar^9 in the absence and presence of SR 140333 and SR 48968 (n=8)	Sar^9*	-	Sar^9*	SR 140333 (10nM)	Sar^9*	SR 48968 (10nM)	-	-
6: Lys^5 in the absence and presence of SR 140333, SR 48968 (n=6)	Lys^5*	-	Lys^5*	SR 140333 (10nM)	Lys^5*	SR 48968 (10nM)	Lys^5*	SR 142801 (0.3μM)
7: SP analogues in the presence of peptidase inhibitors (n=6)	SP*	-	$[Glu^5]$SP*	-	$[Glu^6]$SP*	-	$[Glu^{5,6}]$SP*	-
8: SP in the absence and presence of indomethacin, ranitidine and mepyramine (n=11)	SP*	-	SP*	Indomethacin (10μM)	SP*	Indomethacin (10μM) & ranitidine (10μM)	SP*	Indomethacin (10μM) & mepyramine (0.1μM)

Sar^9 = $[Sar^9Met(O_2)^{11}]$SP, Lys^5 = $[Lys^5MeLeu^9Nle^{10}]$NKA(4-10)

* indicates peptidase inhibitors present (thiorphan (3μM), captopril (10μM) and bestatin (10μM))

n = number of animals

- = no treatment

4.2.4 Drugs

The drugs used included all those previously described in section 2.7. Appropriate vehicle controls were carried out in the presence of 0.001% ethanol, the solution used for dissolving the antagonists.

4.3 Results

Uterine tissue used throughout this study was obtained from 69 oestrogen-treated mice (200μg/kg; s.c) weighing between 20-25g. Histological examination of vaginal smears confirmed cornification of the vaginal epithelium. The mean weight of each tissue preparation was 28.9 ± 0.5mg (n = 268 preparations). The mean response to KPSS was 16.9 ± 0.4g.s/mg tissue (n = 268 preparations). The mean response to MCh (10μM) was 46.6 ± 1.4% of the response to KPSS (n = 268 preparations). Spontaneous activity was observed in nearly all tissue preparations and ranged from 1 – 12 contractions over any 15min period, this would gradually decrease over the duration of the equilibration period. Figure 4.1 shows a representative trace of responses to increasing concentrations of SP in the absence and presence of the NK_1 receptor-selective antagonist SR 140333.

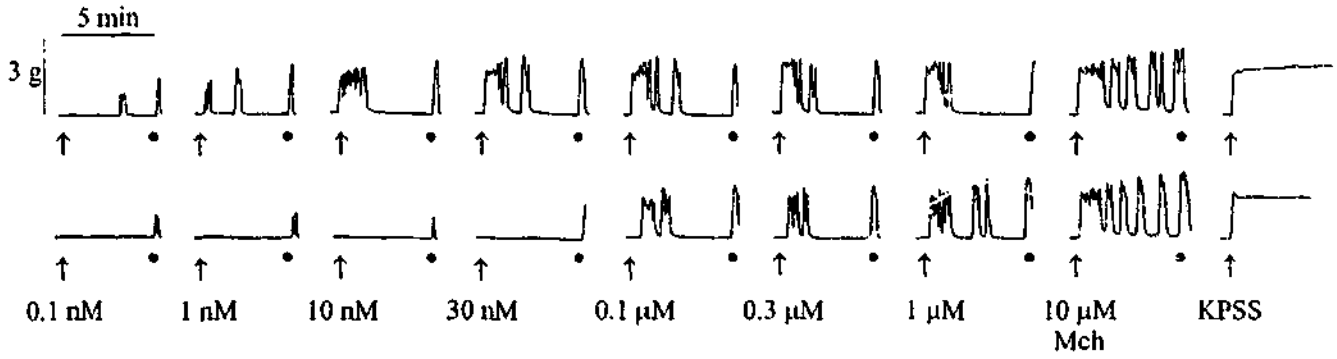


Figure 4.1: Representative trace showing response of longitudinal myometrium from an oestrogen-treated mouse to SP in the absence (upper panel) and presence of SR 140333 (10nM; lower panel) together with responses to MCh (10μM) and KPSS. All responses are in the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM). The arrows indicate agonist addition, the dots represent washes.

4.3.1 Uterotonic activity of substance P analogues

No difference was seen in the potencies of [Glu^5]SP (potency ratio = 1.9, 95% CL = 0.4, 9.8; d.f. = 39), [Glu^6]SP (potency ratio = 4.3, 95% CI = 0.9, 114.9; d.f. = 36) and [Glu^{5,6}]SP (potency ratio = 2.6, 95% CI = 0.9, 9.4; d.f. = 31) compared to SP (Figure 4.2). All three analogues acted as full agonists and the mean pD_2 and E_{max} values were similar to those for SP (one-way ANOVA, P>0.05). Responses to MCh and KPSS were similar in all four groups (Table 4.2).

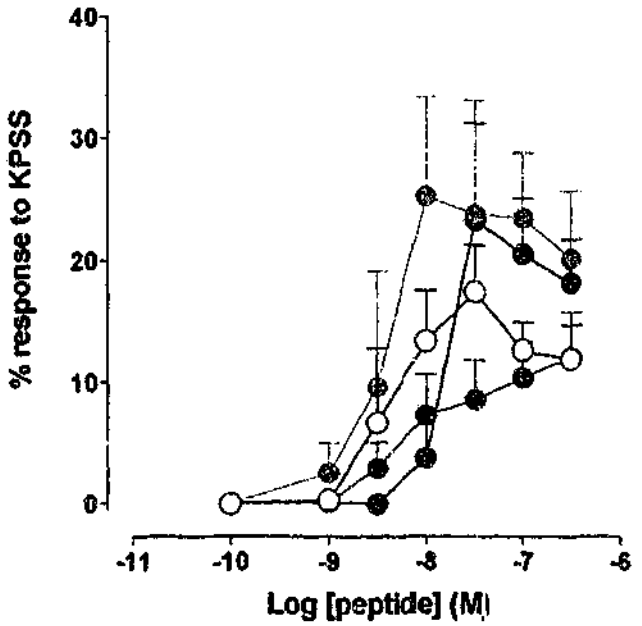


Figure 4.2: Log concentration-response curves (CRCs) to SP (○), [Glu^5]SP (◉), [Glu^6]SP (●) and [Glu^{5,6}]SP (●) on uterine preparations from oestrogen-treated mice in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). Note that log CRCs to SP, [Glu^5]SP and [Glu^{5,6}]SP reached a clear ceiling. Each point is the mean ± SEM, n=6 animals. In this and subsequent figures, a significant difference between log CRCs as indicated by two-way ANOVA followed by Student Newman Keuls is shown by *.

Table 4.2: Effects of peptides, MCh (10μM) and KPSS on myometrium from oestrogen-primed mice (n=6).

Peptide	Response to MCh (%KPSS ± SEM)	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (%KPSS ± SEM)	Peptide Potency (pD_2 ± SEM)
SP	38.0 ± 4.4	20.8 ± 3.6	22.5 ± 4.4	8.21 ± 0.25
[Glu^5]SP	40.4± 6.7	18.1 ± 2.9	33.5 ± 7.7	8.26 ± 0.26
[Glu^6]SP	32.2± 2.5	17.6 ± 2.2	18.5 ± 4.5	7.97 ± 0.16
[Glu^{5,6}]SP	34.0± 8.2	17.3± 2.3	29.3 ± 6.0	7.71 ± 0.23

4.3.2 Uterotonic activity of substance P, neurokinin A, neurokinin B, [Sar^{9}Met(O$_2$)^{11}]SP and [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10)

In the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM) SP was the most potent mammalian tachykinin and was 4.9 and 5.5 fold more potent than NKA (95% CL = 1.7, 18.1; d.f. = 82) and NKB (95% CL = 1.5, 42.8; d.f. = 55) respectively. [Sar^{9}Met(O$_2$)^{11}]SP was equipotent with SP (potency ratio = 2.6, 95% CL = 0.9, 11.0; d.f. = 68). [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) had minimal agonist activity at the concentrations examined (Figure 4.3).

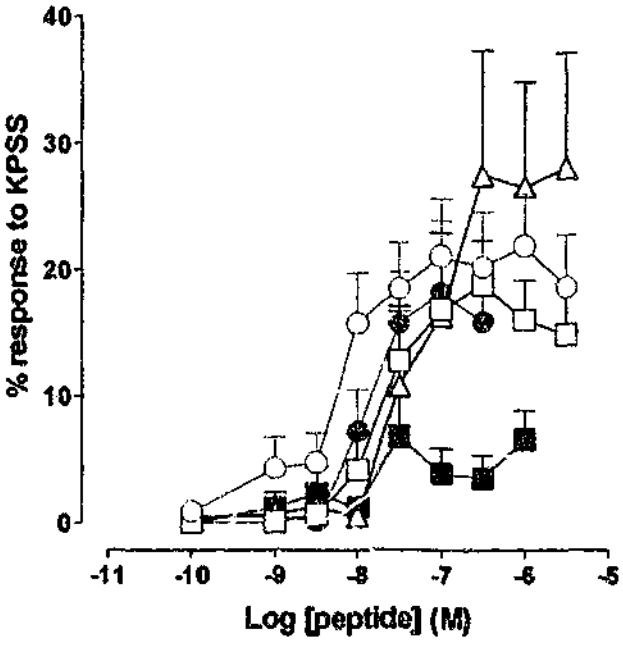


Figure 4.3: Log CRCs to SP (○), NKA (□), NKB (△), [Sar^{9}Met(O$_2$)^{11}]SP (●) and [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) (■) on uterine preparations from oestrogen-treated mice in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). Note that all log CRCs have reached a clear ceiling. Each point is the mean ± SEM, n=6-10 animals. Note that these CRCs are the control curves shown in Figures 4.4, 4.5, 4.6, 4.7 and 4.8.

4.3.3 Effect of tachykinin antagonists on the uterotonic effects of tachykinin peptides

4.3.3.1 Substance P with SR 140333 and SR 48968

SR 140333 (10nM) alone and SR 140333 (10nM) combined with SR 48968 (10nM) significantly attenuated the response to SP (two-way ANOVA, $P<0.05$; d.f. = 324). This attenuation corresponded to 108.3 and 599.9 fold rightward shifts of the log concentration-response curve to SP in the presence of SR 140333 (10nM) (95% CL =

34.6, 1049.3; d.f. = 64) and SR 140333 (10nM) together with SR 48968 (10nM) (95% CL = 105.3, 55555.5; d.f. = 65) respectively. SR 48968 (10nM) alone was without significant effect on the log concentration-response curve to SP (potency ratio = 2.6, 95% CL = 0.5, 8.2; d.f. = 67) (Figure 4.4).

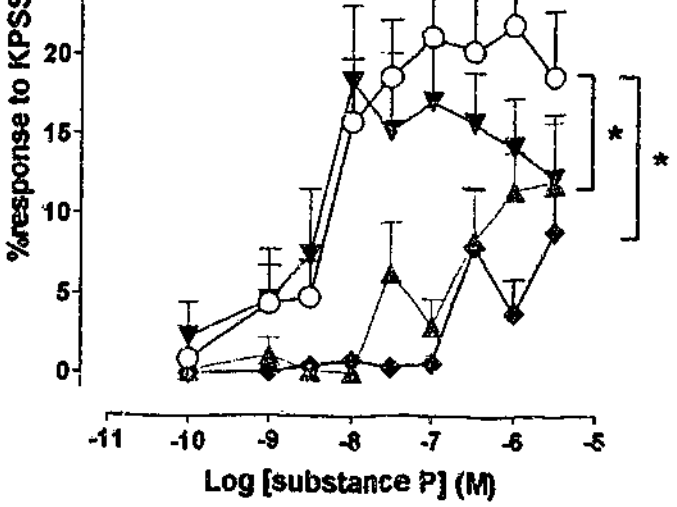


Figure 4.4: Log CRCs to SP in the absence of antagonist (O) and in the presence of SR 140333 (10nM) (▲), SR 48968 (10nM) (▼) and SR 140333 (10nM) combined with SR 48968 (10nM) (◆) on uterine preparations from oestrogen-treated mice. CRCs were constructed in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). 0.001% ethanol is present in the control CRC. Each point is the mean ± SEM, n=10 animals.
* indicates significant difference from control, two-way ANOVA, P<0.05; d.f. = 324.

4.3.3.2 Neurokinin A with SR 140333 and SR 48968

SR 140333 (10nM), SR 48968 (10nM) and SR 140333 (10nM) combined with SR 48968 (10nM) significantly attenuated the response to NKA (two-way ANOVA, P<0.05; d.f. = 285). This attenuation corresponded to a 4.7 fold rightward shift of the log concentration-response curve to NKA in the presence of SR 48968 (95% CL = 1.7, 14.8; d.f. = 87) with no depression of the maximum response. A potency ratio could not be obtained for NKA in the presence of SR 140333 alone as there were insufficient points on the linear component of the concentration-response curve, nor in the presence of SR 140333 together with SR 48968 as the shift obtained was not parallel. However, there was an indication that the maximum response to NKA was reduced in the presence of SR 140333 alone and in combination with SR 48968 (Figure 4.5).

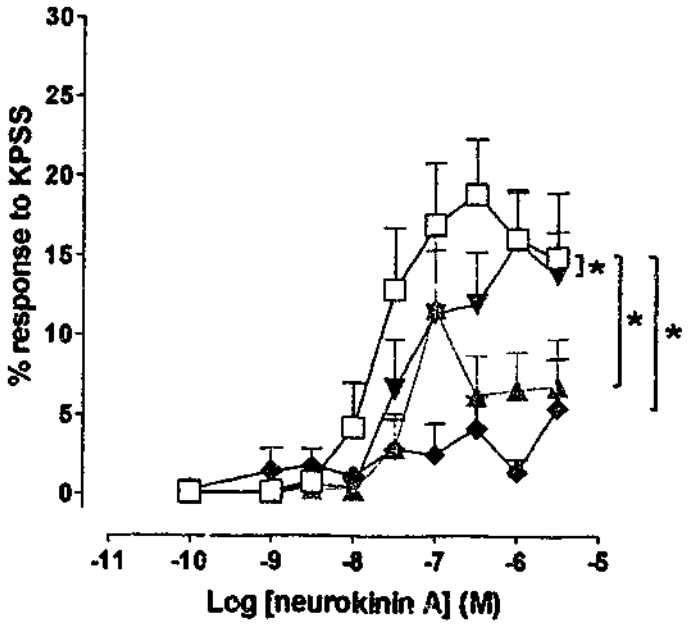


Figure 4.5: Log CRCs to NKA in the absence of antagonist (□) and in the presence of SR 140333 (10nM) (▲), SR 48968 (10nM) (▼) and SR 140333 (10nM) combined with SR 48968 (10nM) (◆) on uterine preparations from oestrogen-treated mice. CRCs were constructed in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). 0.001% ethanol is present in the control CRC. Each point is the mean ± SEM, n=9 animals.

* indicates significant difference from control, two-way ANOVA, $P<0.05$; d.f. = 285

4.3.3.3 Neurokinin B with SR 140333 and SR 48968

SR 140333 (10nM), SR 48968 (10nM) and SR 140333 (10nM) combined with SR 48968 (10nM) significantly attenuated the response to NKB (two-way ANOVA, $P<0.05$; d.f. = 288). This attenuation corresponded to 63.6 and 53.6 fold rightward shifts of the log concentration-response curve to NKB in the presence of SR 140333 (95% CL = 7.9, 3663.0; d.f. = 47) and SR 48968 (95% CL = 14.1, 513.1; d.f. = 59) respectively. A potency ratio could not be obtained for NKB in the presence of SR 140333 together with SR 48968 as the shift obtained was not parallel (Figure 4.6).

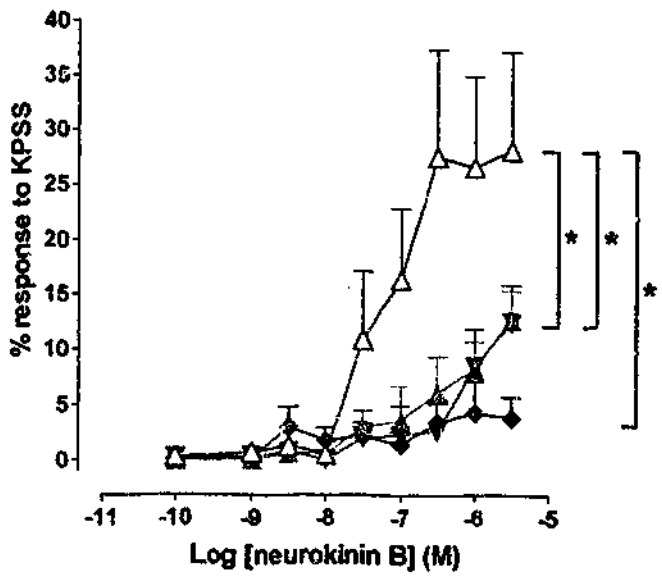


Figure 4.6: Log CRCs to NKB in the absence of antagonist (□) and in the presence of SR 140333 (10nM) (▲), SR 48968 (10nM) (▼) and SR 140333 (10nM) combined with SR 48968 (10nM) (◆) on uterine preparations from oestrogen-treated mice. CRCs were constructed in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). 0.001% ethanol is present in the control CRC. Each point is the mean ± SEM, n=9 animals.

* indicates significant difference from control, two-way ANOVA, $P<0.05$; d.f. = 288

4.3.3.4 [Sar^{9}Met(O$_2$)^{11}]SP with SR 140333 and SR 48968

Responses to [Sar^{9}Met(O$_2$)^{11}]SP were significantly attenuated in the presence of SR 140333 (10nM) (two-way ANOVA, $P<0.05$; d.f. = 147) however a potency ratio was not able to be obtained as the shift was not parallel. SR 48968 (10nM) was without effect on the log concentration-response curve to [Sar^{9}Met(O$_2$)^{11}]SP (potency ratio = 1.6, 95% CL = 0.5, 7.0; d.f. = 35) (Figure 4.7).

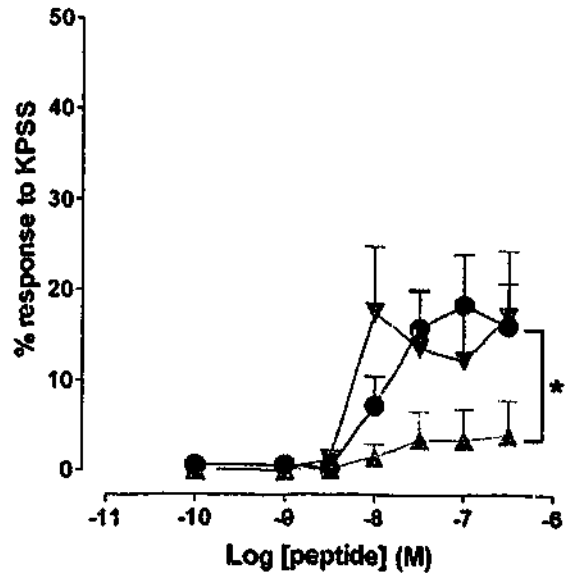


Figure 4.7: Log CRCs to [Sar^{9}Met(O$_2$)^{11}]SP in the absence of antagonist (●) and in the presence of SR 140333 (10nM) (▲) and SR 48968 (10nM) (▼) on uterine preparations from oestrogen-treated mice. CRCs were constructed in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). Each point is the mean ± SEM, n=8 animals.

* indicates a significant difference from control, two-way ANOVA, $P<0.05$; d.f. = 147

Table 4.3: Effects of peptides, MCh (10μM) and KPSS in the absence and presence of tachykinin antagonists on myometrium from oestrogen-primed mice.

N	Agonist	Antagonist	Response to MCh (% KPSS ± SEM)	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (% KPSS ± SEM)	Peptide potency (pD_2 ± SEM)
10	SP		43.6 ± 8.4	18.1 ± 2.0	26.0 ± 4.6	7.71 ± 0.34
10	SP	SR 140333 (10nM)	38.8 ± 6.2	18.5 ± 2.3	18.8 ± 3.3	6.19 ± 0.34
10	SP	SR 48968 (10nm)	41.5 ± 7.3	18.3 ± 1.5	28.7 ± 3.8	8.07 ± 0.28
10	SP	SR 140333 & SR 48968	38.7 ± 3.9	17.5 ± 1.6	12.1 ± 3.5[a]	N/A
9	NKA		36.9 ± 4.5	17.0 ± 2.1	20.2 ± 3.4	7.52 ± 0.16
9	NKA	SR 140333 (10nM)	34.3 ± 5.3	16.1 ± 1.5	12.8 ± 4.5	6.73 ± 0.25
9	NKA	SR 48968 (10nM)	40.9 ± 3.3	15.5 ± 1.5	18.3 ± 2.9	7.27 ± 0.20
9	NKA	SR 140333 & SR 48968	40.3 ± 4.3	15.2 ± 2.0	9.8 ± 3.7	N/A
9	NKB		35.0 ± 8.7	16.6 ± 0.9	32.1 ± 9.4	6.58 ± 0.53
9	NKB	SR 140333 (10nM)	31.1 ± 4.8	18.9 ± 2.4	14.7 ± 2.8	N/A
9	NKB	SR 48968 (10nM)	34.7 ± 4.1	18.0 ± 1.8	16.1 ± 2.8	N/A
9	NKB	SR 140333 & SR 48968	34.9 ± 3.6	18.2 ± 1.9	8.3 ± 2.8[b]	N/A
8	Sar^9		48.0 ± 5.9	19.2 ± 3.0	22.2 ± 5.1	7.69 ± 0.07
8	Sar^9	SR 140333 (10nM)	49.1 ± 4.5	19.6 ± 3.4	5.8 ± 3.8	N/A
8	Sar^9	SR 48968 (10nM)	51.0 ± 7.3	18.9 ± 2.0	21.4 ± 7.5	8.03 ± 0.23
6	Lys^5		48.3 ± 5.9	15.8 ± 1.8	9.4 ± 2.0	N/A
6	Lys^5	SR 140333 (10nM)	45.4 ± 6.5	18.7 ± 2.6	12.2 ± 3.6	N/A
6	Lys^5	SR 48968 (10nM)	51.1 ± 9.1	18.2 ± 2.5	9.8 ± 2.2	N/A
6	Lys^5	SR 142801 (0.3μM)	43.8 ± 5.4	12.4 ± 1.8	13.0 ± 2.9	N/A

N – number of preparations
Sar^9 – [$Sar^9Met(O_2)^{11}$]SP, Lys^5 – [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10)
a – significantly different from the corresponding value obtained in the absence of antagonists
b – significantly different from the corresponding value obtained in the absence of antagonists
N/A – not applicable (could not be estimated as log CRC did not reach a clear maximum)

4.3.3.5 [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) with SR 140333, SR 48968 and SR 142801

SR 140333 (10nM), SR 48968 (10nM) and SR 142801 (0.3μM) were without effect on the response to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) (two-way ANOVA, $P>0.05$; d.f. = 160) (Figure 4.8).

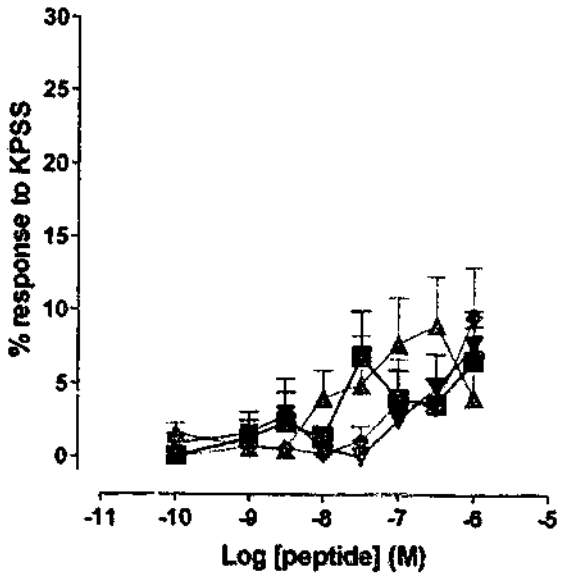


Figure 4.8: Log CRCs to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) in the absence of antagonist (■) and in the presence of SR 140333 (10nM) (▲), SR 48968 (10nM) (▼) and SR 142801 (0.3μM) (◆) on uterine preparations from oestrogen-treated mice. CRCs were constructed in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). Each point is the mean ± SEM, n=6 animals.

No significant differences in the mean responses to MCh or KPSS were observed between treatment groups for each series of experiments (one-way ANOVAs, $P>0.05$; Table 4.3). The maximal responses to peptides (E_{max} % KPSS) did not differ significantly between treatment groups with the exception of both SP and NKB in the presence of SR 140333 (10nM) combined with SR 48968 (10nM) where significantly lower mean E_{max} values were seen (one-way ANOVA, $P<0.05$; Table 4.3). Maximal responses to peptides were either similar or significantly lower than the mean response to MCh (Student's unpaired t-test, $P<0.05$; Table 4.3).

4.3.3.6 Substance P and neurokinin B with SR 142801

Analysis by two-way ANOVA showed that SR 142801 (0.3μM) significantly potentiated the response to SP ($P<0.05$; d.f. = 144) and significantly attenuated the response to NKB ($P<0.05$; d.f. = 162). This effect was small (Figure 4.9) and potency ratio determination did not indicate that the shifts seen with SP (potency ratio = 0.9, 95% CL = 0.3, 2.4; d.f. = 67) or NKB (potency ratio = 2.3, 95% CL = 1.0, 5.2; d.f. = 87) in the presence of SR 142801 (0.3μM) were significant. SR 142801 (0.3μM) had no significant effect on responses to KPSS, MCh nor were mean pD_2 or E_{max} values altered by the presence of the antagonist (Table 4.4).

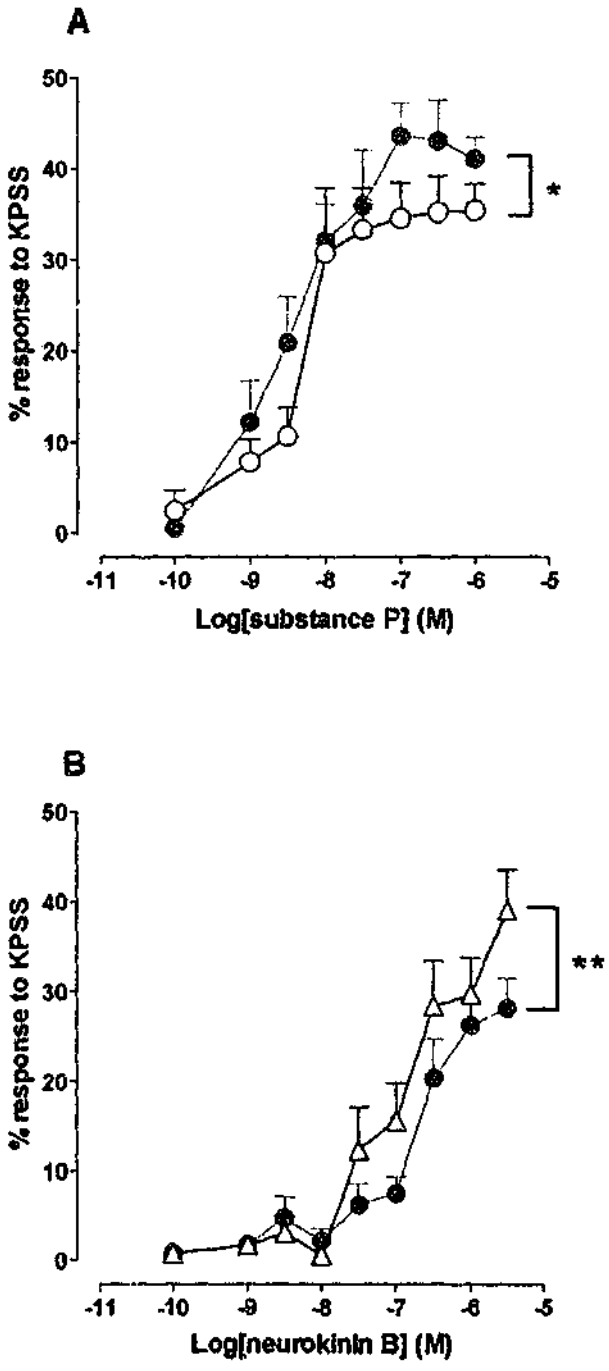


Figure 4.9: Log CRCs to (A) SP and (B) NKB in the absence of antagonist (open symbols) and the presence of SR 142801 (0.3μM) (closed symbols) on uterine preparations from oestrogen-treated mice. CRCs were constructed in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). Each point is the mean ± SEM, n=10 animals.

* indicates significant difference from control, two-way ANOVA, $P<0.05$; d.f. = 144

** indicates significant difference from control, two-way ANOVA, $P<0.05$; d.f. = 162

Table 4.4: Effects of peptides, MCh (10μM) and KPSS in the absence and presence of the tachykinin NK_3 receptor-selective antagonist SR 142801 (0.3μM) on myometrium from oestrogen-treated mice (n=10).

Peptide	Antagonist	Response to MCh (%KPSS ± SEM)	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (%KPSS ± SEM)	Peptide Potency (pD_2 ± SEM)
SP		64.9 ± 11.1	19.7 ± 1.8	42.7 ± 5.9	8.29 ± 0.17
SP	SR 142801	60.5 ± 6.1	19.7 ± 2.0	47.9 ± 4.0	8.28 ± 0.19
NKB		66.5 ± 7.7	19.1 ± 1.5	39.4 ± 4.4	N/A
NKB	SR 142801	51.5 ± 4.3	20.1 ± 1.5	33.4 ± 3.5	N/A

N/A – not applicable (could not be estimated as log CRC did not reach a clear maximum)

4.3.3.7 Methacholine and KPSS with SR 140333, SR 48968 and SR 142801

None of the antagonists used in this study; SR 140333 (10nM), SR 48968 (10nM), SR 140333 (10nM) in combination with SR 48968 (10nM) or SR 142801 (0.3μM) significantly affected the responses to MCh or KPSS (one-way ANOVAs, $P>0.05$) (Figure 4.10).

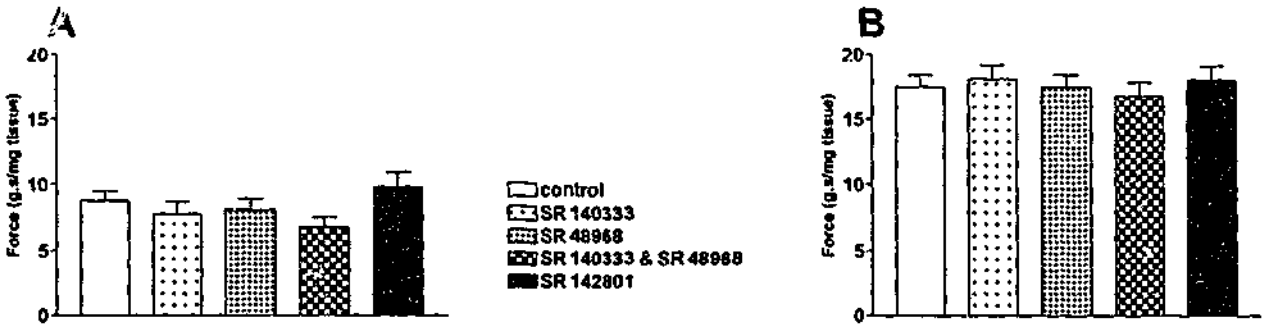


Figure 4.10: Response to (A) MCh (10μM) and (B) KPSS in the absence of antagonists (n=60) and in the presence of SR 140333 (10nM; n=40), SR 48968 (10nM; n=40), SR 140333 (10nM) in combination with SR 48968 (10nM; n=26) and SR 142801 (0.3μM; n=26). These data have been pooled from the response to single concentrations of MCh and KPSS applied at the completion of log CRCs shown in Figures 4.4-4.9 and all responses are in the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM). Each bar is the mean ± SEM, n=number of preparations.

4.3.4 Effect of indomethacin, ranitidine and mepyramine on the response to substance P

Responses to SP were unaffected by indomethacin (10μM) (potency ratio = 1.1, 95% CL = 0.1, 8.1; d.f. = 74), indomethacin (10μM) combined with ranitidine (10μM) (potency ratio = 1.9, 95% CL = 0.4, 12.7; d.f. = 84) or indomethacin (10μM) combined with mepyramine (0.1μM) (potency ratio = 5.9, 95% CL = 0.9, 155.3; d.f. = 84) (Figure 4.11). None of these inhibitors altered the mean responses to MCh or KPSS (one-way ANOVAs, P>0.05; Table 4.5).

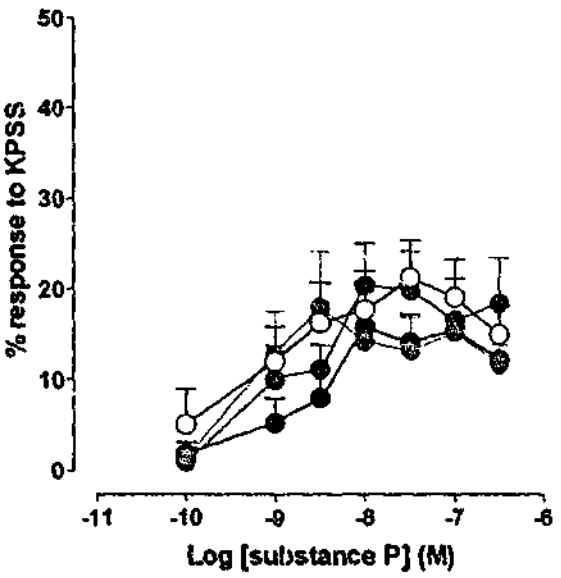


Figure 4.11: Log CRCs to SP in the absence (○) and presence of indomethacin (10μM) (●), indomethacin (10μM) combined with ranitidine (10μM) (●) and indomethacin (10μM) together with mepyramine (0.1μM) (●) on uterine preparations from oestrogen-treated mice. CRCs were constructed in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). Each point is the mean ± SEM, n=11 animals.

Table 4.5: Effects of substance P, MCh (10μM) and KPSS in the absence and presence inhibitors on myometrium from oestrogen-treated mice (n=11).

Inhibitor	Response to MCh (% KPSS ± SEM)	Response to KPSS (gs/mg tissue ± SEM)	Peptide E_{max} (% KPSS ± SEM)	Peptide potency (pD_2 ± SEM)
	55.7 ± 10.6	16.5 ± 2.0	25.6 ± 4.1	8.50 ± 0.43
Indo	58.6 ± 5.7	17.2 ± 1.4	28.2 ± 4.8	8.16 ± 0.20
Indo & Ran	63.8 ± 7.7	14.4 ± 1.2	25.5 ± 4.7	8.71 ± 0.12
Indo & Mep	63.5 ± 9.1	16.0 ± 2.6	21.9 ± 4.3	8.54 ± 0.27

Indo – indomethacin (10μM), Ran – ranitidine (10μM), Mep – mepyramine (0.1μM)

4.4 Discussion

This study was undertaken to investigate the tachykinin receptor types involved in mediating tachykinin-induced contractions in myometrium from oestrogen-treated mice. In the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM), the mammalian tachykinins SP, NKA and NKB and the NK_1 receptor-selective agonist [$Sar^9Met(O_2)^{11}$]SP acted as full agonists while the NK_2 receptor-selective agonist [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) was relatively inactive. Responses to SP, NKA, NKB and [$Sar^9Met(O_2)^{11}$]SP were significantly antagonized by the NK_1 receptor-selective antagonist SR 140333 (10nM) either alone or in combination with the NK_2 receptor-selective antagonist SR 48968 (10nM). SR 48968 significantly antagonized responses to NKA and NKB but was without effect on responses to SP and [$Sar^9Met(O_2)^{11}$]SP.

The effects of tachykinins in regulating myometrial contractility have been studied extensively in the rat (Barr *et al.*, 1991; Candenas *et al.*, 2001; Fisher and Pennefather, 1997; 1998; 1999; Fisher *et al.*, 1993; Hamlin *et al.*, 2000; Magraner *et al.*, 1998; Moodley *et al.*, 1999; Shintani *et al.*, 2000). In the oestrogen-primed rat uterus the three mammalian tachykinins SP, NKA and NKB have been reported to enhance myometrial contractility with an order of potency of NKA>SP≥NKB indicating activation of an NK_2 receptor (Fisher and Pennefather, 1997; Magraner *et al.*, 1998; Pennefather *et al.*, 1993). However, results from the present study and a previous preliminary study conducted in our laboratory (Fleming, 1998) indicated that in the oestrogen-treated mouse uterus the order of agonist potency was SP≥NKA>NKB, which suggests activation of an NK_1 receptor (Henry, 1986).

Given that in high concentrations, SP, NKA and NKB are not totally selective for their preferred tachykinin receptor type (Maggi, 1995a), additional experiments were undertaken using tachykinin receptor-selective agonists. In the presence of peptidase inhibitors the NK_1 receptor-selective [$Sar^9Met(O_2)^{11}$]SP (Drapeau *et al.*, 1987) was approximately equipotent with SP in producing uterine contractions in the oestrogen-treated mouse uterus, although its maximal effect was somewhat less than that of SP. In

contrast, the NK_2 receptor-selective [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) (Chassaing *et al.*, 1991) was relatively inactive, and, as reported in the previous chapter, the NK_3 receptor-selective [$MePhe^7$]NKB (Drapeau *et al.*, 1987) was without effect. These results lend further support to the idea that tachykinins mediate their uterotonic activity in the oestrogen-treated mouse uterus by activation of an NK_1 receptor. Interestingly, these results conflict with those previously obtained in preliminary experiments undertaken in our laboratory using the oestrogen-treated mouse uterus. In these preliminary experiments [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) was reported to be more potent than [$Sar^9Met(O_2)^{11}$]SP, however it was noted that this was unusual given that SP was the most potent mammalian tachykinin in this preparation and was possibly due to a problem with the batch of [$Sar^9Met(O_2)^{11}$]SP used (Fleming, 1998). In contrast to the present study, corresponding studies conducted in the oestrogen-primed rat uterus reported that [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) enhanced myometrial contractions but [$Sar^9Met(O_2)^{11}$]SP and [$MePhe^7$]NKB were without effect (Fisher & Pennefather, 1997; Fisher *et al.*, 1993; Moodley *et al.*, 1999). This finding is consistent with activation of NK_2 rather then NK_1 receptors.

The suggestion that the NK_1 receptor plays a major role in mediating the responses of the tachykinin peptides in the oestrogen-treated mouse uterus is further strengthened by studies using the selective tachykinin receptor antagonists SR 140333, SR 48968 and SR 142801. The non-peptide tachykinin NK_1-selective antagonist SR 140333 (Emonds-Alt *et al.*, 1993b) in a concentration of 10nM, effectively antagonized the actions of SP, NKA, NKB and [$Sar^9Met(O_2)^{11}$]SP. The rightward shift in the response to SP was approximately 100 fold, indicating an apparent pA_2 value for SR 140333 versus SP of approximately 10, which is consistent with the estimates obtained by Emonds-Alt *et al.* (1993b) in a variety of smooth muscle preparations. The present estimate must be interpreted carefully, however, since SR 140333 induced a significant reduction in the maximum response to [$Sar^9Met(O_2)^{11}$]SP. Emonds-Alt *et al.* (1993b) also observed a similar decrease in the maximal response to [$Sar^9Met(O_2)^{11}$]SP in the presence of SR 140333 in various *in vitro* assays and suggested that the antagonism exerted by SR 140333 may be non-competitive. Nevertheless, binding experiments clearly showed that SR 140333 interacted with NK_1 receptors in a competitive manner (Emonds-Alt *et al.*,

1993b). In the present study the rightward shifts produced by SR 140333 in the log concentration-response curves to the mammalian tachykinins as well as [$Sar^9Met(O_2)^{11}$]SP further substantiates the involvement of tachykinin NK_1 receptors in mediating uterotonic effects in the oestrogen-treated mouse uterus.

SR 140333 has also been reported to be a potent antagonist of septide in some tissues (Emonds-Alt *et al.*, 1993b; Oury-Donat *et al.*, 1994). It is unclear though whether this 'septide' variant of the NK_1 receptor, for which NKA and NKB also have affinity (Torrens *et al.*, 2000; Wijkhuisen *et al.*, 1999) is present in mouse tissues.

An interesting finding from this study was that the non-peptide tachykinin NK_2 selective antagonist SR 48968 (Advenier *et al.*, 1992a; Emonds-Alt *et al.*, 1992b), at a concentration of 10nM, significantly antagonized responses to NKA and NKB but not SP or [$Sar^9Met(O_2)^{11}$]SP in the oestrogen-treated mouse uterus. This is in contrast to the study by Fleming (1998) in which SR 48968 (10nM) was reported to have no effect on the response to NKA in the uterus of the oestrogen-treated mouse. Although binding studies have demonstrated that SR 48968 has an affinity for NK_3 binding sites in the guinea-pig and human with an IC_{50} of 330 and 350nM respectively (Chung *et al.*, 1994; Petitet *et al.*, 1993) it has no appreciable affinity for NK_1 binding sites (Advenier *et al.*, 1992a). This indicates that NKA and NKB may exert some of their effects on the oestrogen-treated mouse uterus through activation of a tachykinin NK_2 receptor. This is consistent with the observation that responses to SP, NKA and NKB were shifted slightly further to the right in the combined presence of SR 48968 and SR 140333 than compared to SR 140333 alone.

Unexpectedly, a slight leftward shift was seen in the concentration-response curve to SP in the presence of the non-peptide tachykinin NK_3 antagonist SR 142801 (Emonds-Alt *et al.*, 1995) at a concentration of 0.3μM. SR 142801 has been found to be inactive as an agonist at NK_1 receptors (Patacchini *et al.*, 1995) and no explanation can be offered for this unexpected result. In contrast, SR 142801 caused a slight rightward shift in the concentration-response curve to NKB. It is unlikely that NKB was acting at an NK_3 receptor as it was reported in the previous chapter that the NK_3 receptor-selective agonist [$MePhe^7$]NKB was inactive in the uterus of the oestrogen-treated mouse. SR

142801, at high concentrations (in the micromolar range), has been reported to act as an inhibitor at calcium and sodium channels (Emonds-Alt *et al.*, 1995), however, in this study no reduction was seen in the responses to either SP, MCh or KPSS. Given that SR 142801 is a highly potent antagonist for NK_3 receptors these results indicate that this receptor is not involved in eliciting uterine contractions in the oestrogen-treated mouse. It should be noted however, that the affinity of this antagonist for the tachykinin NK_3 receptor is species dependent (Beaujouan *et al.*, 1997; Patacchini *et al.*, 1995). The affinity of SR 142801 for the mouse tachykinin NK_3 receptor has yet to be established, nevertheless there are reports describing its efficacy as an antagonist of NKB-induced oedema in the mouse ear (Inoue *et al.*, 1996). It is also of interest that Barr *et al.* (1991) reported the presence of a tachykinin NK_3 receptor in the rat uterus, which other investigators have suggested is down-regulated by oestrogen (Pinto *et al.*, 1999). Whether this is also the case for the mouse has yet to be determined.

Analysis of the stability of SP by Auspep, the suppliers of this peptide, revealed that over time aliquots of SP might become contaminated by the hydrolysis of glutamine (Gln) at positions 5 and 6 in the SP sequence. Accordingly, the effects of three SP analogues in which Gln at positions 5 and/or 6 was replaced with Glu were examined. All three analogues acted as full agonists on the uterus of the oestrogen-primed mouse thus providing additional support for the activation of NK_1 receptors in this tissue. No differences in the potencies of these analogues compared to SP was observed this suggests that hydrolysis of Gln at positions 5 and/or 6 does not affect the response of SP in the uterus of the oestrogen-treated mouse.

There have been several reports implicating the involvement of prostanoids and histamine in mediating or modulating the response to SP in various tissues. SP has been shown to cause the release of prostanoids from the rat dorsal spinal cord (Dirig and Yaksh, 1999), the epithelium of rat bronchial smooth muscle (Bodelsson *et al.*, 1999), mast cells in rat cutaneous tissue (Jacques *et al.*, 1989) and the dog iris sphincter smooth muscle (Marathe *et al.*, 1996). It has also been reported that SP-induced contractions in guinea-pig lung parenchymal tissue are due to production of cyclooxygenase metabolites (Gerard, 1987). SP has also been reported to induce histamine release from

human skin mast cells (Barnes *et al.*, 1986; Columbo *et al.*, 1996) and depending on the phase of the reproductive cycle, it may modulate the release of histamine from rat uterine mast cells (Cocchiara *et al.*, 1995). In the present study, neither indomethacin, alone or in the presence of the H_1 histamine receptor antagonist mepyramine, or the H_2 histamine receptor antagonist ranitidine, produced any changes in the uterine response to SP. These results suggest that in the oestrogen-primed mouse uterus neither histamine acting at either H_1 or H_2 histamine receptors nor prostanoids mediate or modulate the uterotonic effects of SP.

In conclusion, the results of the present study using mammalian tachykinins, tachykinin receptor-selective agonists and non-peptide tachykinin antagonists, indicate the importance of the tachykinin NK_1 receptor in mediating the uterotonic effects of tachykinins on the oestrogen-treated mouse uterus. The tachykinin NK_2 receptor may also contribute to the effects of higher concentrations of the mammalian tachykinins NKA and NKB in this tissue. The aim of the next chapter is to investigate whether the uterotonic effects of the tachykinins in the mouse uterus are modulated by pregnancy.

CHAPTER 5

CHARACTERIZATION OF THE UTEROTONIC EFFECTS OF TACHYKININ PEPTIDES ON THE PREGNANT MOUSE UTERUS

Summary

1. The aims were (1) to characterize the receptor type(s) mediating the uterotonic activity of tachykinins in uterus from late-pregnant mice (2) to determine the importance of peptidases in constraining the response to tachykinin peptides in this tissue preparation and (3) to compare the response to tachykinins in uterine preparations in pregnant mice with those from oestrogen-treated mice, described in Chapters 3 and 4.

2. In the absence of peptidase inhibitors, the mammalian tachykinins SP, NKA and NKB produced concentration-related contractions with an order of potency based on the positions of the log concentration-response curves of NKA>SP≥NKB; the NK_1 and NK_2 receptor-selective agonists $[Sar^9Met(O_2)^{11}]SP$ and $[Lys^5MeLeu^9Nle^{10}]NKA(4\text{-}10)$ were equipotent with NKA, however the ceiling effect of the log concentration-response curve for $[Lys^5MeLeu^9Nle^{10}]NKA(4\text{-}10)$ was greater than that for $[Sar^9Met(O_2)^{11}]SP$.

3. The peptidase inhibitor thiorphan (3μM) either alone or in combination with captopril (10μM) or bestatin (10μM) did not potentiate responses to SP, NKA, NKB, $[Sar^9Met(O_2)^{11}]SP$ or $[Lys^5MeLeu^9Nle^{10}]NKA(4\text{-}10)$.

4. The tachykinin NK_1 receptor-selective antagonist SR 140333 (10nM) shifted the log concentration-response curve to SP to the right and attenuated responses to $[Sar^9Met(O_2)^{11}]SP$, but did not affect responses to NKA or $[Lys^5MeLeu^9Nle^{10}]NKA(4\text{-}10)$. The tachykinin NK_2 receptor-selective antagonist SR 48968 (10nM) shifted the log concentration-response curve to NKA to the right and attenuated responses to $[Lys^5MeLeu^9Nle^{10}]NKA(4\text{-}10)$ but did not affect responses to SP and $[Sar^9Met(O_2)^{11}]SP$. The tachykinin NK_3 receptor-selective antagonist SR 142801 (0.3μM) did not affect the responses to any of the peptides tested.

5. The order of potency for the mammalian tachykinins in uterine tissue obtained from pregnant mice differed from those reported in Chapter 4 for non-pregnant mice with the order of agonist potency based on the positions of the log concentration-response curves being NKA>SP≥NKB compared to SP≥NKA>NKB respectively.

6. Taken together, the results of this chapter indicate that in pregnant mouse uterus the contractile effects of the tachykinins are mediated predominantly by the NK_2 receptor. This differs from the oestrogen-treated mouse uterus where responses to tachykinins were mediated mainly by the NK_1 receptor. The peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM), while potentiating responses to the tachykinin peptides in the oestrogen-treated mouse uterus, had no effect when present in similar concentrations in preparations of the pregnant mouse uterus.

5.1 Introduction

In the previous two chapters, the effects of tachykinins on myometrial contractility in the oestrogen-treated mouse were described. Having determined that tachykinin-induced uterine contractions in the oestrogen-treated mouse are mediated primarily by activation of the NK_1 receptor, while the NK_2 receptor plays a lesser role, the aim of this chapter is to examine the contractile effects of these peptides in uterine tissue from pregnant mice.

Preliminary experiments conducted in our laboratory to examine the effects of the mammalian tachykinins and the tachykinin receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP and [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) on uterus from late pregnant mice suggested that the predominant tachykinin receptor type involved in mediating responses to these peptides differed from that of the oestrogen-treated mouse. In the presence of the neprilysin inhibitor thiorphan (10μM, present for experiments with the mammalian peptides only) the rank order of potency was NKA≥[$Lys^5MeLeu^9Nle^{10}$]NKA(4-10)>>SP≅[$Sar^9Met(O_2)^{11}$]SP≅NKB which is consistent with activation of an NK_2 receptor (Fleming, 1998). This apparent difference between myometrium from pregnant and non-pregnant mice is in contrast to the rat, where functional and molecular studies have indicated that the NK_2 receptor is the main receptor in mediating tachykinin-induced uterine contractility in preparations from both non-pregnant and pregnant rats (Candenas *et al.*, 2001; Fisher and Pennefather, 1998; 1999; Magraner *et al.*, 1998; Shintani *et al.*, 2000). However, it is not clear whether the response to tachykinins mediated by activation of the NK_2 receptor is modulated by pregnancy in this species. Shintani and colleagues reported an increased responsiveness of the pregnant rat myometrium to NKA compared to the non-pregnant myometrium and suggested that this could be explained by an increase in NK_2 receptors in this preparation (Shintani *et al.*, 2000). In contrast, a study by Candenas *et al.* (2001) reported that the function and expression of the NK_2 receptor in rat myometrium did not vary during the course of pregnancy.

The specific aim of this chapter was to characterize the tachykinin receptor type(s) mediating uterotonic activity in myometrial preparations from pregnant mice and to

compare these findings with those observed in the oestrogen-treated mouse as reported in Chapters 3 and 4. Receptor characterization was undertaken by determining the effects of SP, NKA, NKB and the NK_1 and NK_2 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP and [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) in the absence and presence of peptidase inhibitors, and by utilizing the selective NK_1, NK_2 and NK_3 non-peptide antagonists SR 140333, SR 48968 and SR 142801.

5.2 Materials and Methods

5.2.1 Animals and tissue preparations

Full details of animals and preparations are given in section 2.1.1 and 2.2.2. Briefly, pregnant female Balb C mice weighing between 35-55g were used on day 17 of the average 19 day gestation period. Eight uterine preparations were obtained from each animal and set up for recording of force from the longitudinal smooth muscle layer.

5.2.2 Experimental protocol

Refer to Chapter 2 (section 2.3). In brief, following the equilibration period, discrete concentration-response curves were constructed. Each agonist concentration remained in contact with the tissue for 5min, was washed out and a higher concentration of agonist was added 15min later (initial concentrations increasing in log units until a response was observed and then increasing in 0.5 log increments). Only one concentration-response curve was generated on each preparation. All preparations were exposed to MCh (10μM) prior to the addition of KPSS to the bath.

5.2.3 Measurement of results and statistical analyses

Responses to all agonists were measured as area under the force-time curve for the duration of agonist exposure as described in section 2.4. Responses were then expressed as a percentage of the response to the KPSS and presented as mean ± SEM.

Statistical analysis was undertaken as described in section 2.6.

E_{max} is defined as the maximum response to an agonist observed over the concentration range used; note that might not in all cases be the greatest response that an agonist could elicit if higher concentrations had been tested.

5.2.4 Drugs

The drugs used included all those previously described in section 2.7, which includes details of stock solutions, vehicles used and vehicle controls as appropriate.

Table 5.1: Overview of experiments undertaken on uterine preparations obtained from pregnant mice. Note that up to eight preparations were obtained from each animal and are allocated as preparation 1, 2, 3, 4, 5, 6, 7 or 8 using a randomized procedure.

Series	Preparation 1		Preparation 2		Preparation 3		Preparation 4		Preparation 5		Preparation 6		Preparation 7		Preparation 8	
	Ag	Tr	Ag	Tr	Ag	Tr	Ag	Tr	Ag	Tr	Ag	Tr	Ag	Tr	Ag	Tr
1: Mammalian TKs in the absence and presence of peptidase inhibitors (n=7)	SP	-	SP	T	SP	T & C	NKA	-	NKA	T	NKA	T & B	NKB	-	NKB	T
2: Sar^9 & Lys^5 in the absence and presence of peptidase inhibitors (n=6)	Sar^9	-	Sar^9	T	Sar^9	T & C	Lys^5	-	Lys^5	T	Lys^5	T & B				
3: SP & NKA in the absence and presence of SR 140333, SR 48968 & SR 142801 (n=5)	SP*	-	SP*	SR 140333 (10nM)	SP*	SR 48968 (10nM)	SP*	SR 142801 (0.3μM)	NKA*	-	NKA*	SR 140333 (10nM)	NKA*	SR 48968 (10nM)	NKA*	SR 142801 (0.3μM)
4: Sar^9 & Lys^5 in the absence and presence of SR 140333, SR 48968 & SR 142801 (n=7)	Sar^9	-	Sar^9	SR 140333 (10nM)	Sar^9	SR 48968 (10nM)	Sar^9	SR 142801 (0.3μM)	Lys^5	-	Lys^5	SR 140333 (10nM)	Lys^5	SR 48968 (10nM)	Lys^5	SR 142801 (0.3μM)

Ag = agonist, Tr = treatment
TK = tachykinins, Sar^9 = [$Sar^9Met(O_2)^{11}$]SP, Lys^5 = [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10)
T = thiorphan (3μM), C = captopril (10μM), B= bestatin (10μM)
* indicates peptidase inhibitors present (thiorphan (3μM), captopril (10μM) and bestatin (10μM))
n = number of animals
- = no treatment

5.3 Results

Uterine tissue used throughout this study was obtained from late-term pregnant mice (day 17 of the average 19 day gestation period) weighing between 35-55g. The marked uterine hypertrophy occurring at this stage of pregnancy allowed for a minimum of four and up to a maximum of eight preparations to be obtained from each uterine horn This is in comparison to non-pregnant mice where a maximum of only two preparations could be obtained from each horn. The mean weight of each tissue preparation was 30.4 ± 0.8mg (n=196 preparations). The mean response to KPSS was 32.7 ± 0.8g.s/mg tissue (n=196 preparations). The mean response to MCh (10μM) was 73.1 ± 1.7% of the response to KPSS (n=196 preparations). Spontaneous activity was observed in nearly all tissue preparations and ranged from one to sixteen contractions over any 15 min period; this gradually decreased over the duration of the equilibration period. Figure 5.1 shows a representative trace of responses to increasing concentrations of the NK_1 and NK_2 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP and [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10). It can be seen that while both receptor-selective agonists cause contractions in the pregnant mouse uterus, contractions produced by [$Sar^9Met(O_2)^{11}$]SP were less sustained then the longer lasting contractions produced by [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10).

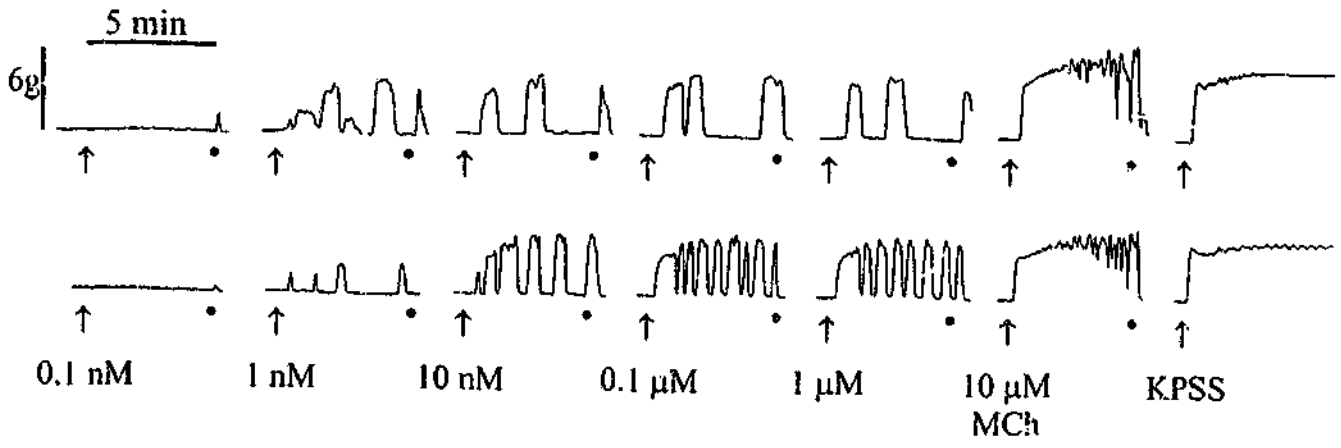


Figure 5.1: Representative trace showing response of longitudinal myometrium from a pregnant mouse to [$Sar^9Met(O_2)^{11}$]SP (upper panel) and [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) (lower panel) together with responses to MCh (10μM) and KPSS. The arrows indicate agonist addition and the dots represent washes.

5.3.1 Influence of peptidase inhibitors on the uterotonic effects of the tachykinin peptides

5.3.1.1 Substance P

Responses to SP were unaffected by thiorpha· (3μM) (potency ratio = 1.1, 95% CL = 0.1, 13.5; d.f = 38) or by thiorphan (3μM) together with captopril (10μM) (potency ratio = 0.7, 95% CL = 0.0, 9.0; d.f. = 31) (Figure 5.2).

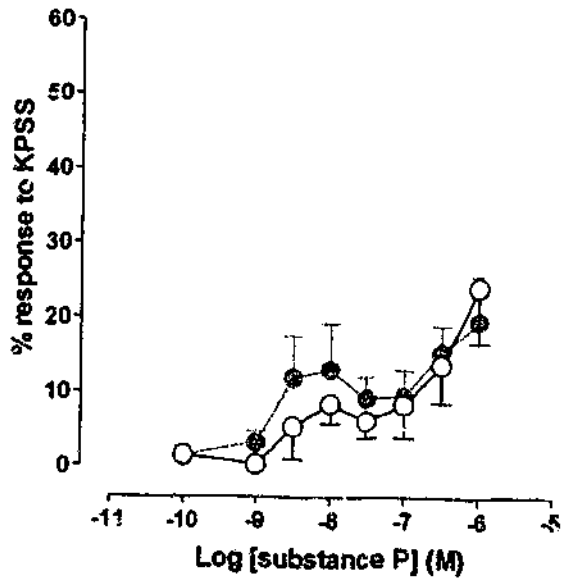


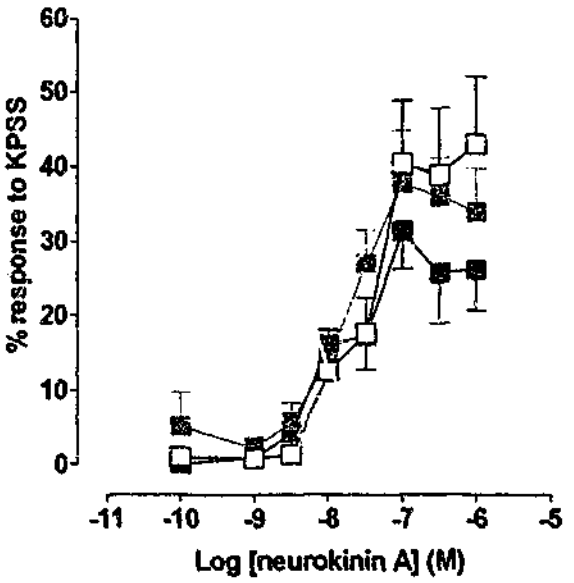
Figure 5.2: Log concentration-response curves (CRCs) to SP in the absence of peptidase inhibitors (O) and in the presence of thiorphan (3μM) (●) and thiorphan (3 μM) together with captopril (10μM) ( ) on uterine preparations from pregnant mice. Each point is the mean ± SEM, n=7 animals. Note that the log CRC to SP did not reach a ceiling over the concentration range of 0.1nM - 1μM.

In this and subsequent figures, a significant difference between log CRCs as indicated by two-way ANOVA followed by Student Newman Keuls is denoted by *.

5.3.1.2 Neurokinin A

Responses to NKA were unaffected by thiorphan (3μM) (potency ratio = 0.5, 95% CL = 0.2, 1.2; d.f. = 43) or by thiorphan (3μM) together with bestatin (10μM) (potency ratio = 0.6, 95% CL = 0.2, 1.4; d.f. = 36) (Figure 5.3).

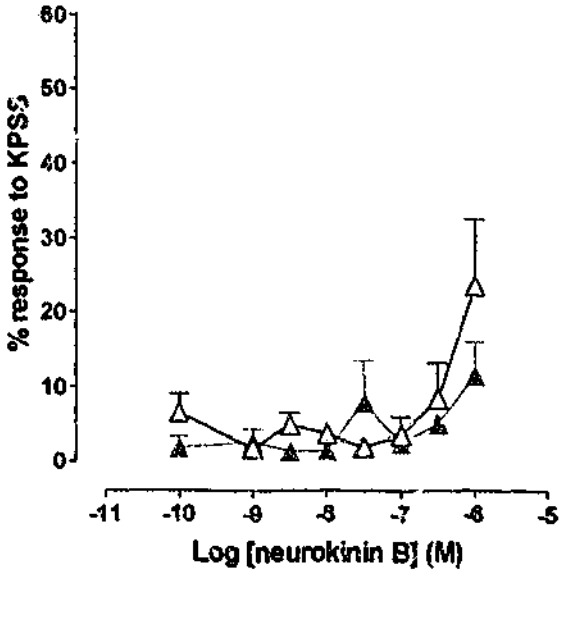
Figure 5.3: Log CRCs to NKA in the absence of peptidase inhibitors (□) and in the presence of thiorphan (3μM) (▩) and thiorphan (3 μM) together with bestatin (10μM) (■) on uterine preparations from pregnant mice. Each point is the mean ± SEM, n=7 animals. Note that the log CRC to NKA reached a ceiling at concentrations over 100nM.



5.3.1.3 Neurokinin B

Responses to NKB were unaffected by thiorphan (3μM) (potency ratio = 0.3, 95% CL = 0.6, 75.3; d.f. = 28) (Figure 5.4).

Figure 5.4: Log CRCs to NKB in the absence of peptidase inhibitors (△) and in the presence of thiorphan (3μM) (▲) on uterine preparations from pregnant mice. Each point is the mean ± SEM, n=7 animals. Note that the log CRC to NKB did not reach a ceiling over the concentration range of 0.1nM - 1μM.



5.3.1.4 [Sar^{9}Met(O$_2$)^{11}]SP

Although the log CRC to [Sar^{9}Met(O$_2$)^{11}]SP reached a ceiling, a potency ratio could not be obtained for the comparison between the control log CRC for [Sar^{9}Met(O$_2$)^{11}]SP in the absence of peptidase inhibitors with corresponding log CRCs in the presence of either thiorphan (3μM) or thiorphan (3μM) together with captopril (10μM) as in the presence of these inhibitors there were insufficient points on the linear part of the curve, however, a two-way ANOVA indicated a slight but statistically significant attenuation in the log CRC to [Sar^{9}Met(O^2)^{11}]SP in the presence of thiorphan compared to the control (3μM) ($p<0.05$; d.f. = 120) (Figure 5.5).

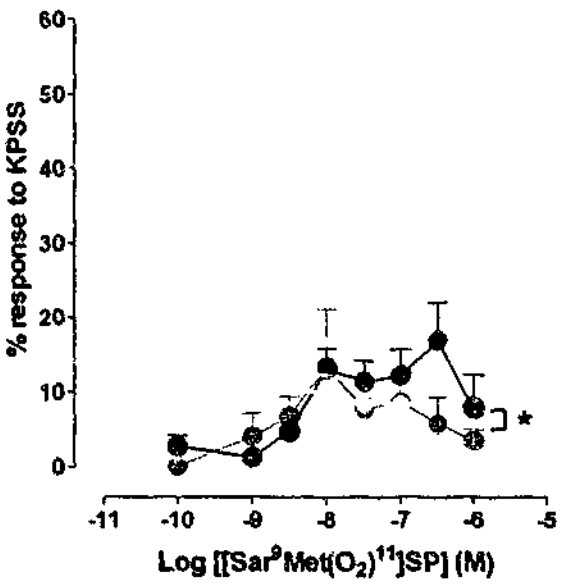


Figure 5.5: Log CRCs to [Sar^{9}Met(O$_2$)^{11}]SP in the absence of peptidase inhibitors (●) and in the presence of thiorphan (3μM) (◉) and thiorphan (3 μM) together with captopril (10μM) ( ) on uterine preparations from pregnant mice. Each point is the mean ± SEM, n=6 animals. Note that the log CRC to [Sar^{9}Met(O$_2$)^{11}]SP reached a ceiling.
* indicates a significant difference in log CRC to [Sar^{9}Met(O$_2$)^{11}]SP in the presence of thiorphan (3μM) compared to control, two-way ANOVA, $P<0.05$; d.f. = 120.

5.3.1.5 [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10)

Responses to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) were unaffected by thiorphan (3μM) (potency ratio = 0.7, 95% CL = 0.4, 1.1; d.f = 44) or by thiorphan (3μM) together with bestatin (10μM) (potency ratio = 1.0, 95% CL = 0.5, 2.2; d.f. = 38) (Figure 5.6).

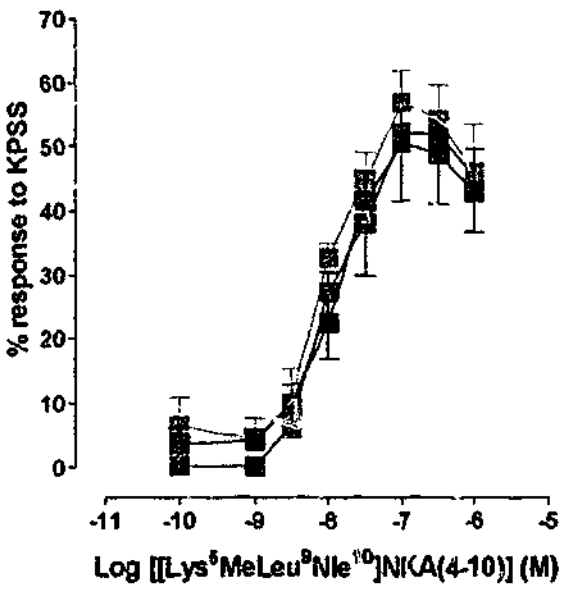


Figure 5.6: Log CRCs to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) in the absence of peptidase inhibitors (■) and in the presence of thiorphan (3μM) (■) and thiorphan (3 μM) together with bestatin (10μM) (■) on uterine preparations from pregnant mice. Each point is the mean ± SEM, n=6 animals. Note that the log CRC to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) reached a ceiling at concentrations over 100nM.

5.3.1.6 Methacholine and KPSS

None of the peptidase inhibitor combinations used in this study; thiorphan (3μM); thiorphan (3μM) and captopril (10μM); thiorphan (3μM) and bestatin (10μM) and thiorphan (3μM), captopril (10μM) and bestatin (10μM) significantly affected the responses to MCh or KPSS (one-way ANOVAs, P>0.05) (Figure 5.7).

Table 5.2: Effects of peptides, MCh (10μM) and KPSS in the absence and presence of peptidase inhibitors on myometrium from pregnant mice.

N	Agonist	Peptidase inhibitors	Response to MCh (% KPSS ± SEM)	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (% KPSS ± SEM)	Peptide potency (pD_2 ± SEM)
7	SP		84.5 ± 3.2	31.1 ± 2.5	29.0 ± 6.1[b]	N/A
7	SP	T	71.7 ± 12.5	34.7 ± 1.5	24.3 ± 5.3[b]	N/A
7	SP	T & C	73.6 ± 10.2	39.1 ± 2.5	26.7 ± 7.4[b]	N/A
7	NKA		86.0 ± 7.1	31.7 ± 2.4	45.3 ± 9.1[b]	7.54 ± 0.11
7	NKA	T	73.4 ± 5.6	31.5 ± 4.7	41.2 ± 6.5[b]	7.78 ± 0.13
7	NKA	T & B	58.2 ± 5.4[a]	40.6 ± 3.4	31.8 ± 5.3[b]	7.95 ± 0.15
7	NKB		72.9 ± 13.3	30.8 ± 2.9	26.3 ± 8.3[b]	N/A
7	NKB	T	64.4 ± 9.3	30.4 ± 3.0	14.2 ± 5.7[b]	N/A
5	Sar^9		75.2 ± 4.6	31.7 ± 4.1	22.5 ± 3.2[b,c]	8.07 ± 0.25
5	Sar^9	T	66.2 ± 7.2	23.3 ± 3.0	18.1 ± 7.3[b]	N/A
5	Sar^9	T & C	80.0 ± 5.0	32.9 ± 3.1	18.9 ± 2.9[b]	N/A
5	Lys^5		70.6 ± 6.4	35.0 ± 3.8	51.5 ± 5.0[b]	7.98 ± 0.14
5	Lys^5	T	72.4 ± 8.1	32.0 ± 2.5	57.5 ± 5.2	8.21 ± 0.18
5	Lys^5	T & B	85.5 ± 8.5	35.0 ± 4.3	59.4 ± 9.6	7.82 ± 0.18

N – number of preparations
Sar^9 – $[Sar^9Met(O_2)^{11}]SP$, Lys^5 – $[Lys^5MeLeu^9Nle^{10}]NKA(4\text{-}10)$
T – thiorphan (3μM), C – captopril (10μM), B – bestatin (10μM)
N/A – not applicable (could not be estimated as log CRC did not reach a clear maximum)
a – significantly different from the corresponding value obtained in the absence of peptidase inhibitors (one-way ANOVA, $P<0.05$)
b – significantly different from the response to MCh (Student's unpaired *t*-tests, $P<0.05$)
c – significantly different from the corresponding maximum response obtained for $[Lys^5MeLeu^9Nle^{10}]NKA(4\text{-}10)$ (Student's unpaired t-test, $P<0.05$)

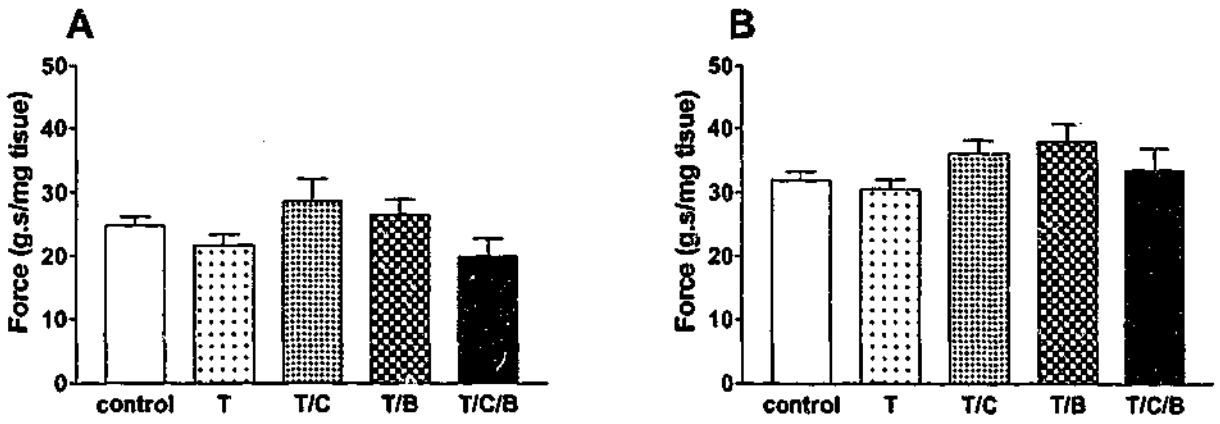


Figure 5.7: Responses to (A) MCh (10μM) and (B) KPSS in the absence of peptidase inhibitors (control; n=33) and in the presence of thiorphan (3μM) (T; n=33), thiorphan (3μM) and captopril (10μM) (T/C, n= 13), thiorphan (3μM) and bestatin (10μM) (T/B, n=13) and thiorphan (3μM), captopril (10μM) and bestatin (10μM) (T/C/B, n=10). These data have been pooled from the responses to single concentrations of MCh and KPSS applied at the completion of log CRCs shown in Figures 5.2-5.6 with the exception of data obtained in the presence of thiorphan, captopril and bestatin which is from the control curves shown in Figures 5.9 and 5.10. Each bar is the mean ± SEM, n = number of preparations.

No significant differences in mean KPSS responses were observed between treatment groups for each series of experiments (one-way ANOVA, $P>0.05$). Responses to MCh (10μM) were similar within treatment groups for each series of experiments with the exception of those in which NKA was used as an agonist in which a significantly smaller response to MCh was seen in tissues when both thiorphan and bestatin were present as compared to the control (one-way ANOVA, $P<0.05$). Maximal responses to peptides were either similar to or significantly lower than the mean response to MCh (Student's unpaired t-test, $P<0.05$) (Table 5.2).

5.3.2 Order of agonist potency

In the absence of peptidase inhibitors the relative order of agonist potency for the mammalian tachykinins based on the positions of the log CRCs was NKA>SP≥NKB. Note that NKA was the only mammalian tachykinin to reach a ceiling over the concentration range used (0.1nM - 1μM). A similar order of potency was also observed in the presence of thiorphan (3μM). Of the receptor-selective agonists [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) was approximately equipotent with NKA. [$Sar^9Met(O_2)^{11}$]SP while having a similar mean pD_2 value had a significantly lower ceiling response than [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) as is evident from the striking difference in the magnitude of the plateaus of the log CRCs for these two selective agonists (one-way ANOVA, $P<0.05$; Table 5.2) (Figure 5.8).

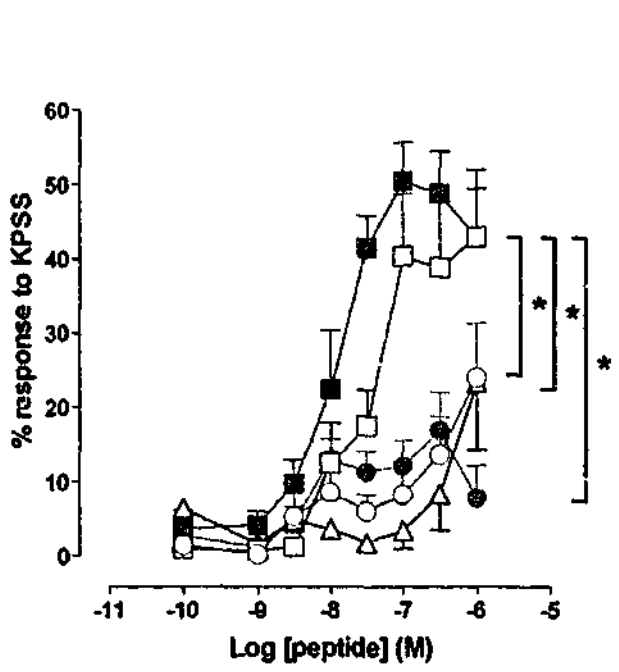


Figure 5.8: Log CRCs to SP (○), NKA (□), NKB (△), [$Sar^9Met(O_2)^{11}$]SP (●) and [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) (■) in the absence of peptidase inhibitors on uterine preparations from pregnant mice. Each point or bar is the mean ± SEM, n=6-7 animals.

* indicates a significant difference in log CRC to SP, NKB and [$Sar^9Met(O_2)^{11}$]SP compared to NKA and [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10), two-way ANOVA, $P<0.05$; d.f. = 216.

5.3.3 Effect of tachykinin antagonists on the uterotonic effects of the tachykinin peptides.

5.3.3.1 Substance P with SR 140333, SR 48968 and SR 142801

SR 140333 (10nM) significantly attenuated the response to SP (two-way ANOVA, $P<0.05$; d.f. = 140). However, a potency ratio could not be obtained as no clear regression for SP was seen with any of the log CRCs. SR 48968 (10nM) and SR 142801 (0.3μM) were without significant effect on the log CRC to SP (Figure 5.9).

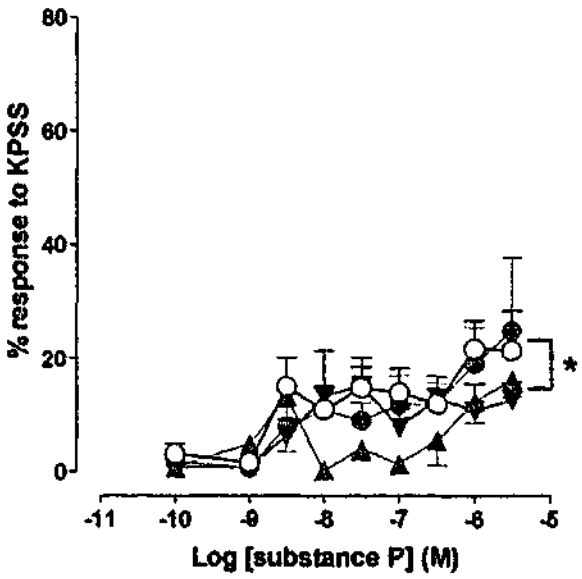


Figure 5.9: Log CRCs to SP in the absence of antagonist (○) and in the presence of SR 140333 (10nM) (▲), SR 48968 (10nM) (▼) and SR 142801 (0.3μM) (●) on uterine preparations from pregnant mice. All CRCs are in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). Each point is the mean ± SEM, n=5.

* indicates a significant difference from control, two-way ANOVA, $P<0.05$; d.f. = 140

5.3.3.2 Neurokinin A with SR 140333, SR 48968 and SR 142801

SR 48968 (10nM) significantly attenuated the response to NKA (two-way ANOVA, $P<0.05$; d.f. = 128) and in its presence there was a marked decrease in the plateau of the log CRC. This attenuation corresponded to an approximate 12 fold rightward shift of the log CRC to NKA in the presence of SR 48968 (95% CL = 2.5, 150.9; d.f. = 25). SR 140333 (10nM) (potency ratio = 0.8, 95% CL = 0.3, 2.3; d.f. = 36) and SR 142801

(0.3μM) (potency ratio = 0.7, 95% CL = 0.2, 2.3; d.f. = 36) were without significant effect on the log CRC to NKA (Figure 5.10).

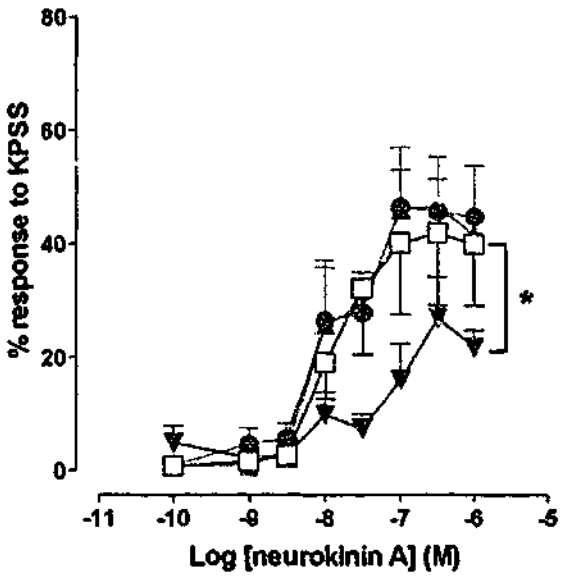


Figure 5.10: Log CRCs to NKA in the absence of antagonist (□) and in the presence of SR 140333 (10nM) (▲), SR 48968 (10nM) (▼) and SR 142801 (0.3μM) (●) on uterine preparations from pregnant mice. All CRCs are in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). Each point is the mean ± SEM, n=5.

* indicates a significant difference from control, two-way ANOVA, P<0.05; d.f. = 128

5.3.3.3 [Sar^{9}Met(O$_2$)^{11}]SP with SR 140333, SR 48968 and SR 142801

SR 140333 (10nM) significantly attenuated the response to the NK_1 receptor-selective agonist [Sar^{9}Met(O$_2$)^{11}]SP (two-way ANOVA, P<0.05; d.f. = 192), however a shift could not be calculated for [Sar^{9}Met(O$_2$)^{11}]SP in the presence of SR 140333 as no clear regression for [Sar^{9}Met(O$_2$)^{11}]SP was seen with any of the log CRCs. SR 48968 (10nM) and SR 142801 (0.3μM) were without significant effect on the log CRC to [Sar^{9}Met(O$_2$)^{11}]SP (Figure 5.11).

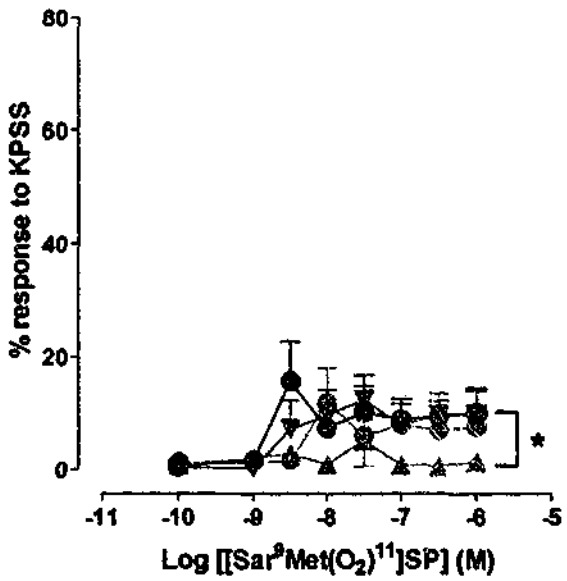


Figure 5.11: Log CRCs to [$Sar^9Met(O_2)^{11}$]SP in the absence of antagonist (●) and in the presence of SR 140333 (10nM) (▲), SR 48968 (10nM) (▼) and SR 142801 (0.3μM) (◉) on uterine preparations from pregnant mice. Each point is the mean ± SEM, n=7.

* indicates a significant difference from control, two-way ANOVA, $P<0.05$; d.f. = 192

5.3.3.4 [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) with SR 140333, SR 48968 and SR 142801

SR 48968 (10nM) significantly attenuated the response to the NK_2 receptor-selective agonist [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) (two-way ANOVA, $P<0.05$; d.f. = 191) however, it was not possible to calculate a potency ratio for the shift obtained in the presence of SR 48968 as the line was not parallel to the control and the maximum response was significantly reduced (one-way ANOVA, $P<0.05$; Table 5.3). SR 140333 (10nM) (potency ratio = 0.7, 95% CL = 0.2, 2.2; d.f. = 52) and SR 142801 (0.3μM) (potency ratio = 2.0, 95% CL = 0.8, 5.7; d.f. = 42) were without effect on the log CRC to [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) (Figure 5.12).

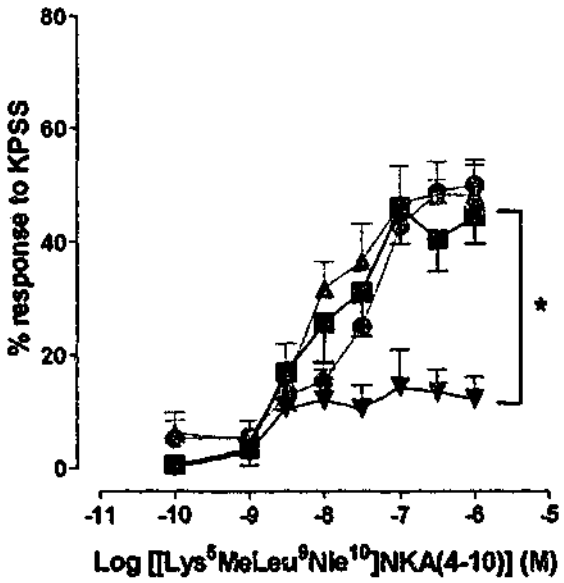


Figure 5.12: Log CRCs to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) in the absence of antagonist (■) and in the presence of SR 140333 (10nM) (▲), SR 48968 (10nM) (▼) and SR 142801 (0.3μM) (●) on uterine preparations from pregnant mice. Each point is the mean ± SEM, n=7.

* indicates a significant difference from control, two-way ANOVA, P<0.05; d.f. = 191

5.3.3.5 Methacholine and KPSS

None of the antagonists used in this study; SR 140333 (10nM), SR 48968 (10nM) or SR 142801 (0.3μM) significantly affected the responses to MCh (10μM) or KPSS (one-way ANOVAs, P>0.05) (Figure 5.13).

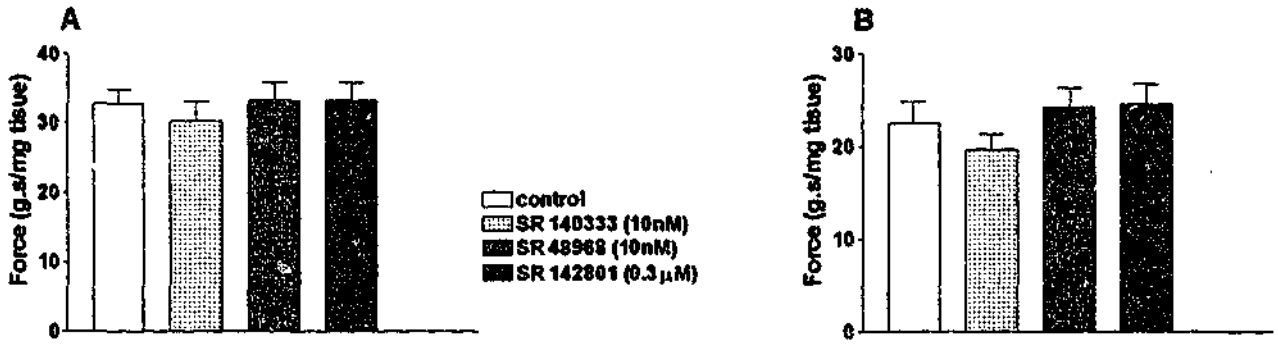


Figure 5.13: Response to (A) MCh (10μM) and (B) KPSS in the absence of antagonists (n=24) and in the presence of SR 140333 (10nM; n=24), SR 48968 (10nM; n=24) and SR142801 (0.3μM; n=24). These data have been pooled from the response to single concentrations of MCh and KPSS applied at the completion of the log CRCs shown in Figures 5.9-5.12. Each bar is the mean ± SEM, n = number of preparations.

Table 5.3: Effect of peptides, MCh (10μM) and KPSS in the absence and presence of tachykinin receptor-selective antagonists on myometrium from pregnant mice.

N	Agonist	Antagonist	Response to MCh (%KPSS ± SEM)	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (%KPSS ± SEM)	Peptide potency (pD_2 ± SEM)
5	*SP		56.4 ± 7.5	33.7 ± 5.4	28.7 ± 4.0[b]	N/A
5	*SP	SR 140333 (10nM)	64.3 ± 10.0	35.2 ± 12.1	25.5 ± 7.0[b]	N/A
5	*SP	SR 48968 (10nM)	77.4 ± 9.0	46.5 ± 9.0	19.7 ± 7.5[b]	N/A
5	*SP	SR 142801 (0.3μM)	76.7 ± 10.2	42.3 ± 8.7	28.8 ± 9.2[b]	N/A
5	*NKA		64.8 ± 12.7	33.4 ± 4.9	42.7 ± 12.1	7.56 ± 0.35
5	*NKA	SR 140333 (10nM)	80.6 ± 12.2	25.7 ± 2.8	50.7 ± 5.2	7.87 ± 0.22
5	*NKA	SR 48968 (10nM)	77.6 ± 4.2	28.0 ± 2.4	35.2 ± 3.8[b]	7.32 ± 0.19
5	*NKA	SR 142801 (0.3μM)	79.7 ± 12.0	33.0 ± 5.9	49.1 ± 9.3	7.85 ± 0.14
7	Sar^9		87.0 ± 6.2	32.5 ± 4.2	24.8 ± 5.1[b]	N/A
7	Sar^9	SR 140333 (10nM)	76.6 ± 8.9	28.0 ± 2.6	9.7 ± 4.1[b]	N/A
7	Sar^9	SR 48968 (10nM)	82.1 ± 12.9	27.1 ± 3.3	18.1 ± 4.2[b]	N/A
7	Sar^9	SR 142801 (0.3μM)	75.7 ± 10.2	29.7 ± 4.5	18.1 ± 5.8[b]	N/A
7	Lys^5		65.3 ± 12.8	31.9 ± 3.3	48.7 ± 6.2	8.08 ± 0.19
7	Lys^5	SR 140333 (10nM)	56.7 ± 10.1	32.1 ± 4.4	52.0 ± 6.0	8.04 ± 0.15
7	Lys^5	SR 48968 (10nM)	66.2 ± 5.5	33.7 ± 2.8	25.9 ± 5.1[a,b]	N/A
7	Lys^5	SR 142801 (0.3μM)	69.4 ± 7.7	30.3 ± 2.4	54.2 ± 3.5	7.23 ± 0.22

Sar^9 – $[Sar^9Met(O_2)^{11}]SP$, Lys^5 – $[Lys^5MeLeu^9Nle^{10}]NKA(4\text{-}10)$

* indicates the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM)

N/A – not applicable (could not be estimated as log CRC did not reach a clear maximum)

N = number of preparations

a = significantly different from the corresponding E_{max} value obtained in the absence of antagonist (one-way ANOVA, $P<0.05$)

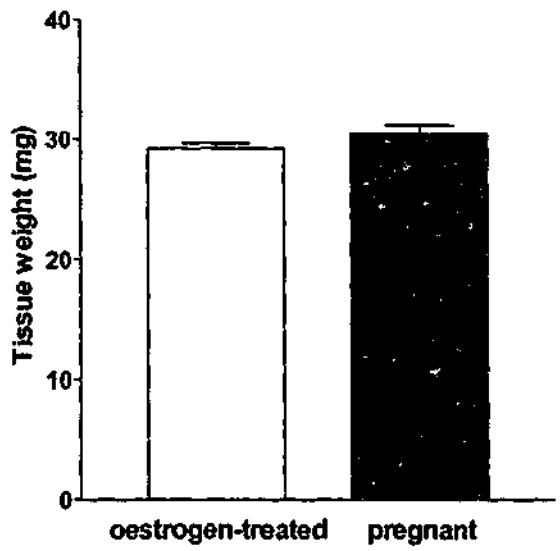
b = significantly different from the corresponding response to MCh (Student's unpaired t-test, $P<0.05$).

No significant differences in mean KPSS responses were observed between treatment groups for each series of experiments (one-way ANOVA, $P>0.05$). Responses to MCh (10μM) were also similar in all series of experiments. The antagonists SR 140333, SR 48968 and SR 142801 had no significant effect on the E_{max} values for the tachykinin peptides with the exception of NKA, which was significantly reduced in the presence of SR 48968 (one-way ANOVA, $P<0.05$) (Table 5.3).

5.3.4 Comparison of the uterotonic effects of tachykinins in uterine preparations from oestrogen-treated and pregnant mice.

Although marked hypertrophy of the uterus occurs during pregnancy, the size of the tissue preparations from the pregnant mice were chosen to be a similar weight to those obtained from non-pregnant mice (Figure 5.14). Responses to both MCh and KPSS, normalized for tissue weight, were significantly increased in tissues obtained from pregnant mice (Figure 5.15 and 5.16, Student's unpaired t-tests, $P<0.05$).

Figure 5.14: Comparison of mean tissue weight of uterine preparations obtained from oestrogen-treated (n=430 preparations) and pregnant mice (n=196 preparations). Each bar is the mean ± SEM.



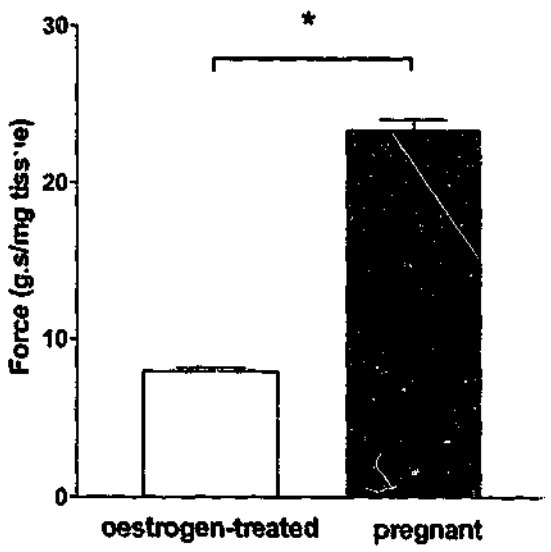


Figure 5.15: Comparison of mean MCh (10μM) response in uterine preparations obtained from oestrogen-treated (n=372 preparations) and pregnant mice (n=196 preparations). Each bar is the mean ± SEM.

* indicates significant difference from preparations obtained from oestrogen-treated mice, Student's unpaired t-test, $P<0.05$; d.f. = 552.

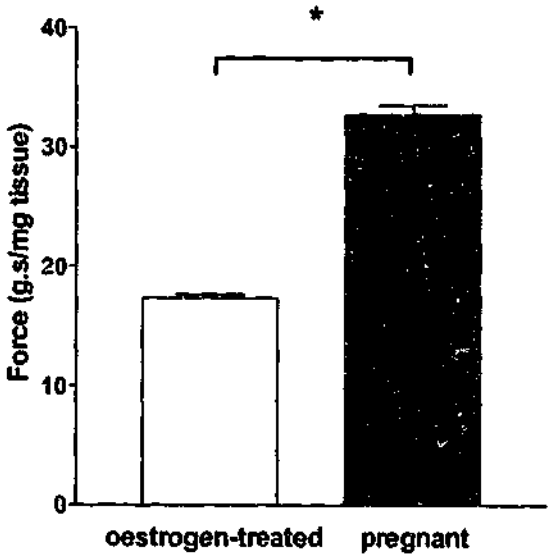


Figure 5.16: Comparison of mean KPSS response in uterine preparations obtained from oestrogen-treated (n=430) and pregnant mice (n=196). Each bar is the mean ± SEM.

* indicates significant difference from oestrogen-treated preparations, Student's unpaired t-test, $P<0.05$; d.f. = 624.

In the oestrogen-treated mouse uterus, the order of agonist potency of the mammalian tachykinins in the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM) was SP≥NKA>NKB. Of the receptor-selective analogues [$Sar^9Met(O_2)^{11}$]SP was approximately equipotent with SP while [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) was relatively ineffective. In contrast, in the pregnant mouse uterus, in the absence of peptidase inhibitors, the order of agonist potency of the mammalian tachykinins based on the relative positions of the log CRCs was NKA>SP≥NKB, however SP and NKB did not reach a ceiling over the concentration range examined (0.1nM - 1μM). [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and [$Sar^9Met(O_2)^{11}$]SP were equipotent with NKA although [$Sar^9Met(O_2)^{11}$]SP was significantly less efficacious. Note that the peptidase inhibitors, at the same concentrations that produced potentiation of the tachykinin agonists on uterine preparations obtained from oestrogen-treated mice, produced no potentiation of responses to tachykinins in uterus from pregnant mice. Figure 5.17 allows comparison of the effects of two receptor-selective agonists on uterine preparations from oestrogen-treated and pregnant mice. Note that [$Sar^9Met(O_2)^{11}$]SP was a full agonist on uterine preparations from oestrogen-treated mice and a partial agonist on preparations from pregnant mice while in contrast [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) was a partial agonist in preparations from oestrogen-treated mice and a full agonist in preparations from pregnant mice.

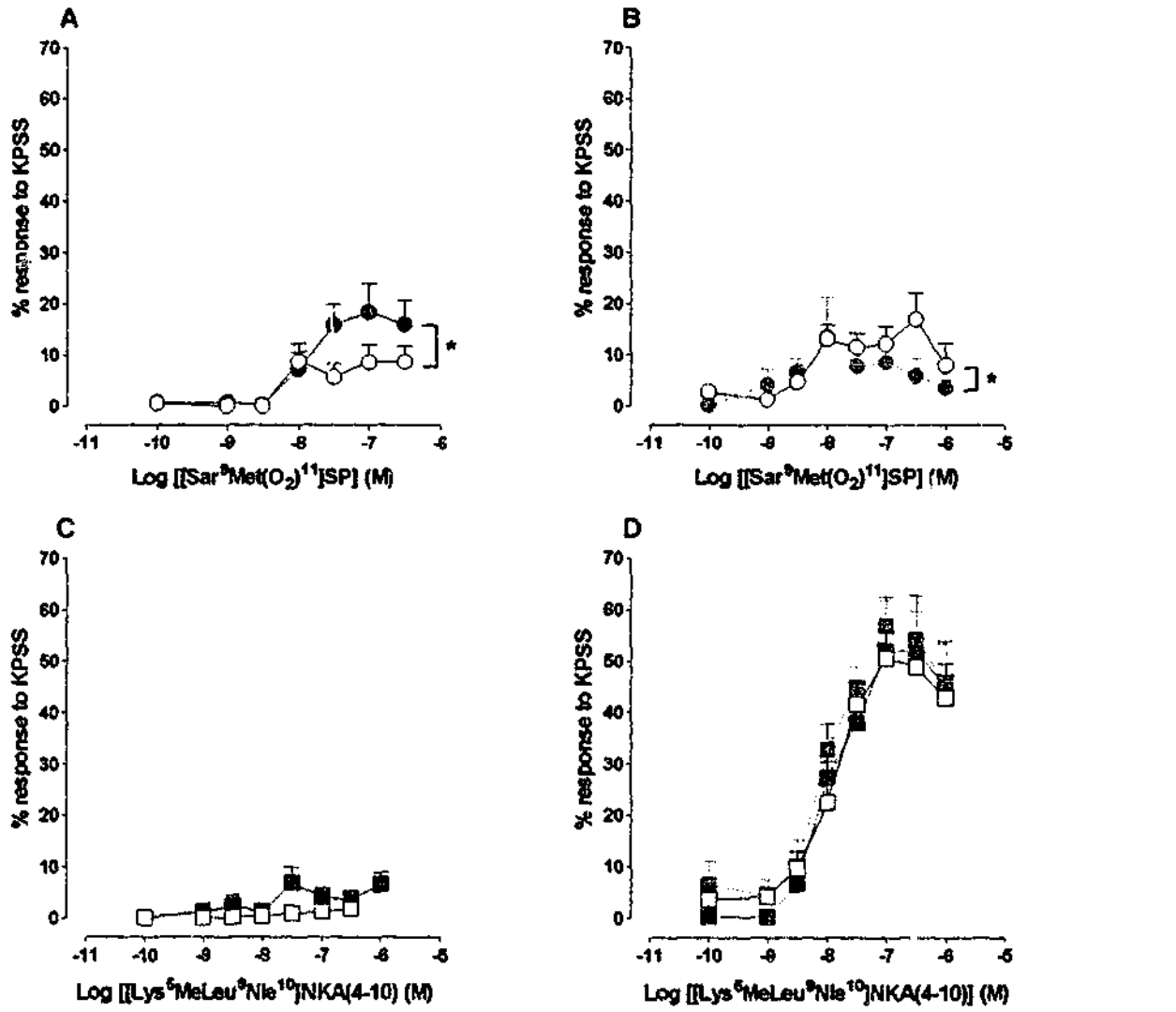


Figure 5.17: Log CRCs to [Sar^{9}Met(O$_2$)^{11}]SP on uterine preparations from (A) oestrogen-treated and (B) pregnant mice and log CRCs to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) on uterine preparations from (C) oestrogen-treated and (D) pregnant mice. CRCs to the receptor-selective agonists are in the absence of peptidase inhibitors (open symbols) and in the presence of thiorphan (3μM) (red), thiorphan (3μM) and captopril (10μM) (orange), thiorphan (3μM) and bestatin (10μM) (green) or thiorphan (3μM), captopril (10μM) and bestatin (10μM) (blue). Each point is the mean ± SEM, n = 6 – 8 animals. Please note that these data were shown previously in Figures 3.8, 3.9, 5.5 and 5.6.
* indicates a significant difference in log CRC compared to control, two-way ANOVA, $P<0.05$.

5.4 Discussion

The present study was undertaken to investigate further the tachykinin receptor type(s) involved in mediating tachykinin-induced activity in the pregnant mouse uterus. It was found that the mammalian tachykinins SP, NKA and NKB all elicited contractions in the pregnant mouse uterus however their efficacies and their order of agonist potency differed from that observed in uterine preparations from the oestrogen-treated mouse.

In the previous chapter it was established that in the oestrogen-treated mouse uterus the order of potency of the mammalian tachykinins is SP≥NKA>NKB. However, in the present study the order of potency on uterine preparations from the pregnant mouse was found to be NKA>SP≥NKB. This indicates that the predominant tachykinin receptor mediating contractions of the pregnant mouse uterus may differ from that of the oestrogen-treated mouse uterus. The results of the present study suggest that in the pregnant mouse uterus, tachykinins mediate contractile activity predominantly by activation of an NK_2 receptor, which is in contrast to the oestrogen-treated mouse uterus where it was found that the NK_1 receptor primarily mediates tachykinin-induced activity. These results are consistent with those obtained previously in preliminary experiments conducted in our laboratory where the order of agonist potency of the mammalian tachykinins in the pregnant mouse uterus was NKA>>SP=NKB (Fleming, 1998). While it has been confirmed that the NK_2 receptor mediates tachykinin-induced contractions in the non-pregnant rat uterus (Fisher and Pennefather, 1997; 1999; Magraner *et al.*, 1998; Pennefather *et al.*, 1993) only two studies to date have investigated the actions of tachykinins on myometrium obtained from pregnant rats. Candenas *et al.* (2001) reported that in the pregnant rat uterus NKA acted as a full agonist whereas maximal responses to both the NK_1 and NK_3 receptor-selective agonists $[Sar^9Met(O_2)^{11}]SP$ and $[MePhe^7]NKB$ were significantly smaller in comparison. Consistent with the observations of Candenas and colleagues, Shintani *et al.* (2000) also reported that NKA acted as a full agonist in myometrium obtained from pregnant rats. This suggests that during pregnancy, as in the non-pregnant rat, the NK_2 receptor appears to be the most important mediator of tachykinin-induced uterine contractility.

Further confirmation of the involvement of the NK_2 receptor in mediating tachykinin-induced activity in the pregnant mouse uterus comes from experiments undertaken with the NK_1 and NK_2 receptor-selective agonists $[Sar^9Met(O_2)^{11}]SP$ and $[Lys^5MeLeu^9Nle^{10}]NKA$. In the present study the NK_2 receptor-selective $[Lys^5MeLeu^9Nle^{10}]NKA(4-10)$ (Chassaing *et al.*, 1991) was equipotent with NKA in producing contractions in the pregnant mouse uterus. Interestingly, the NK_1 selective agonist $[Sar^9Met(O_2)^{11}]SP$ (Drapeau *et al.*, 1987) was also equipotent with NKA, suggesting the presence of functional tachykinin NK_1 receptors in the pregnant mouse uterus. However, log concentration-response curves to $[Sar^9Met(O_2)^{11}]SP$ were bell-shaped which is consistent with literature indicating that cellular responses mediated by the NK_1 receptor are rapidly desensitized (Grady *et al.*, 1997) and $[Sar^9Met(O_2)^{11}]SP$ produced a markedly lower maximum response than either NKA or $[Lys^5MeLeu^9Nle^{10}]NKA(4-10)$. These results are in accordance with those of previous preliminary work conducted in our laboratory on the pregnant mouse uterus (Fleming, 1998) and indicate that during pregnancy tachykinin-induced contractility is mediated primarily through the NK_2 receptor though functionally active NK_1 receptors are also present.

The involvement of both NK_1 and NK_2 receptors in mediating uterotonic responses to tachykinins in the pregnant mouse uterus was further supported by studies using the potent NK_1, NK_2 and NK_3 receptor-selective antagonists SR 140333, SR 48968 and SR 142801. In the presence of the NK_1 receptor-selective antagonist SR 140333 (Emonds-Alt *et al.*, 1993b) responses to both SP and $[Sar^9Met(O_2)^{11}]SP$, but not to NKA or $[Lys^5MeLeu^9Nle^{10}]NKA(4-10)$, were reduced. Conversely, the NK_2 receptor-selective antagonist SR 48968 (Advenier *et al.*, 1992a) reduced responses to NKA and $[Lys^5MeLeu^9Nle^{10}]NKA(4-10)$ but had no effect on responses to SP and $[Sar^9Met(O_2)^{11}]SP$. Interestingly, both SR 140333 and SR 48968 acted as non-competitive antagonists in the pregnant mouse uterus. In the present study the decrease in the maximum response to $[Sar^9Met(O_2)^{11}]SP$ obtained in the presence of SR 140333 is consistent with that seen in the oestrogen-treated mouse uterus and various *in vitro* assays (Emonds-Alt *et al.*, 1993b). The antagonism by SR 48968 of responses to both NKA and $[Lys^5MeLeu^9Nle^{10}]NKA(4-10)$ in the pregnant mouse uterus was also non-

competitive. In different *in vitro* preparations SR 48968 has been reported to act as either a competitive or as a non-competitive antagonist. Croci *et al.* (1995a) observed that SR 48968 competitively antagonised responses to the NK_2 receptor-selective agonist [β-Ala^8]NKA(4-10) in the rat duodenum, whereas it non-competitively antagonised responses to the same agonist in the rat oesophagus. It has been proposed that this difference in antagonist properties could be due to the existence of differing NK_2 receptor subtypes (Croci *et al.*, 1995a) as has also been suggested by recent pharmacological and molecular studies in various tissues in the rat and human (Candenas *et al.*, 2002; Croci *et al.*, 1998; Lippe *et al.*, 1997; Patacchini *et al.*, 2001).

In the present study the NK_3 receptor-selective antagonist SR 142801 (Emonds-Alt *et al.*, 1995) had no effect on responses to SP, NKA, [$Sar^9Met(O_2)^{11}$]SP or [$Lys^5MeLeu^9Nle^10$]NKA(4-10), indicating the non-involvement of NK_3 receptors in mediating uterine contractility in the pregnant mouse.

It is well recognized that uterine contractility is highly dependent upon the hormonal environment and that changes in levels of ovarian steroids or pregnancy can alter the mechanical response of uterine smooth muscle to various agents. Previous studies in our laboratory have described the ability of ovarian steroids and pregnancy to modulate uterine responses elicited by field stimulation and various adrenoceptor agonists in non-pregnant and pregnant guinea-pigs (Adam *et al.*, 1985; Hall and Pennefather, 1990; Handberg *et al.*, 1988; Hartley *et al.*, 1983).

To date there have been few studies investigating the hormonal regulation of uterine contractility bought about by tachykinin receptor activation. In the non-pregnant rat, functional and molecular studies have shown that the three tachykinin receptor types NK_1, NK_2 and NK_3 are present in the uterus and while it appears that their expression and responsiveness are influenced by the uterine hormonal environment, the exact roles that oestrogen and progesterone play in their regulation remains unclear (Barr *et al.*, 1991; Hamlin *et al.*, 2000; Magraner *et al.*, 1998; Moodley *et al.*, 1999; Pinto *et al.*, 1997; 1999). In a study by Moodley *et al.* (1999) which investigated tachykinin-induced uterine contractions in rats during the oestrous cycle no difference was seen in the

relative potencies of SP, NKA, NKB or [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10)] in rats who were in proestrus/oestrus compared to those in metoestrus/dioestrus. In contrast, another study reported that uterine responses to the NK_1, NK_2 and NK_3 receptor-selective agonists SarMet SP, β-Ala NKA and senktide were decreased in tissue from rats in oestrus/proestrus compared to those in metoestrus/dioestrus (Hamlin *et al.*, 2000). In ovariectomised rats, the expression of the three tachykinin receptor types was reported to be selectively and differentially influenced by ovarian steroids (Pinto *et al.*, 1999). In that study neither oestrogen nor progesterone affected NK_2 receptor expression, whereas oestrogen increased NK_1 and strongly decreased NK_3 receptor expression and progesterone decreased NK_1 receptor expression while having no effect on NK_3 receptor expression (Pinto *et al.*, 1999). Pinto *et al.* (1999) also reported that a direct correlation existed between the level of receptor expression and the magnitude of contractile response elicited by activation of that receptor. While Hamlin *et al.* (2000) also observed that oestrogen-treatment caused a decrease in NK_3 receptor-mediated uterine contractility in ovariectomised rats, in contrast to the findings of Pinto *et al.* (1999), oestrogen was also reported to decrease both NK_1 and NK_2 receptor-mediated uterotonic effects. A possible explanation for this reported difference in the effects of oestrogen on tachykinin-induced contractility in the ovariectomised rat could be due to differences in the oestrogen-treatment regime employed by the two studies. While Pinto *et al.* (1999) administered 50μg/kg oestrogen intraperitoneally for two consecutive days before experimentation, Hamlin *et al.* (2000) treated their rats with 100μg/kg oestrogen administered subcutaneously in a single dose 48h before experimentation.

To date, only one study has investigated the effects of pregnancy on tachykinin receptor expression in the uterus (Candenas *et al.*, 2001). This study, conducted in the rat, found that while all three tachykinin receptor types were present in the uterus, the expression of NK_1 and NK_3 receptors varied along the course of pregnancy whereas NK_2 receptor expression remained stable, suggesting that the influence of ovarian steroids on receptor expression is similar to that observed by Pinto *et al.* (1999) in the ovariectomised rat. Candenas *et al.* (2001) also reported a direct correlation between contractile

responsiveness to agonists acting selectively at the three tachykinin receptor types and receptor expression.

In the present study tachykinin receptor function appeared to differ between the non-pregnant and pregnant mouse uterus. This is clearly demonstrated by the observation that while the NK_1 receptor-selective [$Sar^9Met(O_2)^{11}$]SP acted as a full agonist in the non-pregnant mouse uterus it was only a partial agonist in the pregnant mouse uterus. Conversely, the NK_2 receptor-selective [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) acted as a partial agonist in the non-pregnant mouse uterus but was a full agonist in the pregnant mouse uterus. This suggests that in the mouse uterus during pregnancy there may be a change in tachykinin receptor expression, as is reported to occur with NK_1 and NK_3 receptors in the rat uterus (Candenas *et al.*, 2001) and/or receptor-effector coupling. It would therefore be of interest to investigate tachykinin receptor expression in the non-pregnant and pregnant mouse uterus to see whether this is the case.

Since the levels of ovarian steroids were not measured in the present study it was not possible to establish directly any correlation between uterine hormonal environment and the contractile responses elicited by agonists acting at the three tachykinin receptor types. Nevertheless, it is interesting to note though that the mice utilized in this study were used on day 17 of an average 19 day gestation period, which is when oestrogen and progesterone levels are known to peak (Pasqualini and Kincl, 1985). A similar experimental design to that employed by Candenas *et al.* (2001), whereby tachykinin receptor expression and function in the uterus and ovarian steroid levels are measured over several days throughout gestation and post partum, would need to be undertaken before any conclusions could be made concerning hormonal regulation of tachykinin receptors in the mouse uterus during pregnancy.

An interesting finding from the present study is that the neprilysin inhibitor thiorphan (3μM) either alone or in combination with either captopril (10μM) and/or bestatin (10μM) did not potentiate the response to SP, NKA, [$Sar^9Met(O_2)^{11}$]SP nor [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) in the pregnant mouse uterus. In contrast, in preliminary

studies conducted in our laboratory, thiorphan, at the higher concentration of 10μM, significantly potentiated responses to both SP and NKA in the pregnant mouse uterus (Fleming, 1998). This suggests the possibility that thiorphan at a concentration of 3μM is not effective at inhibiting neprilysin in the pregnant mouse uterus. In this context it is of interest that the findings of Chapter 3 showed that thiorphan at a concentration of 3μM enhanced responses to NKA, and in combination with captopril (10μM) and/or bestatin (10μM), potentiated responses to SP and [$Sar^9Met(O_2)^{11}$]SP, in the oestrogen-treated mouse uterus. Furthermore, thiorphan used at the lower concentration of 1uM has also been shown to be effective in preventing tachykinin degradation in several other tissues (Kerr *et al.*, 1997; Maggi *et al.*, 1992; Rouissi *et al.*, 1990). A possible explanation for the apparent ineffectiveness of thiorphan in the present study could be explained by an up-regulation of neprilysin expression in the mouse uterus during pregnancy. The expression of neprilysin, the most effective of the peptidases in cleaving the tachykinins (Matsas *et al.*, 1983; 1985) has been reported to be regulated by ovarian steroids (Pinto *et al.*, 1999) and in the late-term pregnant rat uterus neprilysin activity is 25 times higher than in the non-pregnant rat uterus (Ottlecz *et al.*, 1991). This is consistent with the report that the contractile response elicited by NKA in the pregnant rat uterus is significantly enhanced in the presence of the neprilysin inhibitor phosphoramidon (1μM) whereas no significant potentiation was seen in the non-pregnant rat uterus (Shintani *et al.*, 2000). Unfortunately, the cost of thiorphan prevented it from being used at the higher concentration of 10μM throughout the present study, however it would clearly be of interest to conduct further experiments using increased amounts of thiorphan to clarify the role of peptidases in constraining the contractile response to tachykinins in the pregnant mouse uterus.

It is possible that the order of agonist potency of the mammalian tachykinins in the pregnant mouse uterus could be different in the presence of a higher concentration of thiorphan. However, it is interesting to note that the order of agonist potency of the mammalian tachykinins in the present study was consistent with that obtained in preliminary experiments conducted previously in our laboratory using the higher concentration of thiorphan (Fleming, 1998). The importance of the NK_2 receptor in

mediating tachykinin-induced contractility in the pregnant mouse uterus is also further supported by the use of the NK_2 receptor-selective [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10). [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) which is reported to be metabolically stable (Fisher and Pennefather, 1997; Fisher *et al.*, 1993) was only seen to be a partial agonist in the oestrogen-treated mouse but acted as a full agonist in the pregnant mouse uterus.

In conclusion, the results of the present study using mammalian tachykinins, tachykinin receptor-selective agonists and non-peptide tachykinin antagonists indicate the involvement of functional NK_1 and NK_2 receptors in mediating the contractile activity of tachykinins in the pregnant mouse uterus. In the oestrogen-treated mouse the uterotonic effects of tachykinins were primarily mediated by the NK_1 receptor. In contrast, during pregnancy tachykinin-induced uterine contractility was predominantly mediated by activation of NK_2 receptors. These results suggest that the function of tachykinin receptors in the mouse uterus could be under hormonal regulation, and in contrast to the rat uterus the predominant tachykinin receptor type mediating tachykinin-induced contractile activity appears to be altered during pregnancy.

The aim of the next two chapters is to firstly characterize the tachykinin receptor type(s) mediating the uterotonic activity of tachykinins in the human uterus and secondly to investigate whether the functional responses elicited by activation of tachykinin receptors are modulated by pregnancy as observed in the mouse uterus.

CHAPTER 6

CHARACTERIZATION OF THE UTEROTONIC EFFECTS OF TACHYKININ PEPTIDES ON THE NON-PREGNANT HUMAN UTERUS

Summary

1. The aims were (1) to characterize the receptor type(s) mediating the uterotonic activity of tachykinins in myometrium from non-pregnant women, (2) to examine the influence of peptidases in constraining the response to tachykinin peptides in this tissue preparation and, (3) to investigate the effects of the muscarinic antagonist atropine, the α adrenoceptor antagonist phentolamine and the nerve blocking agent tetrodotoxin (TTX) on uterine responses elicited by tachykinin peptides.

2. In the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM) the mammalian tachykinins SP, NKA and NKB acted as full agonists with an order of potency based on the positions of the log concentration-response curves of NKA>SP≥NKB.

3. The NK_2 receptor-selective agonist [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) acted as a full agonist and was approximately equipotent with NKA. In contrast, the NK_1 and NK_3 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP and [$MePhe^7$]NKB were ineffective.

4. The NK_2 receptor-selective antagonist, SR 48968, produced significant concentration-related rightward shifts in the log concentration-response curve to [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10).

5. Neither of the peptidase inhibitors thiorphan (3μM) nor bestatin (10μM) either alone or in combination potentiated responses to NKA in non-pregnant human uterus.

6. Responses to [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) were unaffected by atropine (0.3μM), phentolamine (1μM) or TTX (1μM) indicating that the receptors activated by [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) are probably located on uterine smooth muscle, rather than on autonomic nerves.

7. The results of this chapter indicate that the contractile effects of the tachykinins in non-pregnant human myometrium are mediated by activation of an NK_2 receptor most likely located on the uterine smooth muscle. The peptidases neprilysin and bestatin-sensitive aminopeptidase do not modulate responses elicited by the tachykinins in this tissue preparation.

6.1 Introduction

The effects of tachykinins on myometrial contractility have largely been studied in rodents. To date, the majority of these studies have been conducted in the rat where it has been confirmed that the tachykinins elicit contractile activity through activation of predominantly an NK_2 receptor (Fisher and Pennefather, 1997; 1998; 1999; Fisher *et al.*, 1993; Magraner *et al.*, 1998; Moodley *et al.*, 1999). It was established in Chapters 3 and 4 that the tachykinins also enhance uterine contractility in the oestrogen-treated mouse uterus, though in this species activity is predominantly mediated by activation of an NK_1 receptor. In contrast, previous reports in the literature regarding the actions of tachykinins on myometrium from non-pregnant women have left the situation unclear, although both SP and the non-mammalian tachykinin eledoisin have been reported to elicit contractions in this tissue (Molina and Zappia, 1976; Ottesen *et al.*, 1983). SP has also been reported to contract isolated preparations of ampullary-isthmic junction obtained from non-pregnant women (Forman *et al.*, 1985).

Immunohistochemical studies have demonstrated the occurrence of tachykinin-immunoreactive nerves in the human uterus (Butler-Manuel *et al.*, 2002; Franco-Cereceda *et al.*, 1987; Fried *et al.*, 1990; Heinrich *et al.*, 1986; Reinecke *et al.*, 1989; Samuelson *et al.*, 1985). The association of these nerves with smooth muscle cells indicates the possibility that tachykinins released from their peripheral terminals may influence myometrial activity. SP has also been reported to be present in neuroendocrine cells in the female reproductive tract (Skrabanek and Powell, 1983). In addition, recent molecular studies conducted by our Spanish collaborators indicate that preprotachykinin B, the gene that encodes for NKB, is expressed in human myometrium (Patak *et al.*, 2003). Furthermore, the presence of the peptidase neprilysin, which constrains the actions of tachykinins in both the rat (Fisher and Pennefather, 1997; Fisher *et al.*, 1993) and mouse uterus (as reported in Chapter 3), has also been reported in the human uterus (Head *et al.*, 1993; Patak *et al.*, 2003). Taken together, these findings are consistent with the hypothesis that tachykinins may exert a modulatory effect upon the contractile activity of the human uterus.

The aims of the experiments described in this chapter were three-fold. The first aim was to characterize the tachykinin receptor type(s) mediating contractile activity in myometrial preparations from non-pregnant women. Receptor characterization was undertaken by determining the effects of the mammalian tachykinins SP, NKA and NKB and the NK_1, NK_2 and NK_3 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP, [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and [$MePhe^7$]NKB. The second aim was to investigate the extent to which peptidases modulated the response to tachykinins in this tissue. The third aim was to elucidate the location of the receptors on which the tachykinins acted to produce their uterotonic effects, by examining the effects of the muscarinic antagonist atropine, the α adrenoceptor antagonist phentolamine and the nerve-blocking agent TTX on uterine responses elicited by tachykinin peptides.

6.2 Materials and Methods

6.2.1 Tissue preparations

Full details of women who gave informed consent to participate in this study are given in Chapter 2 (section 2.1.2 and 2.1.2.1) and Appendix 2. Briefly, uterine tissue was obtained from women (31-65 years old, n=38) undergoing either a total abdominal or vaginal hysterectomy for benign uterine disease. Four preparations were obtained from the outer myometrium (as described in 2.2.3.1) and set up as described in 2.3.1 for recording of force from the longitudinal smooth muscle layer. Please note that of the 38 women who participated in this study, none of the uterine preparations obtained from a subset of 8 women responded to either the agonists or KPSS. Data from this subset of women were not included in the present study.

6.2.2 Experimental protocol

Refer to Chapter 2 (section 2.3).

6.2.3 Measurement of results and statistical analyses

Responses to all agonists were measured as area under the force-time curve for the duration of agonist exposure as described in section 2.4. Responses were then expressed as a percentage of the response to the KPSS and presented as mean ± SEM.
Statistical analysis was undertaken as described in section 2.6.

Correlation between the potencies of NKA and [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and the age of the subject from whom uterine tissue was obtained was determined by a Pearson Product Moment Correlation test.

E_{max} is defined as the maximum response to an agonist observed over the concentration range used; note that might not in all cases be the greatest response that an agonist could elicit if higher concentrations had been tested.

6.2.4 Drugs

The drugs used included all those previously described in section 2.7, which includes details of stock solutions, vehicles used and vehicle controls as appropriate.

Table 6.1: Overview of experiments undertaken on uterine preparations obtained from non-pregnant women. Note that four preparations were obtained from each patient and are allocated as preparation 1, 2, 3 or 4 using a randomized procedure.

Series	Preparation 1		Preparation 2		Preparation 3		Preparation 4	
	Agonist	Treatment	Agonist	Treatment	Agonist	Treatment	Agonist	Treatment
1: Mammalian TKs in the presence of peptidase inhibitors (n=6)	*SP (6)	-	*NKA (6)	-	*NKB (6)	-	-	-
2: Sar^9, Lys^5 and $MePhe^7$ (n=6)	Sar^9 (6)	-	Lys^5 (6)	-	$MePhe^7$ (5)	-	-	-
3: NKA in the absence and presence of peptidase inhibitors (n=5)	NKA (5)	-	NKA (5)	Thiorphan (3μM)	NKA (5)	Bestatin (10μM)	NKA (5)	Thiorphan (3μM) & bestatin (10μM)
4: Lys^5 in the absence and presence of SR 48968 (n=7)	Lys^5 (5)	-	Lys^5 (5)	SR 48968 (1nM)	Lys^5 (7)	SR 48968 (3nM)	Lys^5 (5)	SR 48968 (10nM)
5: Lys^5 in the absence and presence of atropine, phentolamine and TTX (n=6)	Lys^5 (5)	-	Lys^5 (6)	Atropine (0.3μM)	Lys^5 (5)	Phentolamine (1μM)	Lys^5 (5)	TTX (1μM)

TKs = Tachykinins, Sar^9 = [$Sar^9Met(O_2)^{11}$]SP, Lys^5 = [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10), $MePhe^7$ = [$MePhe^7$]NKB

* indicates peptidase inhibitors present (thiorphan (3μM), captopril (10μM) and bestatin (10μM))

n = number of women

Numbers in brackets in agonist column indicate number of viable preparations (*i.e.*, responded to agonist & KPSS)

6.3 Results

Uterine tissue used throughout this study was obtained from 38 women who had undergone either a vaginal or total abdominal hysterectomy (mean age 45.7 years, range 31-65 years; mean parity 2.0, range 0-4). Of these 38 women, uterine preparations obtained from 8 women were not viable (i.e. did not respond to agonist or KPSS) and therefore not included in the present study. The mean weight of all tissue preparations included in the analysis was 104.5 ± 3.5mg (n = 98 preparations). The mean response to KPSS was 15.6 ± 0.9g.s/mg tissue (n = 98 preparations). Spontaneous activity was observed in nearly all tissue preparations and ranged from one to six contractions over any 15min period; this gradually decreased over the duration of the equilibration period. Figure 6.1 shows a representative trace of responses to increasing concentrations of the NK_1, NK_2 and NK_3 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP, [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and [$MePhe^7$]NKB. It can be seen that neither the NK_1 receptor-selective [$Sar^9Met(O_2)^{11}$]SP nor the NK_3 receptor–selective [$MePhe^7$]NKB were effective at eliciting contractile activity in the non-pregnant human uterus.

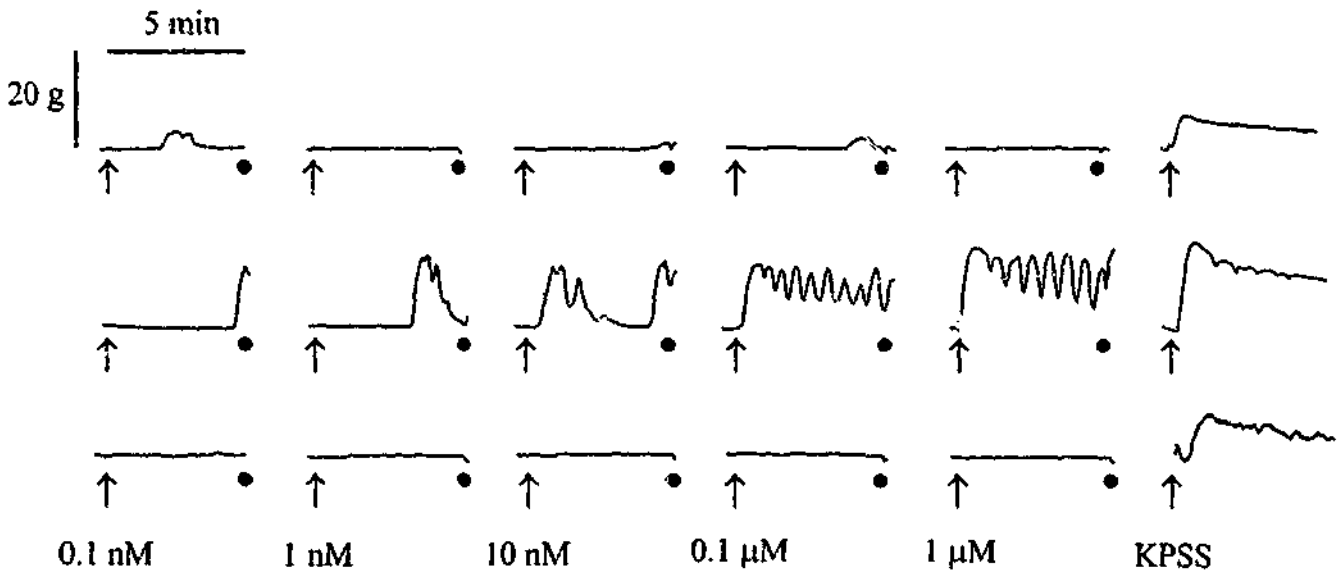


Figure 6.1: Representative trace showing response of outer myometrium from a non-pregnant woman to [$Sar^9Met(O_2)^{11}$]SP (upper panel), [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) (middle panel) and [$MePhe^7$]NKB (lower panel) together with responses to KPSS. The arrows indicate agonist addition and the dots represent washes.

6.3.1 Uterotonic activity of substance P, neurokinin A and neurokinin B

In the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM), NKA was the most potent mammalian tachykinin (two-way ANOVA, $P<0.05$; d.f. = 134) and was 82- and 94-fold more potent than SP (95% C.L. = 35.1, 217.7; d.f. = 57) and NKB (95% C.L. = 35.7, 292.5; d.f. =56) respectively (Figure 6.2).

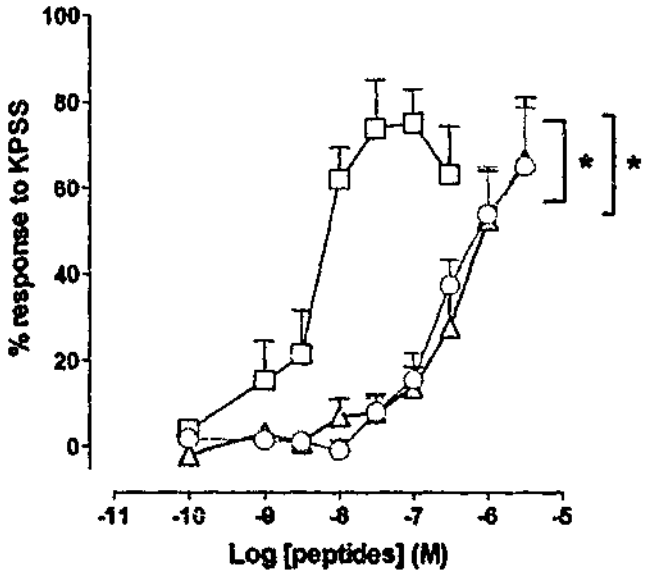


Figure 6.2: Log concentration-response curves (CRCs) to SP (○), NKA (□) and NKB (△) on uterine preparations from non-pregnant women in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). Each point is the mean ± SEM, n=6 women. Note that the log CRC to NKA reached a ceiling at concentrations over 0.1μM. In this and subsequent figures, a significant difference between log CRCs as indicated by two-way ANOVA followed by Student Newman Keuls is shown by *.

* indicates significant difference from NKA, two-way ANOVA, $P<0.05$; d.f. = 134

6.3.2 Uterotonic activity of [Sar^{9}Met(O$_2$)^{11}]SP, [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) and [MePhe^7]NKB

Results of a two-way ANOVA indicated a significant difference in the log CRCs to the tachykinin receptor-selective agonists ($P<0.05$; d.f. = 126). Of the tachykinin receptor-selective agonists only the NK_2 receptor-selective [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) acted as a full agonist while the NK_1 and NK_3 receptor-selective agonists [Sar^{9}Met(O$_2$)^{11}]SP and [MePhe^7]NKB were without effect at the concentrations tested (Figure 6.3).

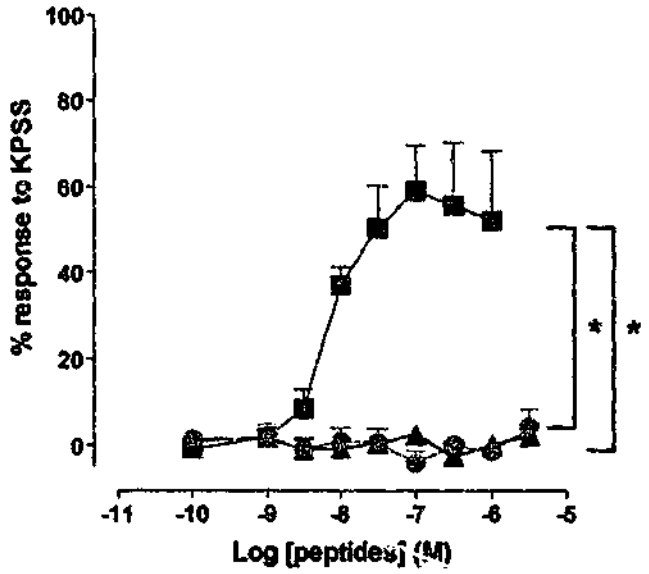


Figure 6.3: Log CRCs to [Sar^{9}Met(O$_2$)^{11}]SP (●), [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) (■) and [MePhe^7]NKB (▲) on uterine preparations from non-pregnant women. Each point is the mean ± SEM, n=5-6 women. Note that the log CRC to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) reached a ceiling at concentrations over 0.1μM.

* indicates significant difference from [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10), two-way ANOVA; d.f. = 126

6.3.3 Order of agonist potency

In the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM) the relative order of agonist potency for the mammalian tachykinins based on the positions of the log CRCs was NKA>SP≥NKB. Of the receptor-selective agonists [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) was 4-fold less potent than NKA (95% CL = 1.86, 8.39; d.f. = 44) while [Sar^{9}Met(O$_2$)^{11}]SP and [MePhe^7]NKB were without effect (Figure 6.4). No significant difference was observed in the maximum responses elicited by the mammalian tachykinins and [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) whereas maximum responses to [Sar^{9}Met(O$_2$)^{11}]SP and [MePhe^7]NKB were significantly smaller (one-way ANOVA, $P<0.05$; Table 6.2). No significant differences were seen in the mean responses to KPSS in experiments for which agonist potencies were compared (one-way ANOVA, $P>0.05$; Table 6.2).

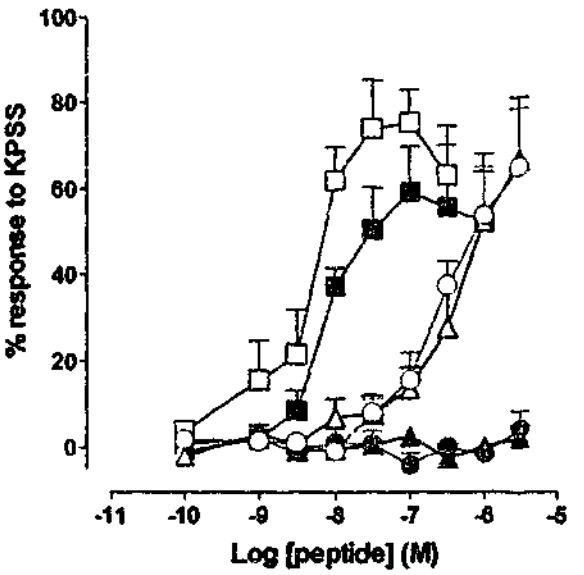


Figure 6.4: Log CRCs to SP (○), NKA (□), NKB (△), [Sar^{9}Met(O$_2$)^{11}]SP (●), [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) (■) and [MePhe^7]NKB (▲) on uterine preparations from non-pregnant women. Each point is the mean ± SEM, n=5-6. CRCs to the endogenous tachykinins were constructed in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). Note that these log CRCs are those shown previously in Figures 6.2 and 6.3.

Table 6.2: Effects of peptides and KPSS on myometrium from non-pregnant women (n=6).

N	Peptide	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (%KPSS ± SEM)	Peptide potency (pD_2 ± SEM)
6	*SP	13.6 ± 2.6	60.1 ± 12.3	N/A
6	*NKA	19.8 ± 3.6	82.5 ± 9.9	8.40 ± 0.14
6	*NKB	16.1 ± 4.0	66.7 ± 14.5	N/A
6	Sar^9	12.6 ± 3.5	9.9 ± 2.2[a]	N/A
6	Lys^5	19.2 ± 5.6	67.3 ± 13.0	8.11 ± 0.11
5	MePhe^7	16.4 ± 5.9	5.7 ± 2.0[a]	N/A

N – number of viable preparations
Sar^9 – [Sar^{9}Met(O$_2$)^{11}]SP, Lys^5 – [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10), MePhe^7 – [MePhe^7]NKB
N/A – not applicable (could not be estimated as log CRC did not reach a clear maximum)
* indicates the presence of peptidase inhibitors (thiorphan (3μM), captopril (10μM) and bestatin (10μM))
a – significantly different from the corresponding maximal responses for NKA and [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) (one-way ANOVA, P<0.05).

6.3.4 Influence of peptidase inhibitors on uterotonic effects of neurokinin A

Responses to NKA were unaffected by thiorphan (3μM) (potency ratio = 1.1, 95% CL = 0.3, 4.1; d.f. = 36), bestatin (10μM) (potency ratio = 1.1, 95% CL = 0.3, 4.3; d.f. = 36) or thiorphan (3μM) together with bestatin (10μM) (potency ratio = 1.0, 95% CL = 0.3, 3.6; d.f. = 36) (Figure 6.5). The peptidase inhibitors also had no significant effect on either the maximal response elicited by NKA or the mean response to KPSS (Table 6.3).

Figure 6.5: Log CRCs to NKA in the absence of peptidase inhibitors (□) and in the presence of thiorphan (3μM) (■), bestatin (10μM) (■) and thiorphan (3μM) together with bestatin (10μM) (■) on uterine preparations from non-pregnant women. Each point is the mean ± SEM, n=5 women.

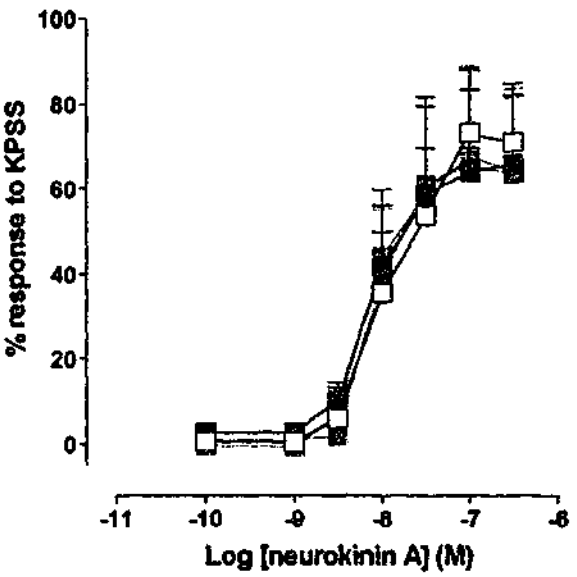


Table 6.3: Effects of NKA and KPSS in the absence and presence of peptidase inhibitors on myometrium from non-pregnant women (n=5).

Peptide	Treatment	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (%KPSS ± SEM)	Peptide Potency (pD_2 tissue ± SEM)
NKA		22.4 ± 4.7	75.6 ± 15.2	7.87 ± 0.13
NKA	T	21.5 ± 1.8	69.2 ± 17.5	8.01 ± 0.21
NKA	B	19.3 ± 2.6	72.0 ± 20.2	8.04 ± 0.24
NKA	T & B	14.9 ± 4.3	73.7 ± 19.1	7.87 ± 0.19

T – thiorphan (3μM); B – bestatin (10μM)

6.3.5 Effect of SR 48968 on the uterotonic response to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10)

SR 48968 at a concentration of 1, 3 and 10nM significantly attenuated the response to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) (two-way ANOVA, $P<0.05$; d.f. =162). This attenuation corresponded to 90-, 154- and 640-fold rightward shifts of the log CRC to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) in the presence of 1nM (95% CL = 8.9, 1964.7; d.f. = 26), 3nM (95% CL = 19.7, 1872.7; d.f. = 27) and 10nM SR 48968 (95% CL = 91.8, 14,705.9; d.f. = 30) respectively (Figure 6.6). Analysis by one-way ANOVA indicated no significant difference in the maximal response to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) in either the absence or presence of SR 48968 ($P>0.05$; d.f = 18), however inspection of the curves indicates that there may be a trend towards depression of the maximum response as the antagonist concentration was increased. Note that the CRCs for [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) in the presence of 1 and 3nM SR 48968 had clearly reached a maximum. Responses to KPSS were unaffected by SR 48968 at the concentrations used (Table 6.4).

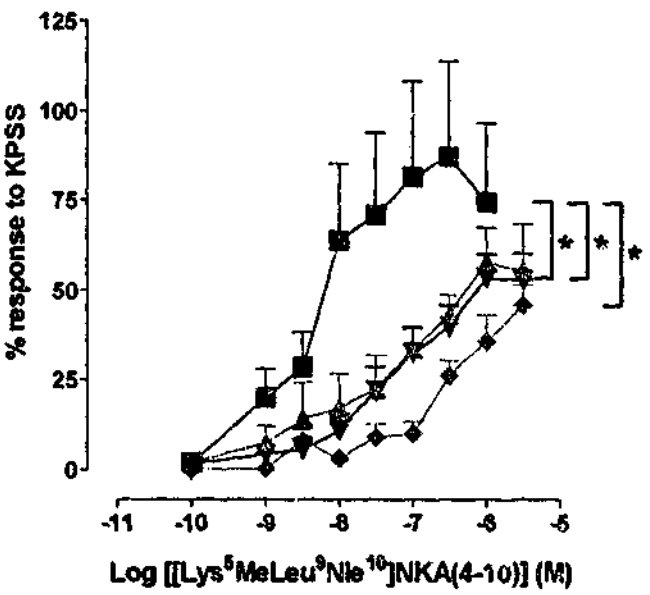


Figure 6.6: Log CRCs to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) in the absence (■) and presence of (1nM; ▲), (3nM; ▼) and (10nM; ◆) SR 48968 on uterine preparations from non-pregnant women. Each point is the mean ± SEM, n=5-7 women.

* indicates significant difference from control, two-way ANOVA, $P<0.05$: d.f. = 162

Table 6.4: Effects of [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) and KPSS in the absence and presence of SR 48968 on myometrium from non-pregnant women (n=5).

SR 48968 concentration	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (%KPSS tissue ± SEM)	Peptide Potency (pD_2 tissue ± SEM)
	13.0 ± 3.4	95.5 ± 26.6	8.31 ± 0.24
1nM	11.6 ± 2.8	64.3 ± 12.3	7.27 ± 0.47
3nM	15.4 ± 4.3	58.6 ± 6.0	5.31 ± 0.72
10nM	18.9 ± 3.5	46.9 ± 6.3	N/A

N/A – not applicable (could not be estimated as log CRC did not reach a clear maximum)

6.3.6 Effect of atropine, phentolamine and tetrodotoxin on the uterotonic response to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10)

Responses to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) were unaffected by atropine (0.3μM) (potency ratio = 2.4, 95% CL = 1.1, 6.1; d.f = 38), phentolamine (potency ratio = 3.0, 95% d.f. = 1.4, 7.7; d.f. = 34) or TTX (potency ratio = 1.0, 95% CL = 0.5, 2.0; d.f. = 35) (Figure 6.7). Their presence had no significant effect on responses to KPSS or the mean pD_2 or E_{max} values for [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) (Table 6.5).

Figure 6.7: Log CRCs to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) in the absence (■) and presence of atropine (0.3μM) (▲), phentolamine (1μM) (▽) and TTX (1μM) ( ). Each point is the mean ± SEM, n=5-6 women.

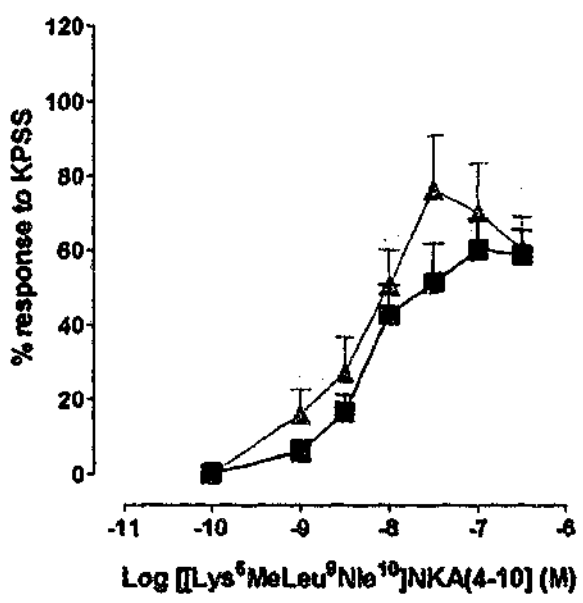


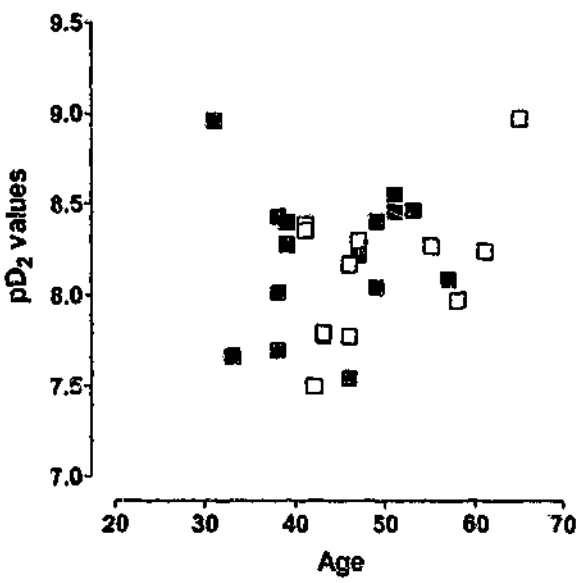
Table 6.5: Effects of [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) and KPSS in the absence and presence of atropine (0.3μM), phentolamine (1μM) and TTX (1μM) on myometrium from non-pregnant women (n=6).

Treatment	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (%KPSS tissue ± SEM)	Peptide Potency (pD_2 tissue ± SEM)
	16.0 ± 4.2	61.6 ± 8.8	8.21 ± 0.18
atrop	8.2 ± 1.7	76.7 ± 14.4	8.47 ± 0.15
pher ne	8.8 ± 2.5	78.6 ± 9.5	8.64 ± 0.15
TTX	13.6 ± 3.2	62.8 ± 10.3	8.25 ± 0.23

6.3.7 Effect of subject's age on the uterotonic response to NKA and [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10)

A Pearson Product Moment Correlation indicated that there was no correlation between the potencies of either NKA (correlation coefficient = 0.51; P = 0.11) or [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) (correlation coefficient = 0.04; P = 0.87) and the age of the subject from whom uterine tissue was obtained (Figure 6.8).

Figure 6.8 Scatter plot showing individual pD_2 values for NKA (□) and [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) (■) versus the age of the subject from whom the uterine tissue was obtained. Note that the individual pD_2 values were obtained from the control log CRCs shown in Figures 6.2, 6.3, 6.5, 6.6 and 6.7.



6.4 Discussion

This study was undertaken to investigate the tachykinin receptor types involved in mediating tachykinin-induced contractions in myometrium from non-pregnant women. It was found that the mammalian tachykinins SP, NKA and NKB and the NK_2 receptor-selective agonist [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) acted as full agonists. In contrast, the NK_1 and NK_3 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP and [$MePhe^7$]NKB were without effect in this tissue. These results indicate that the tachykinins mediate their actions through activation of an NK_2 receptor in the non-pregnant human uterus.

The effects of tachykinins in regulating myometrial activity have been most extensively studied in the rat (Barr *et al.*, 1991; Candenas *et al.*, 2001; Crane *et al.*, 2002; Fisher and Pennefather, 1997; 1998; 1999; Fisher *et al.*, 1993; Hamlin *et al.*, 2000; Magraner *et al.*, 1998; Moodley *et al.*, 1999; Shintani *et al.*, 2000). In this species the mammalian tachykinins were reported to enhance myometrial activity with an order of potency of NKA>SP≥NKB, indicating activation of an NK_2 receptor (Fisher and Pennefather, 1997; Magraner *et al.*, 1998; Pennefather *et al.*, 1993). This order of potency differed to that seen in the oestrogen-treated mouse uterus, as reported in Chapters 3 and 4, where the order of agonist potency was SP≥NKA>NKB which indicated activation of an NK_1 receptor. In the present study the order of agonist potency in the non-pregnant human uterus was NKA>SP≥NKB which suggests, that as in the rat uterus, the tachykinins elicit contractile activity by activation of an NK_2 receptor. These results are consistent with those observed in two earlier studies conducted in the non-pregnant human uterus. Ottesen *et al.* (1983) reported that in the absence of peptidase inhibitors SP (1-10μM) caused dose-dependent contractions of uterine tissue from non-pregnant women. The high concentration of SP needed to elicit contractile activity in that study is consistent with the findings of the present study in which, even in the presence of peptidase inhibitors, SP was significantly less potent than NKA. The non-mammalian tachykinin eledoisin has also been reported to enhance contractile activity in the non-pregnant human uterus (Molina and Zappia, 1976). Although the effects of eledoisin were not examined in the present study, they were however investigated in the pregnant human uterus as described in Chapter 7. In myometrium from pregnant women the uterotonic

effects of eledoisin were antagonised by the NK_2 receptor antagonist SR 48968 but not the NK_1 antagonist SR 140333. This indicates that eledoisin is acting through an NK_2 receptor which is consistent with the report that eledoisin shows preference for the NK_2 receptor due to the nature of the amino acid in position 4 from the C-terminus being aliphatic (Severini *et al.*, 2002).

Additional confirmation of the involvement of the NK_2 receptor in mediating tachykinin-induced activity in the non-pregnant human uterus comes from experiments undertaken with the NK_1, NK_2 and NK_3 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP, [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and [$MePhe^7$]NKB. In the present study only the NK_2 receptor-selective [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) (Chassaing *et al.*, 1991) was effective as an uterotonic agent and was approximately equipotent with NKA. In contrast, the NK_1 receptor-selective [$Sar^9Met(O_2)^{11}$]SP (Drapeau *et al.*, 1987) and NK_3 receptor-selective [$MePhe^7$]NKB (Drapeau *et al.*, 1987) were without effect. This finding is consistent with activation of NK_2 rather than NK_1 or NK_3 receptors and is in accordance with corresponding studies conducted using the oestrogen-treated rat uterus, where it was reported that [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) enhanced myometrial contractions, while [$Sar^9Met(O_2)^{11}$]SP and [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) were relatively ineffective (Fisher and Pennefather, 1997; Fisher *et al.*, 1993).

In the present study responses to [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) were antagonised in a concentration-dependent manner by the non-peptide NK_2 receptor-selective antagonist SR 48968 (Advenier *et al.*, 1992a; Emonds-Alt *et al.*, 1992a). This provides additional support for the involvement of an NK_2 receptor in mediating contractile activity in the non-pregnant human uterus. Interestingly, SR 48968, appeared to cause a decrease in the maximum response of this tissue to [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) although the effect did not attain statistical significance. As was reported in Chapter 5, a similar observation was made using the pregnant mouse uterus. This is not an uncommon occurrence with SR 48968, which has been found to act as either a competitive or as a non-competitive antagonist in different *in vitro* preparations. Croci *et al.* (1995a) observed that SR 48968 competitively antagonised responses to the NK_2 receptor-selective agonist [β-Ala^8]NKA(4-10) in the rat duodenum, whereas it non-competitively

antagonised responses to the same agonist in the rat oesophagus. It has been proposed that this difference in antagonist properties could be due to the existence of differing NK_2 receptor subtypes (Croci *et al.*, 1995a). Interestingly, it has recently been shown that the human and rat uterus express both a wild-type NK_2 receptor and a splice variant of the receptor that has provisionally been termed $NK_2\beta$ (Candenas *et al.*, 2002). The splice variant identified by Candenas *et al.* (2002) would encode a protein with a truncated, six transmembrane domains structure however the authors were unable to clarify whether this splice variant would give rise to a functional protein.

In the rat uterus, Barr *et al.* (1991) reported that contractions to senktide were unaffected by the presence of TTX or the muscarinic antagonist atropine, which suggested that in this tissue the response to senktide was not due to neurogenic mechanisms, but may be due to direct stimulation of post-junctional NK_3 receptors located on smooth muscle cells. In the present study the effects of atropine, the α adrenoceptor antagonist phentolamine and the nerve-blocking agent TTX on uterine responses elicited by [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) was examined. The absence of blockade on the response to [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) by any of these agents indicates that the receptors activated by [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) in the non-pregnant human uterus are also probably located on uterine smooth muscle cells rather than on either noradrenergic or cholinergic nerves.

Neprilysin is the major enzyme that degrades tachykinins (Hooper *et al.*, 1985; Hooper and Turner, 1985; Matsas *et al.*, 1983; 1984). The presence of neprilysin has been detected in the rat uterus (Ottlecz *et al.*, 1991; Pinto *et al.*, 1999) and accordingly studies have reported that in uterine tissue from oestrogen-treated rats the response to tachykinins are potentiated in the presence of the neprilysin inhibitors phosphoramidon and SCH 39370 (Fisher and Pennefather, 1997; Fisher *et al.*, 1993). In the present study the neprilysin inhibitor thiorphan (3μM) either alone or in combination with bestatin (10μM) did not potentiate the response to NKA in the non-pregnant human uterus. This is in contrast to the oestrogen-treated mouse uterus where thiorphan, at the same concentration as used in the present study, potentiated responses to NKA, as described

in Chapter 3. Recent molecular studies have reported that neprilysin expression in the human uterus is low in non-pregnant compared to pregnant women (Patak *et al.*, 2003). Thus the low expression of neprilysin in uterine tissue from non-pregnant women could explain the inability of thiorphan to potentiate the effects of NKA in the present study. In addition, it has also been reported that levels of neprilysin protein were low or negative in women with fibroids (McCluggage *et al.*, 2001). Over 50% of the women who consented to give uterine tissue in the present study were diagnosed with fibroids which could also possibly account for the failure of thiorphan to potentiate the effects of NKA in this study.

It has been reported that the expression of both NKB and the NK_3 receptor in the rat uterus is markedly increased in older animals (Cintado *et al.*, 2001). In the present study uterine tissue was obtained from women whose age ranged from 31-65 years (mean age 45.7 years). However, there was no correlation between the potencies of either NKA or [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and the age of the subject from whom uterine tissue was obtained.

In conclusion, the results of the present study using mammalian tachykinins, tachykinin receptor-selective agonists and the non-peptide tachykinin NK_2 receptor antagonist SR 48968 indicate the involvement of functional NK_2 receptors, most probably located on the uterine smooth muscle cells, in mediating the contractile activity of tachykinins in the non-pregnant human uterus. In addition, the uterotonic effects of NKA in this tissue are not influenced by inhibition of neprilysin and/or bestatin-sensitive aminopeptidases. The aim of the next chapter is to investigate whether the uterotonic effects of the tachykinins in the human uterus are modulated by pregnancy.

CHAPTER 7

CHARACTERIZATION OF THE UTEROTONIC EFFECTS OF TACHYKININ PEPTIDES ON THE PREGNANT HUMAN UTERUS

Summary

1. The aims were (1) to characterize the receptor type(s) mediating the contractile activity of the mammalian tachykinins and the non-mammalian tachykinin eledoisin in myometrial preparations obtained from pregnant women, (2) to investigate whether neprilysin constrained responses to NKA, oxytocin and arginine vasopressin, (3) to examine whether the concentration of calcium used in the physiological salt solution (PPS) had any effect on the response of the mammalian tachykinins and, (4) to compare the response to the tachykinins in uterine tissue from pregnant and non-pregnant women.

2. In the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM) the mammalian tachykinins SP, NKA and NKB acted as full agonists with an order of agonist potency based on the positions of the log CRCs of NKA>SP≥NKB. There was no significant difference in the potency of these peptides when using a modified PPS compared to a non-modified PPS containing 32% more calcium.

3. In the absence of peptidase inhibitors, the NK_2 receptor-selective [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) acted as a full agonist and was equipotent with NKA. The NK_1 and NK_3 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP and [$MePhe^7$]NKB were inactive in this preparation.

4. NKA was significantly more potent in the combined presence of the peptidase inhibitors thiorphan (3μM) and bestatin (10μM). The potencies of oxytocin and AVP were unchanged in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM).

5. The log CRC to eledoisin was shifted to the left in the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM). The uterotonic

effects of this peptide were antagonised by the NK_2 receptor-selective antagonist SR 48968 (1nM) but were unaffected by the NK_1 receptor-selective SR 140333 (1nM).

6. The contractile activity of [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) was antagonised in a concentration-dependent manner by the tachykinin NK_2 receptor-selective antagonist SR 48968.

7. The order of potency of the mammalian tachykinins was NKA>SP≥NKB in both the pregnant and non-pregnant human uterus. NKA and [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) were significantly more potent in the non-pregnant myometrial preparations.

8. Taken together, the results of this chapter indicate that in the pregnant human uterus the contractile effects of the tachykinins are mediated by activation of an NK_2 receptor as was also reported to occur in the non-pregnant uterus. In contrast to the non-pregnant uterus, peptidases were observed to constrain the uterotonic actions of the tachykinins in the pregnant uterus.

7.1 Introduction

In the previous chapter, the effects of tachykinins on myometrial contractility in uterine tissues obtained from the non-pregnant human were described. Having determined that tachykinin-induced contractions in myometrium from the non-pregnant human are mediated by activation of an NK_2 receptor, the aim of this chapter was to examine the contractile effects of these peptides in uterine tissue from pregnant women.

The uterotonic action of tachykinins in the pregnant mouse uterus was previously described in Chapter 5. Tachykinins have also been reported to elicit contractile activity in the pregnant rat uterus (Candenas *et al.*, 2001; Shintani *et al.*, 2000). In both these species, pregnancy-related changes in either the expression and/or actions of tachykinins on the uterus were observed. However, the role that tachykinins play in regulating myometrial contractility in the pregnant human remains unclear. While the mammalian tachykinins and the non-mammalian tachykinin, eledoisin, have been found to elicit contractions of uterine preparations from non-pregnant women (Molina & Zappia, 1976; Ottesen *et al.*, 1983; Chapter 6), the NK_1, NK_2 and NK_3 receptor-selective agonists substance P methyl ester, [β-Ala^8]NKA(4-10) and senktide, respectively, were reported to be without effect on uterine tissue from pregnant women (Barr *et al.*, 1991).

The role that peptidases, chiefly neprilysin, play in constraining responses to the tachykinins in the pregnant human uterus also remains unknown. Neprilysin, the major tachykinin-degrading enzyme (Hooper *et al.*, 1985; Hooper & Turner, 1985; Matsas *et al.*, 1983; 1984) has been detected in the human uterus (Casey *et al.*, 1991; Salamonsen *et al.*, 1999). This enzyme has been reported to constrain responses to the tachykinins in the pregnant rat uterus (Shintani *et al.*, 2000). In contrast, the results of Chapter 5 indicated that the neprilysin inhibitor, thiorphan (1μM), had no effect on the response to tachykinins in the pregnant mouse uterus. These findings suggest the possibility that a species difference in tachykinin degradation could exist.

The aims of the experiments described in this chapter were three-fold. The first aim was to characterize the tachykinin receptor type(s) mediating contractile activity in the

pregnant human uterus. Receptor characterization was undertaken by examining the effects of the mammalian tachykinins, SP, NKA and NKB, the non-mammalian tachykinin eledoisin and the NK_1, NK_2 and NK_3 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP, [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and [$MePhe^7$]NKB. The second aim was to investigate the extent to which peptidases modulated the response to the tachykinins. Peptidase degradation of oxytocin and arginine vasopressin (AVP), two peptides known to be of importance during labour, was also examined as a comparison. The third aim was to compare the findings of this study with those observed in the non-pregnant human uterus as reported in Chapter 6.

In addition, during the undertaking of the present study it was reported that the response to SP *in vitro* relies largely on external Ca^{2+} (Grumann-Junior *et al.*, 2000). The physiological salt solution (PPS) used throughout the functional experiments described in the previous chapters was modified to reduce spontaneous uterine contractions and thus comprised a lower $CaCl_2$ concentration (1.9mM) compared to "non-modified" PPS (2.5mM). Therefore, the effect of $CaCl_2$ concentration on tachykinin activity in the pregnant human uterus was also examined.

7.2 Materials and Methods

7.2.1 Tissue Preparations

Full details of women who gave informed consent to participate in this study are given in Chapter 2 (section 2.1.2 and 2.1.2.2) and Appendix 3. Briefly, uterine tissue was obtained from women (21-38 years old, n=55) who had undergone a lower uterine segment caesarean section (LUSCS) and had given informed consent. Average length of gestation was 38.2 ± 0.2 weeks and none of the women were in labour. Four preparations were obtained from the outer myometrium (as described in 2.2.3.2) and set up as described in 2.3.1 for recording of force from the longitudinal smooth muscle layer. Of the 55 women who participated in this study, three or more uterine preparations obtained from a subset of 8 women did not respond to either the agonists or KPSS and therefore data from this subset of women were not included in the present study.

7.2.2 Additional physiological salt solutions

A subset of experiments was conducted in which the preparations were set up in a "non-modified" Krebs-Henseleit solution containing 2.5mM rather than 1.9mM $CaCl_2$ and 0.5mM rather than 1.1mM $MgSO_4$.

7.2.3 Experimental protocol

Refer to Chapter 2 (section 2.3).

7.2.4 Measurement of results and statistical analyses

Responses to all agonists were measured as area under the force-time curve for the duration of agonist exposure as described in section 2.4. Responses were then expressed as a percentage of the response to the KPSS and presented as mean ± SEM.
Statistical analysis was undertaken as described in section 2.6.

E_{max} is defined as the maximum response to an agonist observed over the concentration range used; note that might not in all cases be the greatest response that an agonist could elicit if higher concentrations had been tested.

Table 7.1: Overview of experiments undertaken on uterine preparations obtained from pregnant women. Note that four preparations were obtained from each patient and are allocated as preparation 1, 2, 3 or 4 using a randomized procedure.

Series	Preparation 1		Preparation 2		Preparation 3		Preparation 4	
	Agonist	Treatment	Agonist	Treatment	Agonist	Treatment	Agonist	Treatment
1: Mammalian TKs in the presence of peptidase inhibitors using a "non-modified" PSS (N=7)	*SP (7)	-	*NKA (7)	-	*NKB (6)	-		
2: Mammalian TKs in the presence of peptidase inhibitors (N=5)	*SP (6)	-	*NKA (6)	-	*NKB (6)	-	-	-
3: Sar^9, Lys^5 and $MePhe^7$ (N=6)	Sar^9 (6)	-	Lys^5 (6)	-	$MePhe^7$ (6)	-	Sar^9 (3)	*
							$MePhe^7$ (3)	*
4: NKA in the absence and presence of peptidase inhibitors (N=7)	NKA (6)	-	NKA (7)	Bestatin (10μM)	NKA (6)	Thiorphan (3μM)	NKA (6)	Bestatin (10μM) & thiorphan (3μM)
5: Oxytocin and AVP in the absence and presence of peptidase inhibitors (N=6)	Oxytocin (6)	-	Oxytocin (6)	*	AVP (6)	-	AVP (6)	*
6: Eledoisin in the absence and presence of peptidase inhibitors, SR 140333 and SR 48968 (N=6)	Eledoisin (6)	-	Eledoisin (6)	*	*Eledoisin (5)	SR 140333 (1nM)	*Eledoisin (5)	SR 48968 (1nM)
7: Lys^5 in the absence and presence of SR 48968 (N=9)	Lys^5 (9)	-	Lys^5 (9)	SR 48968 (1nM)	Lys^5 (9)	SR 48968 (10nM)	Lys^5 (6)	SR 48968 (3nM)
							Lys^5 (3)	SR 48968 (30nM)

TK = Tachykinins, Sar^9 = [$Sar^9Met(O_2)^{11}$]SP, Lys^5 = [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10), $MePhe^7$ = [$MePhe^7$]NKB, AVP = Arginine vasopressin
* indicates in the presence of peptidase inhibitors (thiorphan (3μM), captopril (10μM) and bestatin (10μM))
Unless otherwise stated all experiments were conducted using a modified (1.9mM $CaCl_2$) Krebs-Henseleit solution.
N = number of women
Numbers in brackets in agonist column indicate number of viable preparations (i.e., responded to agonist & KPSS)

7.2.5 Drugs

The drugs used included all those previously described in section 2.7, which included details of stock solutions, vehicles used and vehicle controls as appropriate.

7.3 Results

Uterine tissue used throughout this study was obtained from 55 women who had undergone a lower uterine segment caesarean section (mean age 31.7 years, range 21-38; mean parity 2.0, range 0-6). Of these 55 women, uterine preparations obtained from 8 women were not viable (i.e. did not respond to agonist or KPSS) and therefore not included in the present study. The mean weight of all tissue preparations included in the analysis was 116.3 ± 3.2mg (n = 169 preparations). The mean response to KPSS was 38.4 ± 1.8g.s/mg tissue (n = 169 preparations). Spontaneous activity was observed in nearly all tissue preparations and ranged from one to six contractions over any 15 min period, this gradually decreased over the duration of the equilibration period.

It was noted that of the women who were included in the present study there was a subset of women from which tissue preparations exhibited little to no spontaneous activity though still responded to agonists and KPSS (n = 14 women). A check of their medical background revealed no apparent reasons for this lack of spontaneous activity. The mean age of these 14 women was 33 with a mean gestation of 38 weeks. Indications for the caesarean included breech birth (n=4), twins (n=2), cephalopelvic disproportion (n=1), baby's head moving (n=1) and previous caesarean section (n=6). All women underwent either spinal or epidural anesthesia. Additional medication included: syntocin (n=3), ephedrine (n=5), atropine (n=2) and ventolin (n=1). A Student's unpaired t-test indicated that the mean preparation weight (98.6 ± 5.32mg) for this subset of women was significantly lower than the overall mean preparation weight (116.3 ± 3.2 mg) ($P<0.05$; d.f. = 223). Figure 7.1 shows a representative trace of responses to increasing concentrations of the mammalian tachykinins SP, NKA and NKB. It can clearly be seen that NKA is the most potent of the three mammalian tachykinins in the pregnant human uterus.

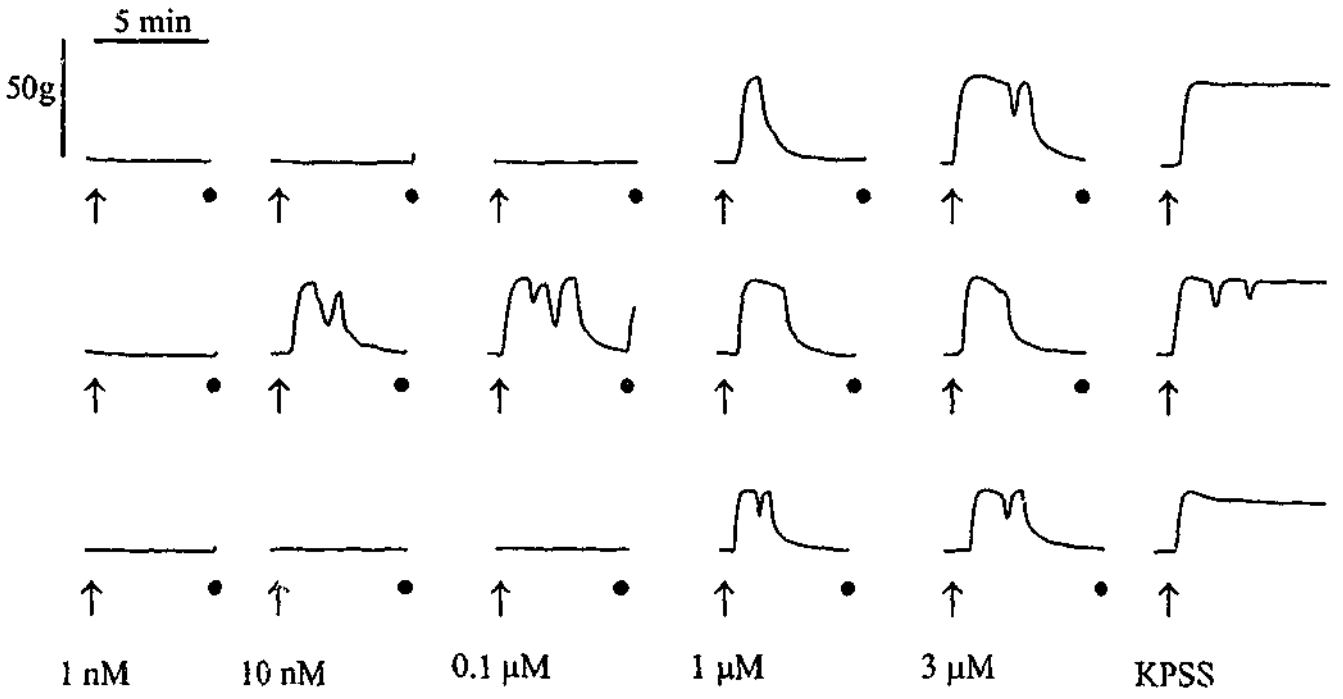


Figure 7.1: Representative trace showing responses of outer myometrium from a pregnant woman to increasing concentrations of SP (upper panel), NKA (middle panel) and NKB (lower panel) together with responses to KPSS. All responses are in the presence of thiorphan (3μM), bestatin (10μM) and captopril (10μM). The arrows indicate agonist addition and the dots represent washes.

7.3.1 Comparison of response to the mammalian tachykinins using 1.9mM and 2.5 mM $CaCl_2$ containing Krebs- Henseleit solutions.

No significant differences were seen in the positions of the concentration-response curves to SP (potency ratio = 1.3, 95% CL = 0.6, 2.7: d.f. = 48), NKA (potency ratio = 1.5, 95% CL = 0.6, 4.6; d.f. = 48) or NKB (potency ratio = 2.7, 95% CL = 0.9, 18.1; d.f. = 28) using the "non-modified" Krebs-Henseleit solution containing 2.5mM $CaCl_2$ as compared to the modified Krebs-Henseleit solution containing 1.9mM $CaCl_2$ (Figure 7.2). The different concentrations of $CaCl_2$ used in the two solutions had no significant effect on either the maximal response elicited by the tachykinin peptides or the mean response to KPSS (Student's unpaired t-tests, $P>0.05$; Table 7.2).

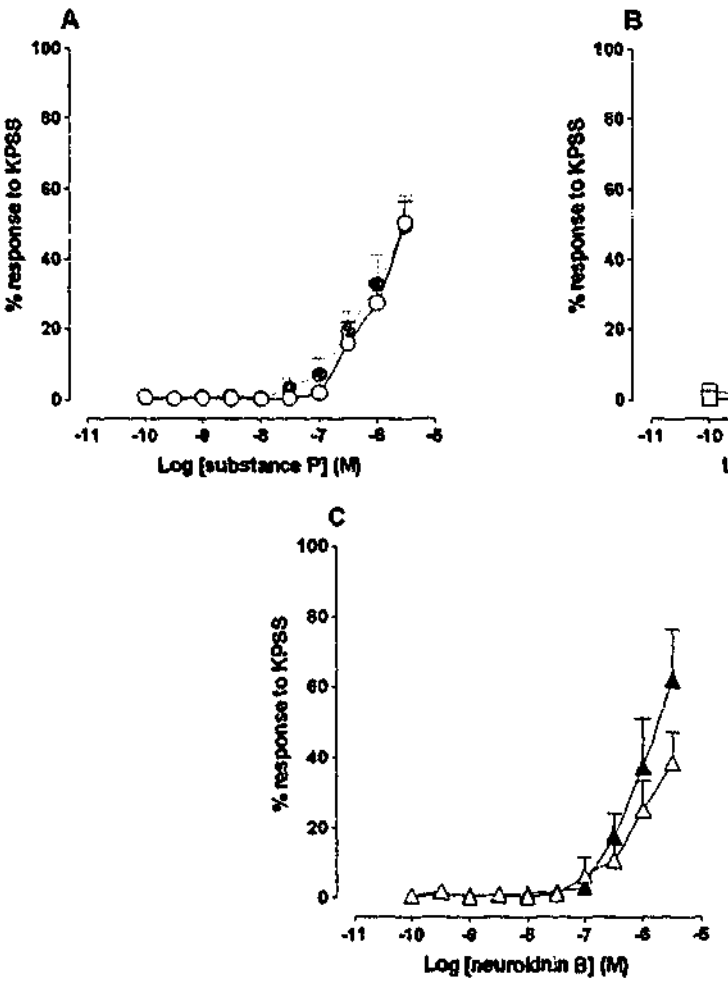


Figure 7.2: Log concentration-response curves (CRCs) to (A) SP, (B) NKA and (C) NKB using either modified PSS containing 1.9mM $CaCl_2$ (open symbols) or "non-modified" PSS containing 2.5mM $CaCl_2$ (closed symbols) on uterine preparations from pregnant women in the presence of thiorphan (3μM), bestatin (10μM) and captopril (10μM). Each point is the mean ± SEM, n= 6-7 women. In this and subsequent figures, a significant difference between log CRCs as indicated by two-way ANOVA followed by Student Newman Keuls is shown by *.

Table 7.2: Effect of peptides and KPSS in the presence of either 1.9mM or 2.5mM $CaCl_2$ containing Krebs-Henseleit solution on myometrium from pregnant women (n= 5-7).

Peptide	[$CaCl_2$] in PSS (mM)	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (%KPSS ± SEM)	Peptide Potency (pD_2 tissue ± SEM)
SP	1.9	50.4 ± 13.5	50.3 ± 6.2	N/A
	2.5	43.2 ± 4.9	49.4 ± 8.9	N/A
NKA	1.9	35.8 ± 8.2	67.8 ± 9.7	7.66 ± 0.06
	2.5	49.2 ± 8.6	72.3 ± 10.3	7.67 ± 0.18
NKB	1.9	47.0 ± 17.5	38.7 ± 8.7	N/A
	2.5	39.9 ± 6.6	62.0 ± 14.5	N/A

N/A - not applicable (could not be estimated as log CRC did not reach a clear maximum)

7.3.2 Uterotonic activity of substance P, neurokinin A, neurokinin B and eledoisin

In the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM), NKA was the most potent mammalian tachykinin (two-way ANOVA, $P<0.05$; d.f. = 221) and was 46- and 68-fold more potent than then SP (95% CL = 23.6, 96.6; d.f. = 62) and NKB (95% CL = 29.7, 178.6; d.f. = 60) respectively. The CRC to the non-mammalian tachykinin eledoisin, which was investigated in a separate subset of experiments (see Figure 7.8), has been included in the present figure in order to permit comparison of its response to those of the mammalian tachykinins. In the presence of peptidase inhibitors eledoisin was 3-fold less potent then NKA (95% CL = 1.51, 6.72; d.f. = 31) (Figure 7.3).

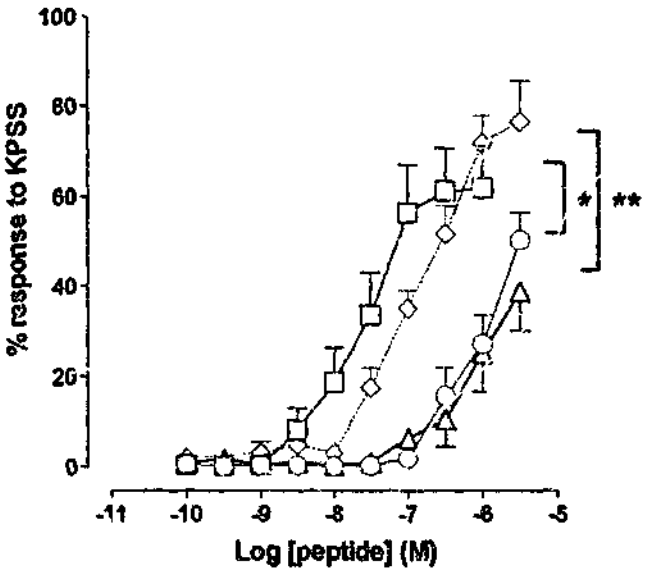


Figure 7.3: Log CRCs to SP (○), NKA (□), NKB (△) and eledoisin (◇) on uterine preparations from pregnant women in the presence of thiorphan (3μM), bestatin (10μM) and captopril (10μM). Note that log CRCs for NKA and eledoisin have reached a clear ceiling. Each point is the mean ± SEM, n=6 women.

*, ** indicates significant difference from NKA and eledoisin, two-way ANOVA, $P < 0.05$, d.f. = 221

7.3.3 Uterotonic activity of [Sar^{9}Met(O$_2$)^{11}]SP, [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) and [MePhe^7]NKB

Analysis by two-way ANOVA indicated a significant difference in the log CRCs to the tachykinin receptor-selective agonists ($P<0.05$; d.f. = 190). Of the tachykinin receptor-selective agonists only the NK_2 selective [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) acted as a full agonist while the NK_1 and NK_3 receptor-selective [Sar^{9}Met(O$_2$)^{11}]SP and [MePhe^7]NKB were without effect at the concentrations tested (Figure 7.4). The presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM) had no significant effect on the log CRCs to [Sar^{9}Met(O$_2$)^{11}]SP and [MePhe^7]NKB (data not shown).

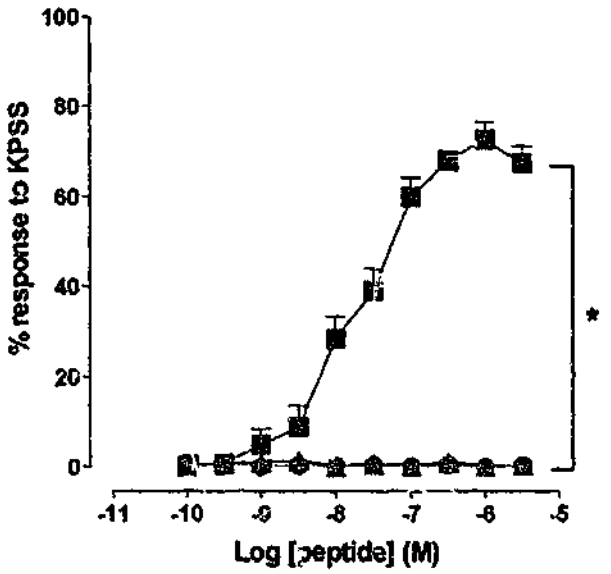


Figure 7.4: Log CRCs to [Sar^{9}Met(O$_2$)^{11}]SP (●), [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) (■) and [MePhe^{7}]NKB (▲) in the absence of peptidase inhibitors on uterine preparations from pregnant women. Note that the log CRC for [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) has reached a clear ceiling. Each point is the mean ± SEM, n = 6 women.

* indicates significant difference from [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10), two-way ANOVA, $P < 0.05$; d.f. = 190

7.3.4 Order of agonist potency

In the presence of the peptidase inhibitors thiorphan (3μM), captopril (10μM) and bestatin (10μM) the relative order of agonist potency for the mammalian tachykinins based on the positions of the log CRCs was NKA>SP≥NKB. Of the receptor-selective agonists [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) was equipotent with NKA (potency ratio = 1.5, 95% = 0.9, 2.8; d.f. = 54) while [Sar^{9}Met(O$_2$)^{11}]SP and [MePhe^{7}]NKB were without effect in this preparation at the concentrations tested. The non-mammalian tachykinin eledoisin was 3-fold less potent then NKA (95% CL = 1.51, 6.72; d.f. =31) (Figure 7.5). Maximum responses to the mammalian tachykinins, eledoisin and [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) were significantly larger than maximum responses to [Sar^{9}Met(O$_2$)^{11}]SP and [MePhe^{7}]NKB. No significant differences were seen in the mean responses to KPSS in experiments for which agonist potencies were compared (one-way ANOVA, $P>0.05$; Table 7.3).

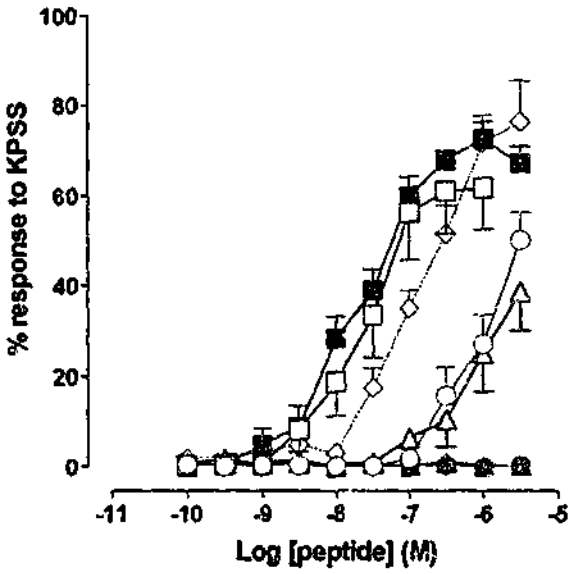


Figure 7.5: Log CRCs to SP (○), NKA (□), NKB (△), [Sar^{9}Met(O$_2$)^{11}]SP (●), [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) (■), [MePhe^7]NKB (▲) and eledoisin (◇) on uterine preparations from pregnant women. Each point is the mean ± SEM, n = 6. CRCs to the mammalian tachykinins and eledoisin were constructed in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). Note that these CRCs are those shown previously in Figures 7.3 and 7.4.

Table 7.3: Effects of peptides and KPSS on myometrium from pregnant women (n=6).

Peptide	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (% KPSS ± SEM)	Peptide Potency (pD_2 ± SEM)
SP	50.4 ± 13.5	50.3 ± 6.2	N/A
NKA	35.8 ± 8.2	67.8 ± 9.6[b]	7.66 ± 0.06
NKB	47.0 ± 17.5	38.7 ± 8.7	N/A
Sar^9	41.8 ± 6.2	1.3 ± 0.4[a]	N/A
Lys^5	64.0 ± 13.4	76.1 ± 2.7[b]	7.72 ± 0.10
MePhe^7	38.7 ± 8.5	2.1 ± 0.6[a]	N/A
eledoisin	28.0 ± 10.2	78.3 ± 8.1	6.81 ± 0.09[c]

Sar^9 – [Sar^{9}Met(O$_2$)^{11}]SP, Lys^5 – [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10), MePhe^7 – [MePhe^7]NKB
N/A – not applicable (could not be estimated as log CRC did not reach a clear maximum)
a – significantly different from maximum responses to SP, NKA, NKB, [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) and eledoisin
b – significantly different from maximum responses to SP and NKB
c – significantly different from pD_2 values obtained for NKA and [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10)

7.3.5 Influence of peptidase inhibitors on the uterotonic effects of neurokinin A

Two-way ANOVA indicated a significant difference in the response to NKA in the presence of all three peptidase inhibitor combinations tested ($P<0.05$; d.f. = 186). This corresponded to a 5-fold leftward shift in the presence of bestatin (10μM) (95% CL =

1.4, 21.7; d.f. = 57). However, potency ratios could not be obtained for NKA in the presence of thiorphan (10μM) and thiorphan together with bestatin as in both cases the lines departed significantly from parallelism (Figure 7.6). The peptidase inhibitors had no significant effect on the mean response to KPSS and though maximum responses to NKA were higher in the presence of the peptidase inhibitors this was not shown to be significant (one-way ANOVA, P>0.05; d.f. = 21).

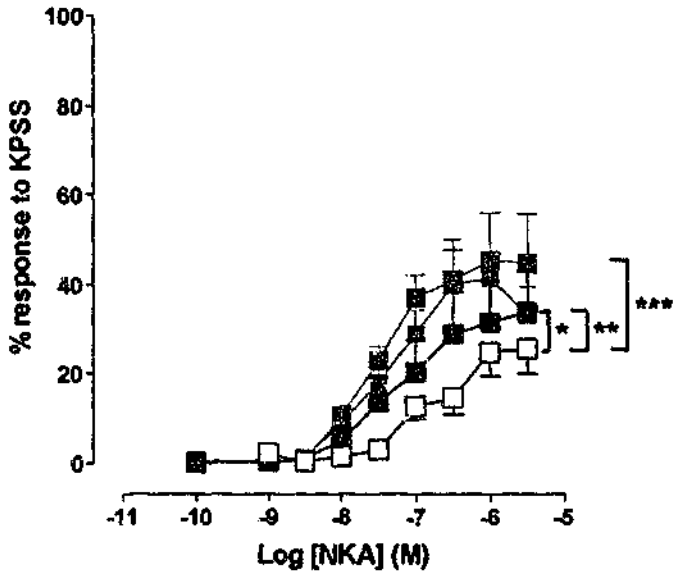


Figure 7.6: Log CRCs to NKA in the absence of peptidase inhibitors (□) and in the presence of bestatin (10μM) (■), thiorphan (3μM) (▣) and thiorphan (3μM) together with bestatin (10μM) (▣) on uterine preparations from pregnant women. Each point is the mean ± SEM, n=6-7 women.

*, **, *** indicates a significant difference in log CRC compared to control, two-way ANOVA, P < 0.05; d.f. = 186

Table 7.4: Effects of NKA and KPSS in the absence and presence of peptidase inhibitors on myometrium from pregnant women (n=6-7).

Peptidase inhibitor	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (%KPSS ± SEM)	Peptide Potency (pD_2 ± SEM)
control	34.6 ± 9.8	29.3 ± 5.3	6.73 ± 0.08
bestatin (10μM)	37.4 ± 11.7	35.3 ± 9.0	7.00 ± 0.29
thiorphan (3μM)	35.7 ± 6.6	47.0 ± 8.4	7.41 ± 0.18
bestatin (10μM) & thiorphan (3μM)	48.3 ± 6.7	48.3 ± 8.7	7.55 ± 0.09[a]

a – significantly different from corresponding value obtained in the absence of peptidase inhibitors, one-way ANOVA, P < 0.05; d.f. = 21

7.3.6 Influence of peptidase inhibitors on the uterotonic effects of oxytocin and arginine vasopressin

Responses to both oxytocin (potency ratio = 0.9, 95% CL = 0.3, 2.8; d.f. = 80) and AVP (potency ratio = 0.7, 95% CL = 0.3, 1.8; d.f. = 61) were unaffected by the addition of thiorphan (3μM), bestatin (10μM) and captopril (10μM) (Figure 7.7). The peptidase inhibitors also had no significant effect on either the maximal responses elicited by oxytocin and AVP or the mean response to KPSS (Student's unpaired t-tests, P>0.05; Table 7.5).

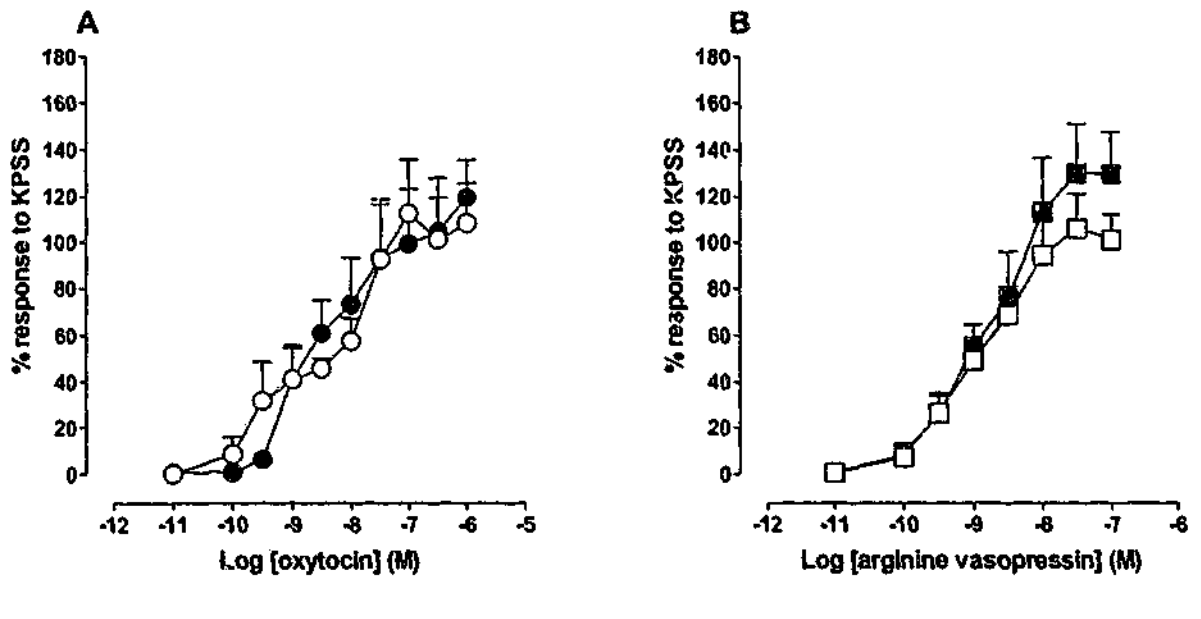


Figure 7.7: Log CRCs to (A) oxytocin and (B) AVP in the absence (open symbols) and presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM) (closed symbols) on uterine preparations from pregnant women. Each point is the mean ± SEM, n=6 women.

Table 7.5: Effects of oxytocin, AVP and KPSS in the absence and presence of peptidase inhibitors on myometrium from pregnant women (n=6).

Peptide	Treatment	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (%KPSS ± SEM)	Peptide potency (pD_2 ± SEM)
oxytocin		33.5 ± 13.4	119.7 ± 22.2	8.48 ± 0.14
	inhibitors	30.6 ± 4.9	124.4 ± 20.4	8.22 ± 0.14
AVP		33.2 ± 5.7	112.4 ± 13.0	8.90 ± 0.16
	inhibitors	37.7 ± 8.5	139.1 ± 18.2	8.82 ± 0.16

inhibitors – thiorphan (3μM) , captopril (10μM) & bestatin (10μM)

7.3.7 Effect of peptidase inhibitors and the NK_1 and NK_2 receptor-selective antagonists SR 140333 and SR 48968 on the uterotonic effects of eledoisin.

In the presence of thiorphan (3μM), bestatin (10μM) and captopril (10μM) there was a 5-fold leftward shift in the log concentration-response curve to eledoisin (95% CL = 3.0, 9.9; d.f. = 19). In the presence of the peptidase inhibitors, responses to eledoisin were unaffected by 1nM SR 140333 (potency ratio = 1.35, 95% CL = 0.7, 2.9; d.f. = 40) while 1nM SR 48968 caused a 9-fold rightward shift (95% CL = 4.9, 14.6; d.f. =42) (Figure 7.8). The peptidase inhibitors, SR 140333 and SR 48968 had no significant effect on either the maximal response elicited by eledoisin or the mean response to KPSS (one-way ANOVA, P>0.05; Table 7.6).

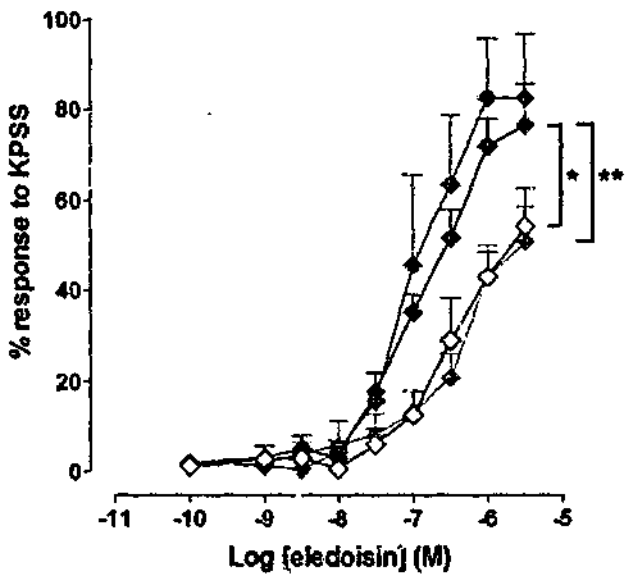


Figure 7.8: Log CRCs to eledoisin in the absence (◇) and presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM) (peptidase inhibitors) (◆), peptidase inhibitors together with SR140333 (1nM) (▲) and peptidase inhibitors together with SR 48968 (1nM) (▼) on uterine preparations from pregnant women. Each point is the mean ± SEM, n= 5-6 women.

*, ** indicates a significant difference in log CRC compared to eledoisin in the presence of peptidase inhibitors, two-way ANOVA, $P < 0.05$; d.f. = 162

Table 7.6: Effect of eledoisin and KPSS in the absence and presence of peptidase inhibitors and tachykinin antagonists on myometrium from pregnant women (n=5-6).

Treatment	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (%KPSS ± SEM)	Peptide Potency (pD_2 ± SEM)
control	21.4 ± 10.0	55.4 ± 7.6	N/A
inhibitors	28.0 ± 10.2	78.3 ± 8.1	6.81 ± 0.09
inhibitors & SR 140333 (1nM)	25.1 ± 5.7	84.0 ± 13.9	6.82 ± 0.13
inhibitors & SR 48968 (1nM)	33.0 ± 10.3	51.0 ± 7.6	N/A

inhibitors – thiorphan (3μM), captopril (10μM) and bestatin (10μM)

N/A – not applicable (could not be estimated as log CRC did not reach a clear maximum)

7.3.8 Effect of the NK_2 receptor-selective antagonist SR 48968 on the uterotonic effect of [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10)

SR 48968 at a concentration of 1, 3, 10 and 30nM significantly attenuated the response to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) (two-way ANOVA, $P<0.05$; d.f. = 279). This attenuation corresponded to 5-, 15-, 81- and 200-fold rightward shifts of the log concentration-response curve to [Lys^{5}MeLeu^{9}Ne10]NKA(4-10) in the presence of 1nM, (95% CL = 2.5, 9.0; d.f. = 96), 3nM (95% CL = 8.1, 28.7; d.f. = 81), 10nM (95% CL = 49.0, 138.8; d.f. = 80) and 30nM SR 48968 (95% CL = 106.5, 421.4; d.f. = 9) respectively (Figure 7.9). A depression in maximum response to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) was seen with all four concentrations of the antagonist but statistical significance was only reached with 10nM and 30nM (one-way ANOVA, $P<0.05$; d.f. = 31). Responses to KPSS were unaffected by SR 48968 at the concentrations used (Table 7.7).

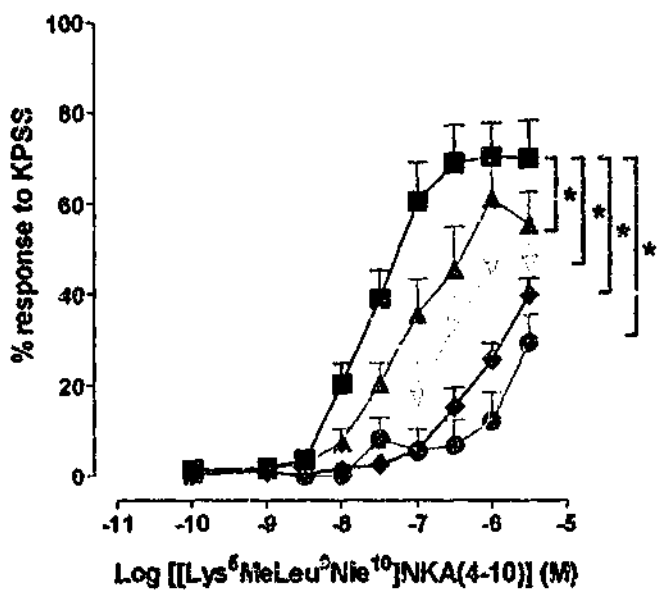


Figure 7.9: Log CRCs to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) in the absence (■) and presence of 1nM (▲), 3nM ( ), 10nM (◆) and 30nM SR 48968 (●) on uterine preparations from pregnant women. Note that the log CRC to [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) did not reach a ceiling in the presence of 10 and 30nM SR 48968. Each point is the mean ± SEM, n=3-9 women.

* indicates significant difference from control CRC, two-way ANOVA, $P < 0.05$; d.f = 279

Table 7.7: Effect of [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) and KPSS in the absence and presence of the NK_2 receptor-selective antagonist SR 48968 on myometrium from pregnant women (n=3-9).

N	[SR 48968] (mM)	Response to KPSS (g.s/mg tissue ± SEM)	Peptide E_{max} (%KPSS ± SEM)	Peptide potency (pD_2 ± SEM)
9	0	27.9 ± 5.1	77.4 ± 8.0	7.50 ± 0.10
9	1	39.4 ± 7.7	63.6 ± 9.5	6.57 ± 0.44
6	3	36.3 ± 8.5	51.0 ± 5.4	6.71 ± 0.19
9	10	37.5 ± 7.5	40.0 ± 3.8[a]	N/A
3	30	36.1 ± 6.4	29.5 ± 6.2[a]	N/A

N – number of preparations
N/A – not applicable (could not be estimated as CRC did not reach a clear maximum)
a – significantly different from control, one-way ANOVA, $P < 0.05$; d.f. = 31

7.3.9 Comparison of the uterotonic effects of tachykinins in uterine preparations from non-pregnant and pregnant women.

Myometrium used throughout the experiments described in this Chapter was obtained from pregnant women undergoing LUSCS, and that described in Chapter 6 was obtained from non-pregnant women undergoing hysterectomy. It was observed that the non-pregnant women were significantly older than the pregnant women (Figure 7.10, Student's unpaired t-test, $P<0.05$).

As stated in Chapter 5, marked hypertrophy of the uterus occurs during pregnancy. A slight but significant difference in the size of the tissue preparations obtained from pregnant as compared to non-pregnant women was observed (Figure 7.11; Student's unpaired t-test, $P<0.05$), despite a concerted effort to keep the size of the tissue preparations relatively consistent between the two groups. Responses to KPSS, normalized for tissue weight, were significantly increased in myometrium obtained from pregnant women (Figure 7.12, Student's unpaired t-test, $P<0.05$).

In myometrium obtained from non-pregnant women, the order of agonist potency of the mammalian tachykinins in the presence of the peptidase inhibitors thiorphan (3μM),

captopril (10μM) and bestatin (10μM) based on the relative positions of the log CRCs was NKA>SP≥NKB. Of the receptor-selective analogues only [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) acted as a full agonist while [Sar^{9}Met(O$_2$)^{11}]SP and [MePhe^7]NKB were ineffective. This is consistent with what was found in tissue from the pregnant women, indicating that the NK_2 receptor mediates the uterotonic effect of tachykinins in both the non-pregnant and pregnant uterus. With the exception of [Sar^{9}Met(O$_2$)^{11}]SP, no significant differences in the mean peptide E_{max} values for the mammalian tachykinins and the receptor-selective analogues were observed between the two groups (Figure 7.13, Student's unpaired t-tests, P>0.05). However, both NKA and [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) were significantly more potent in myometrium obtained from non-pregnant compared to pregnant women (Table 7.8, Student's unpaired t-test, P<0.05; d.f. =10).

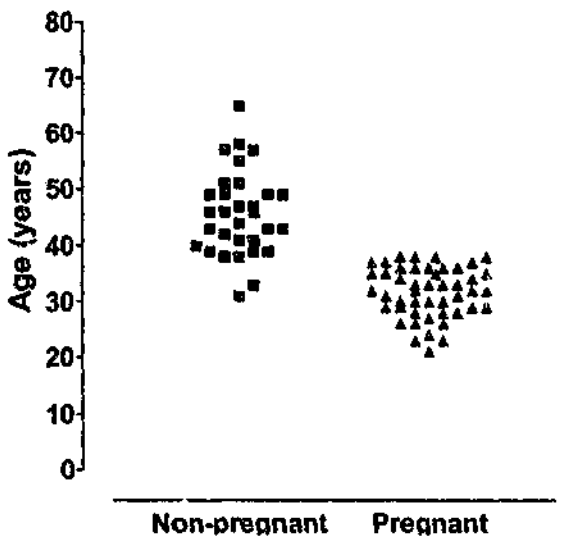


Figure 7.10: Scatter plot showing the ages of non-pregnant (■, n=30 women) and pregnant women (▲, n=47 women) from whom myometrium was obtained and included in the present study.

Figure 7.11: Comparison of mean tissue weight of uterine preparations obtained from non-pregnant (n=98 preparations) and pregnant women (n=169 preparations) included in the present study. Each bar is the mean ± SEM.

* indicates significant difference from non-pregnant preparations, Student's unpaired t-test, $P<0.05$; d.f. =265

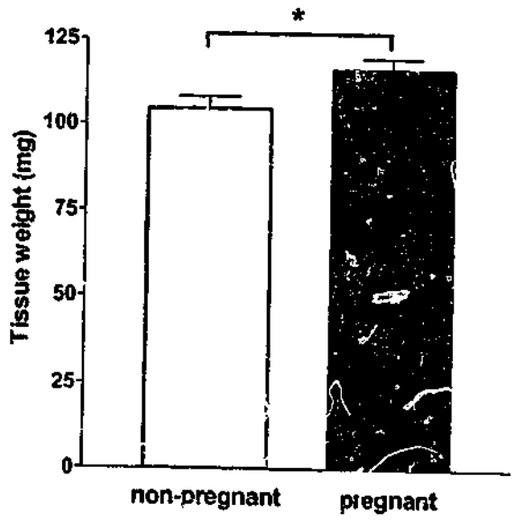
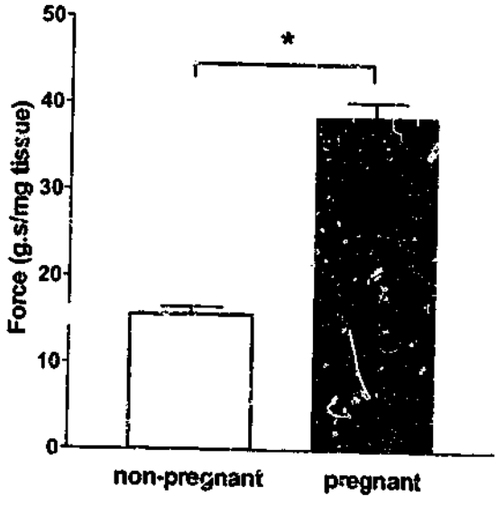


Figure 7.12: Comparison of the mean KPSS response normalized for tissue weight in uterine preparations obtained from non-pregnant (n=98 preparations) and pregnant women (n=169 preparations) included in the present study. Each bar is the mean ± SEM.

* indicates significant difference from non-pregnant preparations, Student's unpaired t-test, $P<0.05$; d.f. = 265.



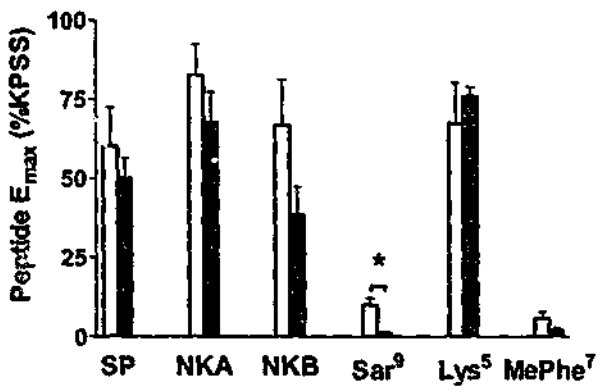


Figure 7.13: Peptide E_{max} values for SP, NKA, NKB, [Sar^{9}Met(O$_2$)^{11}]SP (Sar^9), [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) (Lys^5) and [MePhe^7]NKB (MePhe^7) in uterine preparations from non-pregnant (open bars) and pregnant women (closed bars). SP, NKA and NKB are in the presence of thiorphan (3μM), captopril (10μM) and bestatin (10μM). Each point is the mean ± SEM. Please note that these data were previously shown in Tables 6.2 and 7.3.

* indicates significant difference between non-pregnant and pregnant women, Student's unpaired t-test, $P<0.05$; d.f. = 10.

Table 7.3: Mean pD_2 values for NKA and [Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10) on uterine preparations from non-pregnant and pregnant women (n=6).

Subject	Peptide	Peptide potency ($pD_2 \pm SEM$)
Non-pregnant	NKA	8.40 ± 0.14
Pregnant		7.66 ± 0.06[a]
Non-pregnant	[Lys^{5}MeLeu^{9}Nle^{10}]NKA(4-10)	8.11 ± 0.11
Pregnant		7.72 ± 0.10[a]

a – significantly different from the corresponding value obtained in the non-pregnant preparations (Student's unpaired t-test, $P<0.05$; d.f. = 10).

7.4 Discussion

The present study clearly demonstrates for the first time that the mammalian tachykinins SP, NKA and NKB elicit contractile activity in the pregnant human uterus. This finding is in contrast to a previous report that tachykinins were without effect on myometrial tissue obtained from pregnant women (Barr *et al.*, 1991).

In the previous chapter it was established that in the non-pregnant human uterus the order of agonist potency of the mammalian tachykinins is NKA>SP≥NKB, suggesting activation of an NK_2 receptor, which is consistent with the order of agonist potency reported in the present chapter. This indicates that in the human the tachykinin receptor type mediating myometrial contractility is the same in both the pregnant and non-pregnant uterus. Corresponding studies conducted using rat myometrial preparations reported a similar finding, with the NK_2 receptor being the predominant receptor type mediating contractile activity of both the non-pregnant (Fisher and Pennefather, 1998; 1999; Magraner *et al.*, 1998; Pennefather *et al.*, 1993) and pregnant rat uterus (Candenas *et al.*, 2001; Shintani *et al.*, 2000). This is in contrast to the mouse, where it was reported in Chapters 4 and 5 that the predominant tachykinin receptor type mediating contractions of the non-pregnant mouse uterus is the NK_1 receptor, while in pregnant mice it is an NK_2 receptor type. These findings suggest that there are species-related differences in the actions of tachykinins in the mammalian uterus.

While the present study was in progress, Grumann-Junior and co-workers reported that the response to SP *in vitro* relies largely on external Ca^{2+} (Grumann-Junior *et al.*, 2000). Consistent with previous studies in this laboratory the PPS used throughout the functional experiments described in Chapters 3 to 6 had been modified to reduce spontaneous uterine contractions and thus contained a lower $CaCl_2$ concentration (1.9mM) than "non-modified" PPS (2.5mM). A subset of experiments was therefore conducted to determine whether the concentration of $CaCl_2$ in the bathing solution would affect the uterotonic activity elicited by the mammalian tachykinins in the pregnant human uterus. The results of these experiments indicated that the responses of this preparation elicited by SP, NKA and NKB in this preparation were not different in the two bathing solutions. This finding suggests that the "modified" PPS contained

enough Ca^{2+} for the tachykinins to elicit contractile activity and reaffirmed its suitability for use in functional experiments in which the effects of the tachykinin peptides were being examined.

Further support for the involvement of the NK_2 receptor in mediating tachykinin-induced activity in the pregnant human uterus was obtained from experiments undertaken with the NK_1, NK_2 and NK_3 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP, [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) and [$MePhe^7$]NKB. In the present study only the NK_2 receptor-selective [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) (Chassaing *et al.*, 1991) produced concentration-related contractile responses and was equipotent with NKA, whereas the NK_1 and NK_3 receptor-selective [$Sar^9Met(O_2)^{11}$]SP (Drapeau *et al.*, 1987) and [$MePhe^7$]NKB (Drapeau *et al.*, 1987) were without effect.

Antagonist studies confirmed that the uterotonic effects of tachykinins on the pregnant human uterus was mediated through an NK_2 receptor. In the presence of the potent non-peptide NK_2 receptor-selective antagonist SR 48968 (Emonds-Alt *et al.*, 1992a), responses to the NK_2 receptor-selective [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) were reduced. Interestingly, SR 48968 acted as a non-competitive antagonist in this tissue as was also previously reported to occur in both the non-pregnant human uterus and pregnant mouse uterus. SR 48968 has been reported to act as either a competitive or as a non-competitive antagonist in different *in vitro* preparations. Croci *et al.* (1995a) observed that SR 48968 competitively antagonised responses to the NK_2 receptor-selective agonist [β-Ala^8]NKA(4-10) in the rat duodenum, whereas it non-competitively antagonised responses to the same agonist in the rat oesophagus, prompting speculation that this may be due to the existence of differing NK_2 receptor subtypes. This suggestion is supported by recent pharmacological and molecular studies conducted using various tissues from the rat and human (Candenas *et al.*, 2002; Croci *et al.*, 1998; Lippe *et al.*, 1997; Patacchini *et al.*, 2001). Of particular interest is the study by Candenas *et al.* (2002), which reported that the human uterus expresses both a wild-type NK_2 receptor type and a splice variant of the receptor that has provisionally been termed $NK_{2\beta}$. However, the authors were unable to clarify whether the $NK_{2\beta}$ variant would give rise to a functional protein.

In the present study the effects of the non-mammalian tachykinin eledoisin were also examined. Eledoisin produced concentration-related contractions of myometrium from pregnant women, which is consistent with its effects in the non-pregnant human uterus (Molina and Zappia, 1976). Eledoisin was only marginally less potent than NKA which suggested that this peptide was acting through an NK_2 receptor in the pregnant human uterus. This is consistent with the finding that eledoisin shows preference for the NK_2 receptor due to the nature of the amino acid in position 4 from the C-terminus being aliphatic (Severini *et al.*, 2002). Further support for the role of the NK_2 receptor in mediating the response to this peptide was obtained when the NK_2 receptor-selective antagonist SR 48968 reduced responses to eledoisin while the potent NK_1 receptor-selective antagonist SR 140333 (Emonds-Alt *et al.*, 1993b) was without effect.

A possible explanation for the difference in results obtained between the present study and that of Barr *et al.* (1991), which reported that tachykinins had no effect in myometrium from pregnant women, could be attributed to the lack of peptidase inhibitors in the latter study. It has been extensively documented that tachykinins are susceptible to hydrolysis by numerous peptides. Neprilysin is the most effective of the tachykinin-degrading enzymes (Hooper *et al.*, 1985; Hooper and Turner, 1985; Matsas *et al.*, 1983; 1984) and its presence has been reported in the uterus (Head *et al.*, 1993; Ottlecz *et al.*, 1991; Pinto *et al.*, 1999; Riley *et al.*, 1995). In the present study experiments involving the mammalian tachykinins were conducted in the presence of a peptidase inhibitor cocktail comprising the neprilysin inhibitor thiorphan (3μM), the ACE inhibitor captopril (10μM) and the bestatin-sensitive aminopeptidase inhibitor bestatin (10μM). Peptidase inhibitors were not used when the NK_2 receptor-selective [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) was tested since it has been shown to be metabolically stable (Fisher and Pennefather, 1997; Fisher *et al.*, 1993). In contrast [β-Ala^8]NKA(4-10], the NK_2 receptor-selective agonist used by Barr *et al.* (1991), has been reported to be a substrate for neprilysin (Medeiros and Turner, 1995). Thus this may explain why Barr *et al.* (1991) failed to observe enhancement of uterine contractions in the presence of this peptide. Although responses to both the NK_1 and NK_2 receptor-selective agonists [$Sar^9Met(O_2)^{11}$]SP and [$MePhe^7$]NKB have been reported to be potentiated in the

presence of peptidase inhibitors (Fisher *et al.*, 1993; Kerr *et al.*, 1997; Chapter 3), this was not seen to be the case in the present study (data not shown). This indicates that the lack of activity of these two receptor-selective agonists was not due to their inactivation by peptidases, but rather to their lack of affinity for NK_2 receptors. In contrast, responses to NKA were significantly potentiated in the combined presence of thiorphan (3μM) and bestatin (10μM), confirming reports that NKA is a substrate for both neprilysin (Hooper *et al.*, 1985; Hooper and Turner, 1985; Matsas *et al.*, 1983; 1984) and bestatin-sensitive aminopeptidases (Hooper and Turner, 1985). In contrast, these peptidase inhibitors at the same concentrations failed to potentiate the response to NKA in the non-pregnant human uterus (Chapter 6). This difference is consistent with molecular data that shows that the expression of neprilysin is lower in the non-pregnant myometrium than in the pregnant myometrium (Patak *et al.*, 2003). Interestingly, a similar observation was reported by Shintani *et al.* (2000), who failed to observe potentiation of the response to NKA by phosphoramidon in the non-pregnant rat uterus, whereas the same peptidase inhibitor produced significant potentiation in the pregnant rat uterus. Furthermore this finding is consistent with the observation that neprilysin activity is 25 times higher in the late-term pregnant rat uterus compared to the non-pregnant rat uterus (Ottlecz *et al.*, 1991).

It was of interest that in the present study, the presence of the same peptidase inhibitor cocktail used with the tachykinins did not potentiate uterotonic responses to either oxytocin or arginine vasopressin (AVP). Although oxytocin has been reported to be a substrate for neprilysin (Ottlecz *et al.*, 1991) the lack of potentiation of responses to this peptide in the presence of the peptidase inhibitors supports the finding that tachykinins are better substrates for neprilysin (Katayama *et al.*, 1991; Matsas *et al.*, 1984). The hydrolysis of AVP by human placental subcellular fractions and pregnancy sera has been reported to be significantly inhibited by bestatin (1mM) but not phosphoramidon (1mM) (Mizutani *et al.*, 1995). This finding suggests the active involvement of bestatin-sensitive aminopeptidases but not neprilysin in the degradation of AVP. The lack of potentiation of the response to AVP in the present study may possibly be explained by the low concentration of bestatin used (10μM) compared to the study by Mizutani *et al.* (1995).

The excitatory effect of tachykinins on the near-term pregnant uterus could indicate a role for these peptides in the initiation of parturition, where the release of tachykinins could contribute to the cascade of events leading to labour. This suggestion is consistent with reports that the placenta is a source of tachykinins during late pregnancy (Graf *et al.*, 1996; Page *et al.*, 2000; Sastry *et al.*, 1981), that SP is detected in amniotic fluid (Sanfilippo *et al.*, 1992) and that maternal immunoreactive SP concentrations in plasma are low during pregnancy and found to be higher immediately postpartum (Skrabanek *et al.*, 1980). In addition to their excitatory effect on myometrial smooth muscle tachykinins have also been implicated in regulating uteroplacental blood flow (Bodelsson and Stjernquist, 1992; Hansen *et al.*, 1988; Skrabanek *et al.*, 1980), pre-eclampsia (Page and Lowry, 2000; 2001; Page *et al.*, 2000; 2001) and cervical ripening (Collins *et al.*, 2002).

The results of the present study indicate that NKA and [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) were more potent in the non-pregnant than in the pregnant uterus. This may reflect a pregnancy-related decrease in NK_2 receptor levels and/or pregnancy-induced changes in the activity of peptidases. The former possibility is consistent with the report that NK_2 receptors are more strongly expressed in uterine tissue from non-pregnant compared to pregnant women (Patak *et al.*, 2003). Recent molecular studies have also reported low levels of neprilysin in the non-pregnant uterus (Patak *et al.*, 2003), which may explain why peptidase inhibitors potentiated the effects of NKA in pregnant but not non-pregnant uterine preparations. The high potency of tachykinins in the non-pregnant uterus together with the low expression of neprilysin, the main enzyme involved in their degradation, suggests that tachykinins may be implicated in some physiological or pathophysiological role in the reproductive tract of non-pregnant women, for example in menstrual disorders such as dysmenorrhoea and menorrhagia.

In conclusion, the results of the present study using the mammalian tachykinins and tachykinin receptor-selective agonists and antagonists indicate the involvement of functional NK_2 receptors in mediating the contractile activity of tachykinins in the pregnant human uterus. They also indicate that in contrast to the mouse uterus, the

predominant tachykinin receptor type mediating uterine contractility is not modulated by pregnancy.

The aim of the next chapter is to investigate the presence of SP- and NKA-immunoreactivity in the human and mouse uterus and to determine whether this is modulated by pregnancy.

CHAPTER 8

PILOT IMMUNOHISTOCHEMICAL STUDY OF SUBSTANCE P, NEUROKININ A AND CALCITONIN GENE-RELATED PEPTIDE IN THE MOUSE AND HUMAN UTERUS

Summary

1. The aims of this pilot study were firstly to investigate the presence of tachykinin peptides and calcitonin gene-related peptide (CGRP) within the mouse and human uterus and the mouse cervix, and secondly to examine the effects of pregnancy on their distribution.

2. Immunohistochemical studies demonstrated the presence of sparse SP-, NKA- and CGRP- immunoreactive (IR) fibres distributed throughout both the uterus and cervix of the oestrogen-treated mouse. In contrast, peptidergic fibres were not visualised in the uterus and cervix of the pregnant mouse.

3. SP- and tyrosine hydroxylase (TH)-, but not NKA- or CGRP-IR fibres were sparsely present in the outer layer of myometrium from non-pregnant women. No peptide or TH-IR fibres were observed in myometrial tissue obtained from the site of excision in pregnant women.

4. These pilot results demonstrate a sparse distribution of tachykinin-containing nerves in both the non-pregnant mouse and human uterus, the location of which suggests an association with blood vessels. The absence of tachykinin- and CGRP-IR fibres in both these species during pregnancy is of interest but further studies will need to be undertaken before it can be established whether this is due to pregnancy-induced sensory nerve degeneration.

8.1 Introduction

In the previous chapters the ability of tachykinins to modulate myometrial contractility in both non-pregnant and pregnant mice and humans was described. Having determined that the NK_2 receptor was the predominant tachykinin receptor type involved in eliciting tachykinin-induced uterine contractions in the human and the pregnant mouse, it was of particular interest to examine whether NKA, the mammalian tachykinin that shows a preference for the NK_2 receptor, was present in the mouse and human uterus.

Previous immunohistochemical studies have reported the presence of SP-containing primary sensory neurones in the female genital tract of numerous species including the human (Butler-Manuel *et al.*, 2002; Franco-Cereceda *et al.*, 1987; Fried *et al.*, 1990; Heinrich *et al.*, 198[illegible], Reinecke *et al.*, 1989; Samuelson *et al.*, 1985), rat (Huang *et al.*, 1984; Papka *et al.*, 1985; 1987; Shew *et al.*, 1991; Traurig *et al.*, 1984; 1988), guinea-pig (Alm *et al.*, 1978; Alm and Lundberg, 1988; Franco-Cereceda *et al.*, 1987; Gibbins *et al.*, 1985; Heinrich *et al.*, 1986; Huang *et al.*, 1984; Mitchell and Ahmed, 1992), horse (Bae *et al.*, 2001), pig (Majewski *et al.*, 1996), cat (Alm *et al.*, 1978; Huang *et al.*, 1984; Kawatani and de Groat, 1991), cow (Lakomy *et al.*, 1995) and mouse (Huang *et al.*, 1984). In these species SP-immunoreactive (IR) fibres were demonstrated in the myometrium and endometrium, close to uterine glands and around blood vessels. In addition, regional differences in the number of SP-IR fibres were observed with the number of immunostained fibres being more numerous in the cervix and vagina compared to the uterus and ovary.

The colocalisation of SP with CGRP in a subset of primary sensory neurones in the female reproductive tract has been demonstrated in the rat, cat, guinea-pig, horse and human (Alm and Lundberg, 1988; Bae *et al.*, 2001; Franco-Cereceda *et al.*, 1987; Gibbins *et al.*, 1985; Kawatani and de Groat, 1991; Samuelson *et al.*, 1985; Shew *et al.*, 1991; Traurig *et al.*, 1988). However, very few studies have investigated the presence of NKA-immunoreactivity in the female genital tract; consequently NKA-IR fibres have only been reported in the rat (Dalsgaard *et al.*, 1985; Traurig *et al.*, 1988; 1991) and guinea-pig uterus (Alm and Lundberg, 1988). In these two species NKA was observed to be colocalised with either SP or SP together with CGRP.

It is unclear whether as with the noradrenergic innervation of the uterus (Alm *et al.*, 1979; Owman, 1981; Sjöberg, 1968; Thorbert *et al.*, 1979; Wikland *et al.*, 1984) these neuropeptide-containing nerves degenerate as pregnancy progresses. Alm and Lundberg (1988) reported the disappearance of all neuropeptide-immunoreactivity in the guinea-pig uterus at full term pregnancy. In contrast, in the rat uterus one study observed that no comparable degeneration occurred during pregnancy (Traurig *et al.*, 1984) while another reported hypertrophy of the afferent innervation during pregnancy (Amira *et al.*, 1995).

The aim of this pilot study was to investigate the occurrence of SP-, NKA- and CGRP-immunoreactivity in both the human and mouse uterus and the mouse cervix, particularly that of NKA whose presence has not been established in the female genital tract of either species. Tissue used in this study was obtained from both non-pregnant and pregnant mice and humans so that the effect of pregnancy on neuropeptide-immunoreactivity could also be examined and compared with tyrosine-hydroxylase-immunoreactivity, which was used as a control in the human tissues. Note that the techniques employed in this study were not designed to allow any quantitative analysis of immunoreactivity.

8.2 Materials and Methods

8.2.1 Tissue preparations

Uterine horns and cervices from oestrogen-treated and pregnant mice (refer to 2.1.1 for full animal details), and outer myometrial tissue from non-pregnant and pregnant women (refer to 2.1.2 for full patient details) were fixed in phosphate-buffered saline (mM; NaCl, 136.90; KCl, 2.68; KH_2PO_4, 1.46; Na_2HPO_4, 8.10) (PBS) containing 4% paraformaldehyde for 2h. Tissues were then washed in PBS containing 7% sucrose and 0.01% sodium azide four times for 10min each time. Tissues were then left in this solution and refrigerated at 4°C for between 48-72h. Tissues were then embedded in "Tissue Tek" (OCT embedding compound) in vinyl specimen moulds (cryomold) and snap-frozen with liquid iso-pentane quenched to the temperature of liquid nitrogen and maintained at -70°C. 14µm sections of tissue were cut using a cryostat (Reichert-Jung CM 1800, -21°C) and thawed onto gelatin-coated slides. Slides were maintained at -70°C until staining.

8.2.2 Immunohistochemistry

Slides were incubated for 18-20h at room temperature with rabbit polyclonal antibodies for SP (Serotec), NKA (Phoenix) and CGRP (Serotec). Human tissue was also incubated with a mouse monoclonal antibody for tyrosine hydroxylase (Boehringer Mannheim). Antibodies were diluted in an antibody-diluting medium containing 0.1% w/v sodium azide, 0.01% w/v bovine serum albumin, 0.1% w/v lysine and 0.1% v/v Triton in phosphate-buffered solution. For experiments using mouse tissue all primary antibodies were diluted 1:1000 while the following dilutions were used for human tissue (SP, 1:1000; NKA, 1:1000; CGRP, 1:2000; tyrosine hydroxylase, 1:100). For control sections, the primary antibody was omitted. Tissue sections were then washed with PBS four times for 10min each time before a one hour incubation with a secondary antibody at room temperature. Fluorescein isothiocyanate (FITC-)-conjugated anti-rabbit immunoglobulin (1:500 for mouse tissue, 1:250 for human tissue) (Vector) was used for tissues that had been incubated with SP, NKA or CGRP. Texas Red-conjugated anti-mouse immunoglobulin (1:500, Vector) and streptavidin (1:100, Vector) was used for

tissues that had been incubated with tyrosine hydroxylase. Tissues were then washed in PBS four times every 10min, mounted in "Vectashield" (Vector), cover slipped and examined under an Olympus fluorescence photomicroscope.

To prevent cross-reactions with endogenous immunoglobulins in experiments using human tissue, primary and secondary antibody incubations were done in antibody diluting medium containing 2% human serum. Human serum was obtained from blood that was refrigerated at 4°C for 48h and then centrifuged for 4min at 3000 rpm. Supernatant was pipetted off and centrifuged again for 4min at 3000 rpm. The serum was then pipetted into eppendorf tubes and frozen at -70°C until further use.

8.2.3 Materials

The materials used were: mouse polyclonal antibody for tyrosine hydroxylase (Boehringer Mannheim); fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin (Vector); rabbit polyclonal antibody for CGRP (Serotec), NKA (Phoenix) and SP (Serotec); Streptavidin (vector); Texas Red-conjugated anti-mouse immunoglobulin (Vector); Vectashield (Vector). All other reagents were of analytical grade.

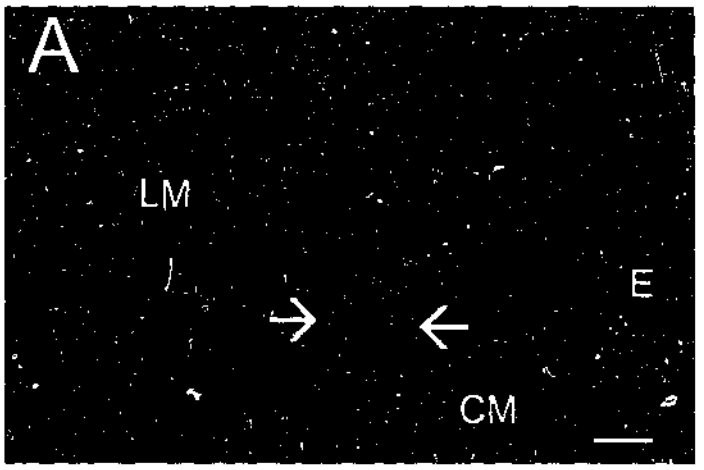
All primary antibodies were supplied in lyophilized form, reconstituted in distilled water, aliquoted into eppendorf tubes and stored at -70°C. Secondary antibodies were supplied in solution and stored at 4°C.

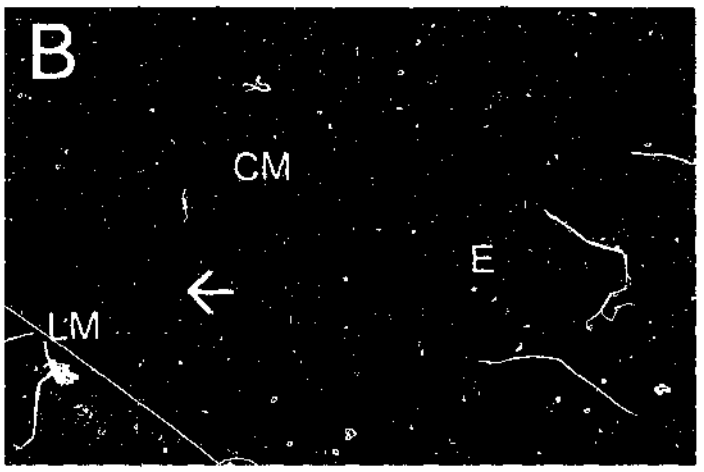
8.3 Results

All results are based on tissue obtained from 4 animals/patients and every specimen was examined in 6-8 sections. No immunostaining was observed in control sections from which the primary antibody had been omitted.

In the oestrogen-treated mouse uterus SP-, NKA- and CGRP-immunostaining was observed between the circular and longitudinal muscle layers. In the cervix, peptidergic-IR fibres were demonstrated between the circular muscle layer and the endometrium. A similar distribution pattern was seen with the three peptides (Figures 8.1 and 8.2). In contrast, no peptidergic-immunoreactivity was seen in either the uterus or cervix in tissue obtained from pregnant mice (Figures 8.3 and 8.4).

Sparse SP- and TH-immunoreactivity but not NKA- or CGRP-immunoreactivity was observed in the outer myometrium obtained from non-pregnant women (Figure 8.5). However, no immunostaining was seen in corresponding tissue obtained from the upper edge of the excision in pregnant women (Figure 8.6).





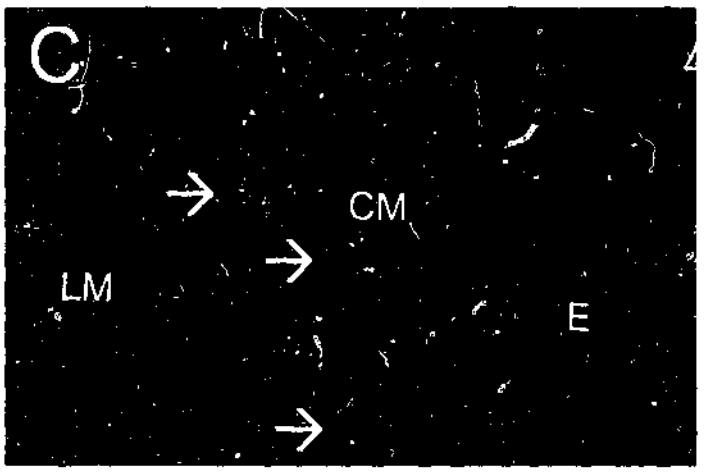


Figure 8.1: Representative photomicrographs showing cross sections of oestrogen-treated mouse uterus stained with (A) SP, (B) NKA and (C) CGRP. Nerve fibres immunoreactive for peptides are indicated by arrows. Cross sections of mouse uterus show sparse immunostaining to both tachykinins and CGRP running between the circular and longitudinal muscle layers. CM, circular muscle; E, endometrium; LM, longitudinal muscle. Objective x40. The scale bar represents 6μm.

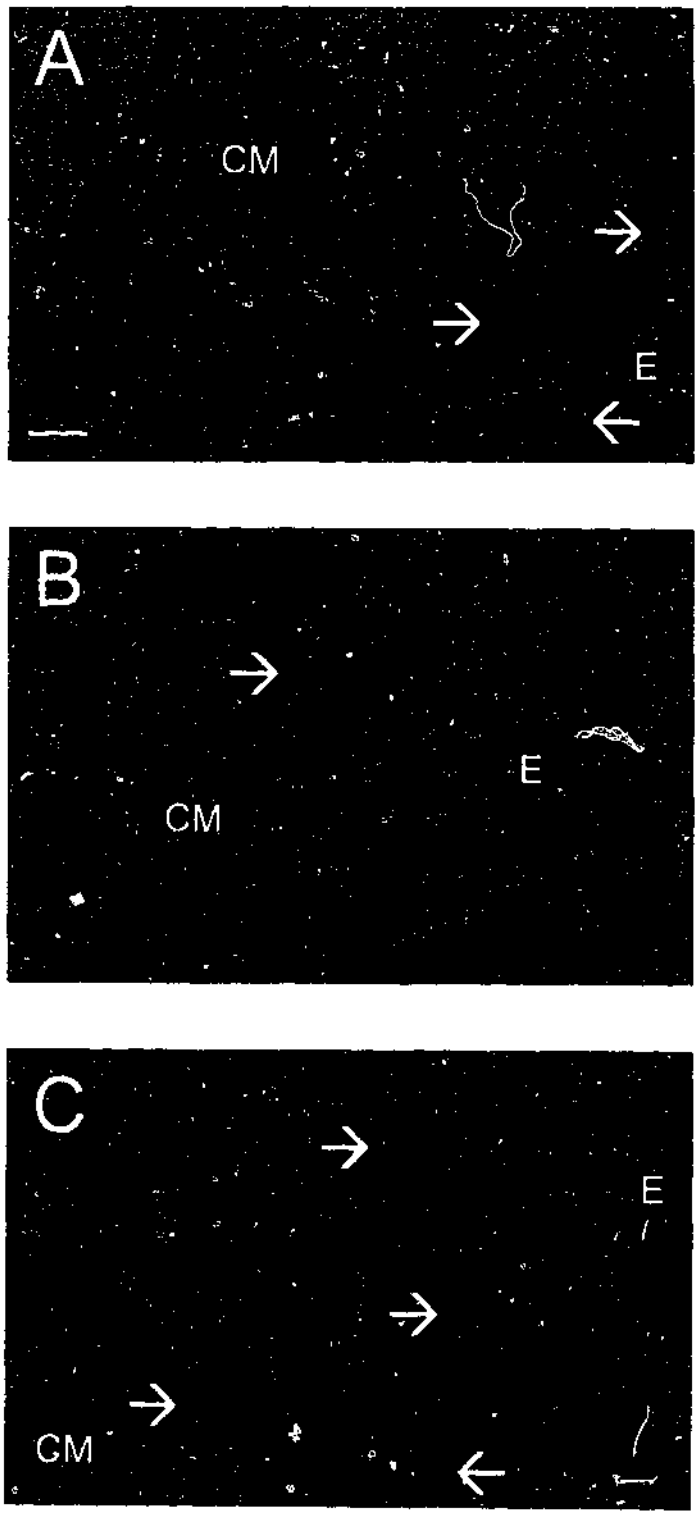
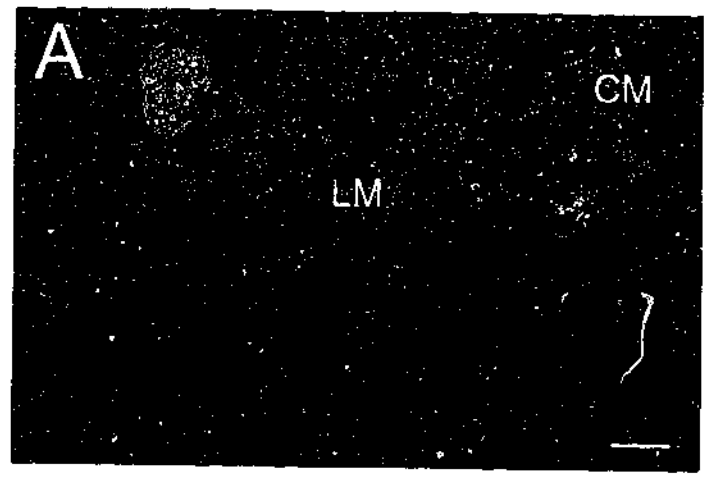
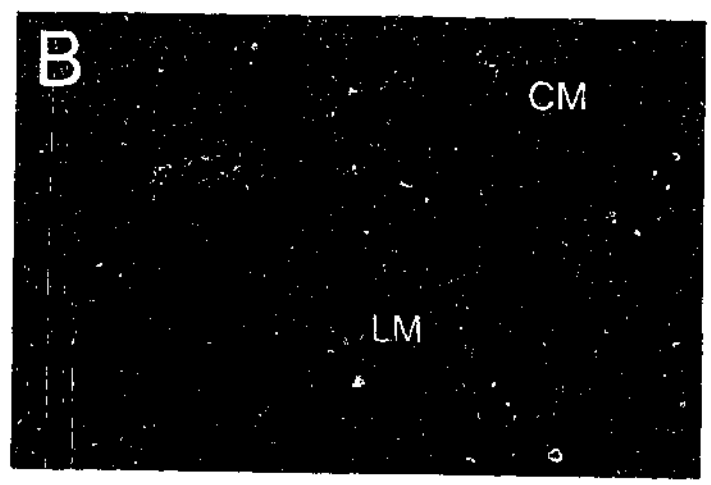


Figure 8.2: Representative photomicrographs showing cross sections of oestrogen-treated mouse cervix stained with (A) SP, (B) NKA and (C) CGRP. Nerve fibres immunoreactive for peptides are indicated by arrows. Cross sections of mouse cervix show sparse immunostaining to both tachykinins and CGRP running between the circular muscle layer and the endometrium. CM, circular muscle; E, endometrium. Objective x40. The scale bar represents 6μm.





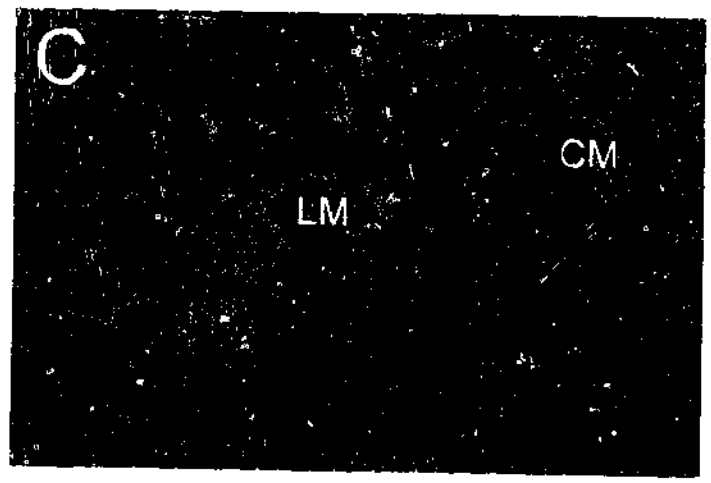


Figure 8.3: Representative photomicrographs showing cross sections of pregnant mouse uterus stained with (A) SP, (B) NKA and (C) CGRP. No immunoreactivity was seen with any of the peptides. CM, circular muscle; LM, longitudinal muscle. Objective x40. The scale bar represents 6μm.

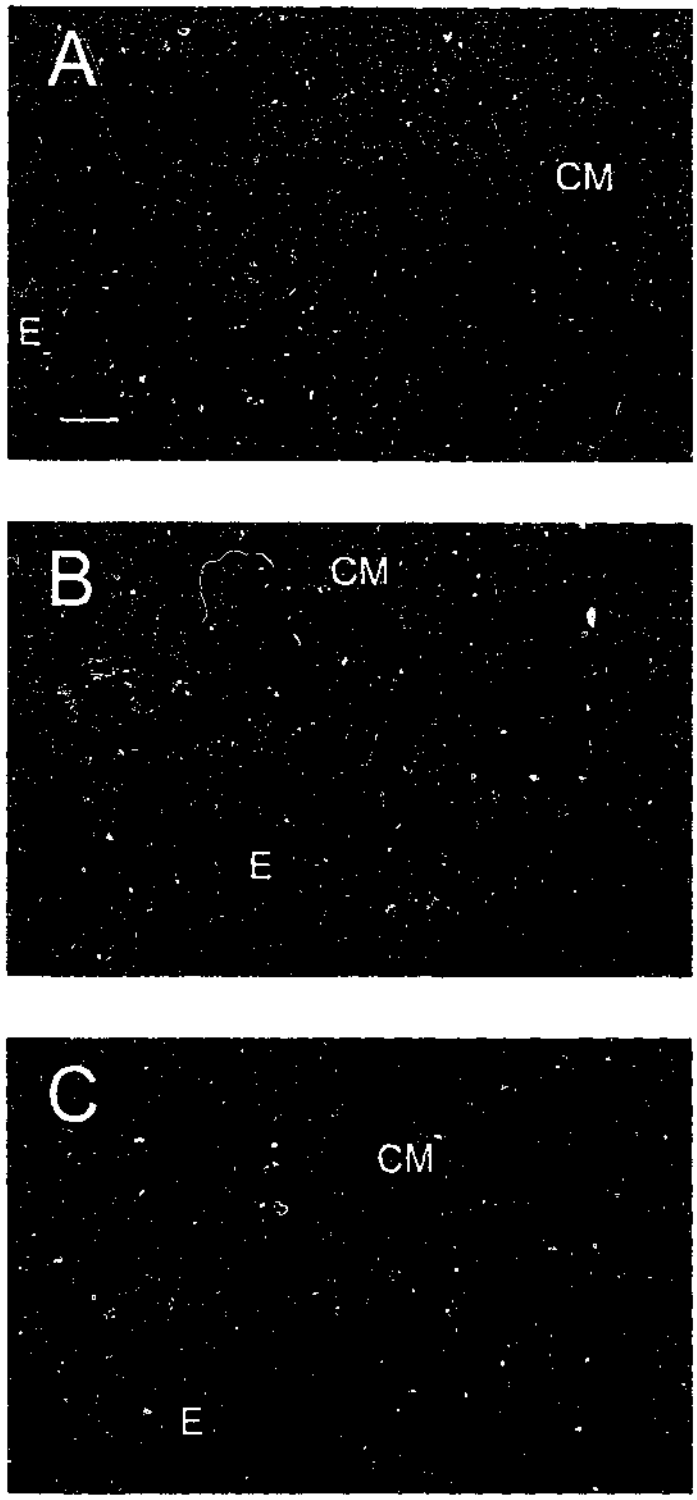


Figure 8.4: Representative photomicrographs showing cross sections of pregnant mouse cervix stained with (A) SP, (B) NKA and (C) CGRP. No immunoreactivity was seen with any of the peptides. CM, circular muscle; E, endometrium. Objective x40. The scale bar represents 6μM.

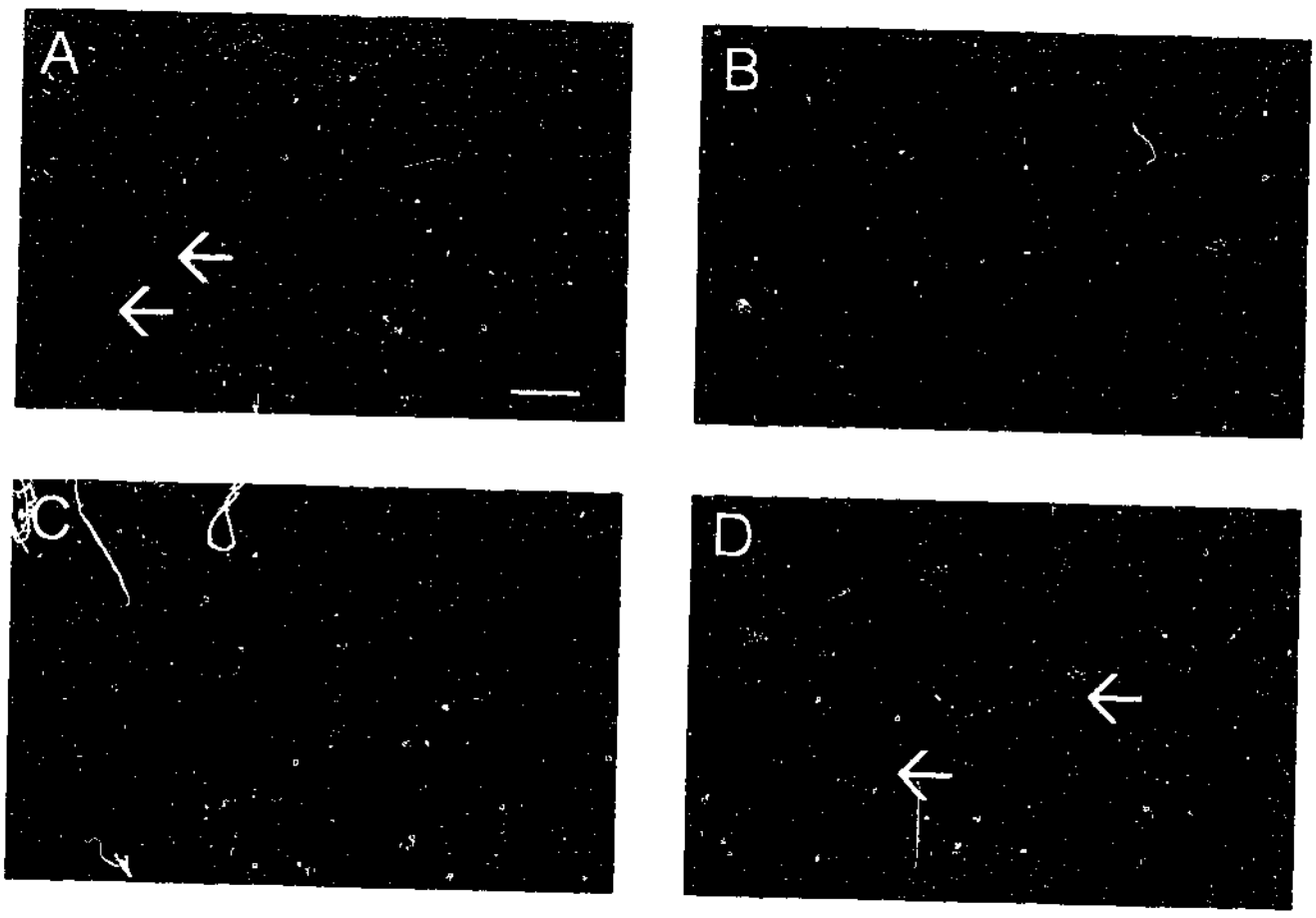


Figure 8.5: Representative photomicrographs showing cross sections of human non-pregnant outer myometrium stained with (A) SP, (B) NKA, (C) CGRP and (D) TH. Nerve fibres immunoreactive for peptides are indicated by arrows. Sparse immunostaining was observed with both SP and TH. Objective x40. The scale bar represents 9μm.

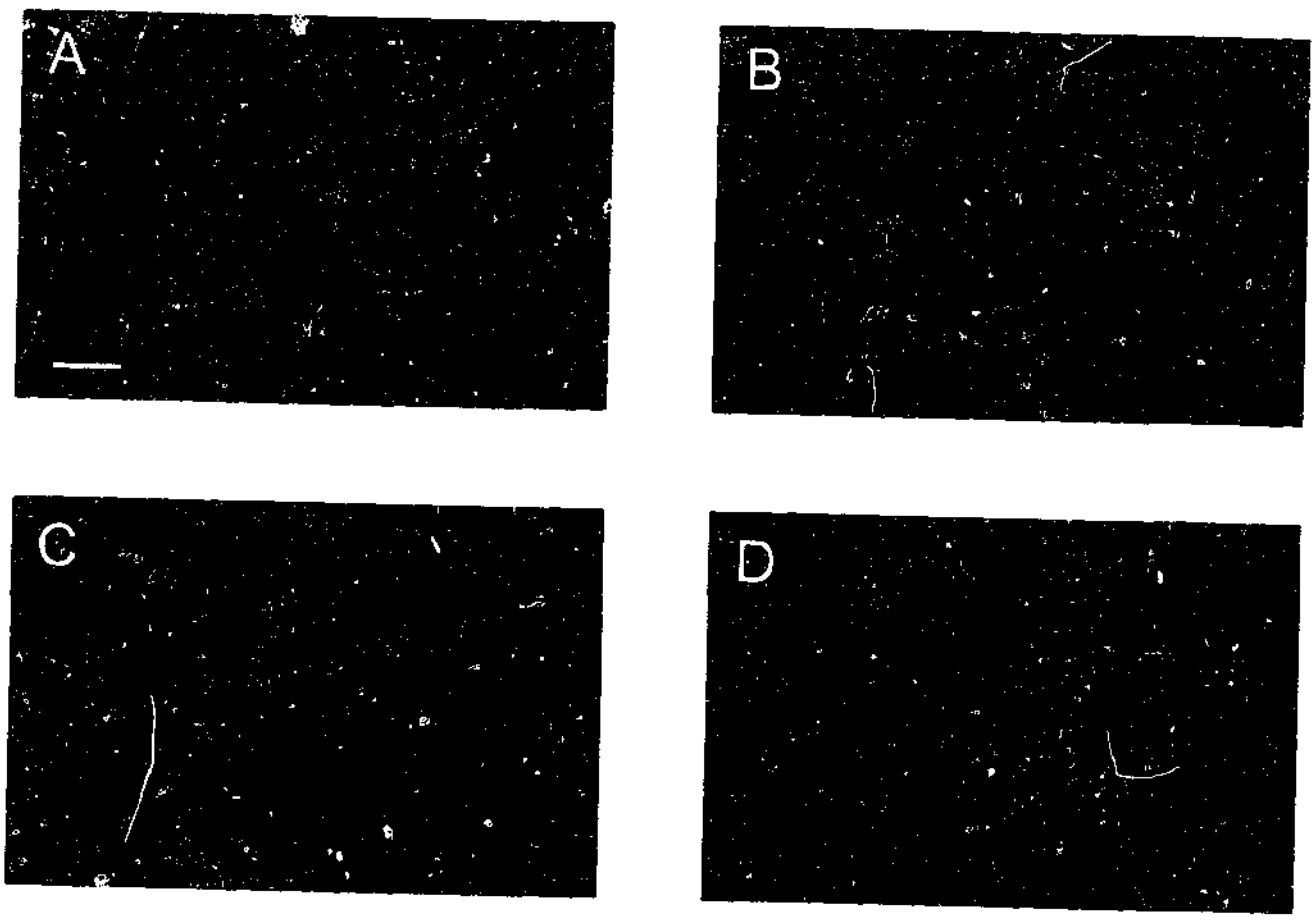


Figure 8.6: Representative photomicrographs showing cross sections of human pregnant outer myometrium stained with (A) SP, (B) NKA, (C) CGRP and (D) TH. No immunoreactivity was seen with any of the peptides. Objective x40. The scale bar represents 9μm.

8.4 Discussion

The aim of this pilot study was to investigate the presence of tachykinin peptides and CGRP within the mouse and human uterus. SP-, NKA- and CGRP-IR fibres were demonstrated running between the circular and longitudinal muscle layers of the oestrogen-treated mouse uterus and the circular muscle layer and endometrium of the mouse cervix, which suggests that they were associated primarily with blood vessels. Sparse SP and TH-IR fibres were observed in between muscle fibres of human outer myometrium, however neither NKA- nor CGRP-immunoreactivity could be demonstrated in this tissue. In contrast, SP-, NKA- and CGRP-immunoreactivity was not visualized in uterine tissue obtained from either pregnant mice or humans.

The presence of SP-IR fibres in the mouse uterus has previously been reported (Huang *et al.*, 1984). However, the present study is the first to demonstrate the presence of both NKA- and CGRP-IR fibres in the genital tract of the female mouse. In the present study, SP-IR fibres were observed running between either the circular and longitudinal muscle layers of the uterus or between the circular muscle layer and the endometrium of the cervix. This distribution suggests an association with blood vessels. This is consistent with the study by Huang *et al.* (1984), which reported that SP-IR fibres were associated with blood vessels in the mouse reproductive tract. The authors also reported that SP-immunoreactivity was associated with smooth muscle. This was not observed in the present study. However, both studies reported that the number of SP-IR fibres were sparse in the uterus, thus Huang *et al.* (1984) would have had a greater probability of demonstrating positive immunostaining as their study used a larger number of animals (n=10 compared to n=4 used in the present study). In addition, the present study and that of Huang and colleagues employed different immunohistochemistry protocols, which could also possibly account for any reported differences.

In the present study, NKA- and CGRP-IR fibres in the mouse uterus were observed to have a distribution similar to that of the SP-IR fibres, which suggests the possibility that these three peptides are colocalised. The colocalisation of SP with CGRP in a subset of primary sensory neurones in the female reproductive tract has been demonstrated in the rat, cat, guinea-pig, horse and human (Alm and Lundberg, 1988; Bae *et al.*, 2001;

Franco-Cereceda *et al.*, 1987; Gibbins *et al.*, 1985; Kawatani and de Groat, 1991; Samuelson *et al.*, 1985; Shew *et al.*, 1991; Traurig *et al.*, 1988). The coexistence of SP with NKA has also been reported in the rat uterus (Dalsgaard *et al.*, 1985; Traurig *et al.*, 1991) while Alm and Lundberg (1988) have demonstrated the coexistence of SP, NKA and CGRP in the guinea-pig uterus. It would therefore be of interest to conduct further studies using double-immunostaining to confirm whether SP, NKA and CGRP coexisted in the mouse uterus.

The presence of SP-, NKA- and CGRP-IR fibres was also demonstrated in the mouse cervix. IR- fibres were seen to run between the circular muscle layer and the endometrium, again suggesting an association with blood vessels. Whilst this study was not quantitative, it appeared that IR-fibres, particularly those expressing CGRP-immunoreactivity, were more frequent in the cervix compared with the uterus. This is consistent with other studies that have reported the existence of regional differences in neuropeptide-immunoreactivity in the female reproductive tract. The distribution of both SP- and CGRP-IR fibres has been reported to be more frequent in the cervix compared to that of the uterus in numerous species including the mouse, rat, guinea-pig, cat and horse (Alm and Lundberg, 1988; Bae *et al.*, 2001; Huang *et al.*, 1984; Traurig *et al.*, 1984).

Both SP- and CGRP-IR fibres have previously been reported to be present in the human uterus (Franco-Cereceda *et al.*, 1987; Heinrich *et al.*, 1986; Reinecke *et al.*, 1989; Samuelson *et al.*, 1985). In the present study, sparse SP-IR fibres were observed in the outer myometrium but NKA- and CGRP-IR fibres were not detected. To date, no other study has investigated the presence of NKA-immunoreactivity in the human uterus, therefore it is not possible to confirm whether the absence of NKA-immunoreactivity observed in the present study was due to a genuine lack of NKA-IR fibres in this tissue or to methodological problems. However, CGRP-IR fibres were also not detected in the present study whereas their presence in the human uterus has previously been reported by other authors (Franco-Cereceda *et al.*, 1987; Reinecke *et al.*, 1989; Samuelson *et al.*, 1985). This suggests the possibility of limitations within the current study. One such limitation is that the present study only examined the outer myometrium for

immunoreactivity as this corresponded to the tissue preparations used in functional experiments described in Chapter 6. Previous studies of SP- and CGRP-immunoreactivity in this tissue reported that not only were these IR-fibres rarely found in the human uterus but that they also displayed a differential distribution between regions (Heinrich *et al.*, 1986; Samuelson et al., 1985). It would therefore be of interest to conduct further experiments using uterine tissue of full thickness and from differing regions in order to extend the results obtained in the present study.

It is well documented that a decrease in the uterine noradrenergic nerve supply occurs during pregnancy (Alm *et al.*, 1979; Owman, 1981; Sjöberg, 1968; Thorbert *et al.*, 1979; Wikland *et al.*, 1984). The results of the present study in which tyrosine hydroxylase-immunoreactivity was demonstrated in the non-pregnant human myometrium but was absent in corresponding tissue from pregnant women are consistent with such pregnancy-induced denervation. However, it still remains unclear whether the sensory innervation undergoes a similar decrease as pregnancy progresses. In the present study SP-, NKA- and CGRP-IR fibres were not detected in myometrium obtained from pregnant mice or humans. Due to the limitations of the current study as mentioned previously, it is not clear whether this is indicative of a genuine decrease in neuropeptide levels, especially since neither NKA- nor CGRP-immunoreactivity had been detected in the non-pregnant human uterus. The disappearance of all neuropeptide-immunoreactivity has been reported to occur in the guinea-pig uterus at full term pregnancy (Alm and Lundberg, 1988). In contrast, conflicting results have been reported in the rat uterus, with one study observing no comparable degeneration of primary afferent fibres during pregnancy (Traurig *et al.*, 1984) while another reported the occurrence of hypertrophy (Amira *et al.*, 1995). These conflicting results, together with those of the present study, suggest that further studies will need to be undertaken before a conclusion can be drawn.

The presence of peptidergic-immunoreactivity in the reproductive tract of both the mouse and human as described by the present study and others, could imply a functional role for these peptides in various reproductive processes including menstruation, conception and parturition. Association of SP-, NKA- and CGRP-IR fibres with

vascular smooth muscle may indicate a role in the regulation of blood flow in this tissue. Though an association with myometrial smooth muscle was not seen in the present study, previous studies have demonstrated this in both the mouse (Huang *et al.*, 1984) and human uterus (Franco-Cereceda *et al.*, 1987; Heinrich *et al.*, 1986; Reinecke *et al.*, 1989; Samuelson *et al.*, 1985). Taken together with the results of the previous chapters, which demonstrated that tachykinins had a uterotonic effect in both the mouse and human uterus suggests it is possible that these peptides may play a physiological or pathophysiological role involving modulation of myometrial contractility in both these species.

CHAPTER 9

CONCLUDING REMARKS

Peripheral nerves containing the tachykinins SP and NKA have been observed in the female reproductive tract of several mammalian species. However, the function of these peptide-containing nerves in reproduction has yet to be elucidated. Previous work from this laboratory has shown that the tachykinins elicit smooth muscle activity in the rat uterus (Fisher and Pennefather, 1997; 1998; 1999; Fisher *et al.*, 1993; Moodley *et al.*, 1999; Pennefather *et al.*, 1993). It is therefore probable that the release of tachykinins from peripheral nerves could effect coordination of uterine muscular activity, important for facilitating emptying of uterine contents during menstruation, transport of sperm to the fallopian tubes, implantation of the conceptus and expulsion of the fetus during parturition.

To date, the majority of studies investigating the actions of tachykinins on myometrial contractility have been conducted using the rat uterus. In this species, the tachykinin receptor type involved in eliciting the uterotonic activity of these peptides has been established. In addition, the importance of peptidases in constraining responses to the tachykinins in the rat uterus has been demonstrated. However, very little is known about their actions on myometrium in other species. Two early studies reported that SP and the non-mammalian tachykinin eledoisin both elicited contractile activity in myometrium obtained from non-pregnant women (Molina and Zappia, 1976; Ottesen *et al.*, 1983). In contrast, agonists selective for the three established tachykinin receptor types NK_1, NK_2 and NK_3 were without effect in myometrium obtained from pregnant women (Barr *et al.*, 1991). In addition, pilot studies conducted in this laboratory had demonstrated that the tachykinins could elicit contractile activity in the non-pregnant and pregnant mouse uterus (Fleming, 1998).

The aim of this thesis was therefore:

- to determine the susceptibility of tachykinins to various peptidases in the mouse and human uterus;
- to characterize the tachykinin receptor type(s) involved in mediating myometrial contractility in the mouse and human;
- to attempt to demonstrate the presence of tachykinin-containing nerves in myometrium from mice and humans using immunohistochemical methods;

- to investigate the effect of pregnancy on the phenomena associated with the above stated aims.

Previous functional experiments undertaken in this laboratory using the tachykinin peptides have reported the need for peptidase inhibitors when examining the effects of these peptides in the rat uterus. The main enzyme responsible for tachykinin degradation is believed to be neprilysin (Hooper *et al.*, 1985; Hooper and Turner, 1985; Matsas *et al.*, 1983; 1984). However, SP is also a substrate for angiotensin converting enzyme (ACE) (Hooper and Turner, 1985; Skidgel *et al.*, 1984; Yokosawa *et al.*, 1983) while NKA and NKB are substrates for bestatin-sensitive aminopeptidases (Hooper and Turner, 1985; Nau *et al.*, 1986). Initial experiments described in Chapter 3 suggested that as in the rat uterus (Fisher and Pennefather, 1997; Fisher *et al.*, 1993; Magraner *et al.*, 1998), neprilysin plays a key role in inactivating tachykinins in the oestrogen-treated mouse uterus. In comparison, ACE and bestatin-sensitive aminopeptidases appear to have relatively minor roles in contributing to tachykinin degradation in this tissue. It was however decided that all subsequent functional experiments using uterine preparations obtained from both mice and humans would be conducted in the presence of a "peptidase inhibitor cocktail". This "cocktail" consisted of the neprilysin inhibitor thiorphan (3μM), the ACE inhibitor captopril (10μM) and the bestatin-sensitive aminopeptidase inhibitor bestatin (10μM).

The tachykinin receptor type(s) involved in mediating the effects of the tachykinins in the oestrogen-treated and pregnant mouse uterus were investigated in Chapters 4 and 5. In myometrial preparations from the oestrogen-treated mouse it was concluded, from the rank order of agonist potency of the mammalian tachykinins, and from further studies using the tachykinin receptor-selective agonists and antagonists, that responses were predominantly mediated by the NK_1 receptor, although a component of the response to NKA and NKB may also be mediated by the NK_2 receptor. Corresponding experiments conducted using myometrial preparations from pregnant mice indicated that tachykinin-induced myometrial contractility was mediated predominantly by the NK_2 receptor. The significance of such a change in the predominant tachykinin-receptor

type mediating tachykinin responses during pregnancy is unclear. It would be of interest to investigate this phenomenon further, by undertaking experiments to confirm whether this was a reflection of a change in receptor-effector coupling and/or in tachykinin receptor type numbers. It was also observed that the same concentrations of peptidase inhibitors seen to potentiate responses to the mammalian tachykinins in the oestrogen-treated mouse uterus had no effect in the pregnant mouse uterus. This may reflect a pregnancy-induced up-regulation of peptidases as has been demonstrated in the rat uterus (Ottlecz *et al.*, 1991) and it would clearly be of interest to investigate this further to clarify the role of peptidases in constraining the uterotonic effects of tachykinins in the pregnant mouse.

Similar functional experiments described in Chapters 6 and 7, have for the first time, confirmed that tachykinins produce uterotonic effects on myometrium from non-pregnant and pregnant women, and determined the tachykinin receptor type mediating such effects. The results of these experiments indicated that the uterotonic effects of the tachykinins in both non-pregnant and pregnant women were mediated by the NK_2 receptor. The absence of blockade by atropine, phentolamine and TTX of the effect of [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) on myometrium from non-pregnant women also suggests that these NK_2 receptors may be located on the uterine smooth muscle.

In contrast to the mouse, peptidase inhibitors were seen to potentiate the response to the tachykinins in myometrial tissue from pregnant but not non-pregnant women. This observation is consistent with recent molecular studies conducted by our Spanish collaborators, which reported that the expression of neprilysin in the human uterus is lower in non-pregnant compared to pregnant women (Patak *et al.*, 2003). Furthermore, it has been reported that neprilysin levels are low or negative in women with fibroids (McCluggage *et al.*, 2001) diagnosed as present in a large majority of women participating in the present study, in addition to experiencing various menstrual disorders. The low activity of neprilysin observed in myometrial tissue from non-pregnant women diagnosed with benign uterine disorders contrasts with the situation in both the non-pregnant rat and mouse. This suggests the possibility that reduced neprilysin levels may be implicated in human uterine dysfunction.

It was also of interest that the two most effective tachykinins, the NK_2-preferring NKA and the NK_2 receptor-selective [$Lys^5MeLeu^9Nle^{10}$]NKA(4-10) were significantly more potent in myometrium from non-pregnant than in tissues from pregnant women. Consistent with this observation is the report that levels of NK_2 receptor expression are higher in the uterus of non-pregnant compared to pregnant women (Patak *et al.*, 2003). In contrast, no difference was observed in the potency of NKA on uterine preparations obtained from non-pregnant and pregnant rats (Candenas *et al.*, 2001; Shintani *et al.*, 2000). In addition, the level of expression of the NK_2 receptor in the rat uterus did not alter during pregnancy (Candenas *et al.*, 2001). The high expression of myometrial NK_2 receptors in non-pregnant women together with the low expression of the peptidase neprilysin could indicate a physiological or pathophysiological role for tachykinins in the non-pregnant uterus. A decrease in NK_2 uterine receptor numbers during pregnancy could also help to promote uterine quiescence. These are important questions that warrant further investigation.

In respect to tachykinin receptor types involved in mediating uterotonic activity, the present study has established that the human is similar to the rat. In both these species the NK_2 receptor mediates tachykinin-induced contractile activity in both the non-pregnant and pregnant uterus. In contrast, the NK_1 receptor is the predominant receptor type mediating the uterotonic effects of tachykinins in non-pregnant mice whereas during pregnancy the NK_2 receptor is the predominant receptor type mediating the contractile activity of these peptides. These observations suggest that when investigating pregnancy-induced effects upon the actions of tachykinins on the uterus, the rat may be a better model for the human compared to the mouse.

The finding that the NKA-preferring NK_2 receptor was involved in mediating contractile activity in both the non-pregnant and pregnant human uterus as well as the pregnant mouse uterus was the impetus for the pilot immunohistochemical study presented in Chapter 8. Although previous studies have confirmed the presence of SP-immunoreactive (IR) fibres in the myometrium of various species, the presence of NKA–IR fibres had yet to be demonstrated in either the mouse or human. This present study is the first to demonstrate the presence of sparse NKA-IR fibres located between

the circular and longitudinal muscle layers in the oestrogen-treated mouse uterus. However, no peptide immunoreactivity could be visualized in uterine tissue obtained from pregnant mice. In the human only sparse SP- and tyrosine hydroxylase-IR fibres were observed between muscle fibres in samples of outer myometrium obtained from non-pregnant women. This finding is consistent with previous reports that the distribution of SP-IR nerves in the human uterus was sparse (Fried *et al.*, 1990; Heinrich *et al.*, 1986; Reinecke *et al.*, 1989; Samuelson *et al.*, 1985). The expression of PPT-A, the gene that encodes for SP and NKA, has also been reported as being relatively sparsely expressed in the non-pregnant human uterus (Patak *et al.*, 2003). The authors suggested this low expression of PPT-A might be due to the absence of cell bodies in the myometrium, as in sensory nerves, the tachykinin precursor PPT-A is processed into SP and NKA in the cell soma and the peptides are then transported along the axon to sites of release in the nerve terminals (Maggi, 1997). In contrast, no peptide-immunoreactivity was visualized in outer myometrium from pregnant women. The disappearance of tyrosine hydroxylase immunoreactivity in myometrium from pregnant women is consistent with the reports of a pregnancy-induced decrease in uterine noradrenergic nerves (Alm *et al.*, 1979; Owman, 1981; Sjöberg, 1968; Thorbert *et al.*, 1979; Wikland *et al.*, 1984). The disappearance of tachykinin-IR nerves in uterine tissue during pregnancy has been reported to occur in the guinea-pig (Alm and Lundberg, 1988) but not the rat (Amira *et al.*, 1995; Traurig *et al.*, 1984) suggesting that species differences may exist. However, further experiments are needed to clarify whether the lack of peptide-immunoreactivity in uterine tissue obtained from both pregnant humans and mice was due to a genuine lack of IR-nerve fibres or methodological limitations within the present study.

It is also important to note that the tachykinin-containing peripheral nerves are not the only source of these peptides in the female reproductive tract. SP has been reported in neuroendocrine cells present on the epithelial surface of the urogenital tract (Skrabanek and Powell, 1983) and the placenta (Graf *et al.*, 1996; Sastry *et al.*, 1981). The placenta has been reported to strongly express NKB (Page *et al.*, 2000) and more recently the gene that encodes for NKB has been demonstrated in the human uterus (Patak *et al.*, 2003). In addition the presence in the human placenta of a tachykinin gene transcript

that leads to the prediction of four unique tachykinins named endokinin (EK)A-D has just been reported (Page *et al.*, 2003). Synthesized EKA/B was reported to act as a full agonist at SP-preferring NK_1 receptors prompting the authors to propose that EKA/B is the peripheral SP-like endocrine/paracrine agonist where SP is not expressed (Page *et al.*, 2003).

In conclusion, experiments from the present study have established that:

1. tachykinins mediate myometrial activity in the oestrogen-treated mouse by activation of predominantly the NK_1 receptor, and that during pregnancy the NK_2 receptor is the predominant receptor type mediating the uterotonic effects of these peptides;
2. tachykinins elicit contractile activity in myometrium from non-pregnant and pregnant women through activation of the NK_2 receptor;
3. peptidases constrain the effects of tachykinins in the oestrogen-treated mouse and the pregnant human uterus but have little effect in the pregnant mouse and the non-pregnant human uterus;
4. sparse tachykinin-immunoreactive fibres are present in the oestrogen-treated mouse and non-pregnant human uterus but were unable to be visualized in uterine preparations from the pregnant mouse and human.

Results from this study suggest that tachykinins may participate in the regulation of myometrial contractility in both the non-pregnant and pregnant mouse and human. Furthermore these peptides have also been implicated in various other reproductive processes including regulating uterine blood flow, pre-eclampsia and cervical ripening. Taken together, this suggests that the tachykinins may play important roles in various aspects of reproductive function and that further studies should be carried out to elucidate their precise role(s), especially since tachykinin receptor antagonists are undergoing clinical trials as possible therapeutic agents.

APPENDICES

Appendix 1:

PARTICIPANT INFORMATION STATEMENT

Project No 99/46

Lay Title of Project
Regulation of the effer nerve mediators on the muscle of the uterus.

Thank you for ta' he time to read this Information Statement.
This information statement is 2 pages long. Please make sure you have all the pages.

You are invited to participate in a Research Project that is explained below.

What is the Research Project about?
The Project investigates the regulation of uterine contraction and relaxation in an attempt to understand the processes. As these processes are intimately involved with the development of the foetus and the initiation of labour we need to better understand the actions and what controls them. A better understanding of these processes may lead to better treatment of premature labour and uncoordinated labour at term.

Who are the Researchers?
Dr. Alison Lilley, Mr. Sebastian Ziccone

Why am I being asked to be in this research project?
You are asked to participate because you are pregnant and have carried to near term or you are having a hysterectomy. The tissue you will provide is ideal for our experiments.

What do I need to do to be in this research project?
Simply consent to the surgeon taking a small piece of your uterus (approximately 2cm x 1cm)

Is there likely to be a benefit to me?
There will be no direct benefit to you.

Is there likely to a benefit to other people in the future?
There will be a benefit to pregnant women in the future as more is learnt about the processes that control the uterus during pregnancy. This investigation may lead to a better treatment for premature labour and better control of the labouring uterus at delivery.

What are the possible risks?
There are no risks involved apart from those incurred from the operation you have already agreed to and which is clinically necessary. All experiments are carried out on your uterine tissue once it has been removed from your body.

What are the possible discomforts?
There is no added discomfit associated with being involved with this project.

What are the possible side effects?
There are no side effects associated with this project.

What are the likely things that could be an inconvenience?
None

What will be done to make sure the information is confidential?
All reference to you in any publication of the results will be by coded number only.

Will I be informed of the results when the research project is finished?
If you wish to be informed of the results of the project please make that wish known to the scientist who will forward to you the results in lay terms at the end of the project.

You can decide whether or not to take part in this research project.

You can decide whether or not you would like to withdraw at any time without explanation.

You may like to discuss participation in this research project with your family and with your doctor. You can ask for further information before deciding to take part.

The name and telephone number of the person to contact for more information or in an emergency.

As there are no consequences (side effects, discomfit) with participation in this project there is no need for contact numbers.

<u>For people who speak languages other than English</u>
If you would also like information about the research and the Consent Form in your language, please ask for it to be provided.

What are my rights as a Participant?

1. I am informed that no information regarding my medical history will be released. This is subject to legal requirements.

2. I am informed that the results of any tests involving me will not be published so as to reveal my identity. This is subject to legal requirements.

3. The detail of the procedure proposed has also been explained to me. This includes how long it will take, how often the procedure will be performed and whether any discomfort will result.

4. It has also been explained that my involvement in the research may not be of any benefit to me personally. I understand that the purpose of this research project is to improve the quality of medical care in the future.

5. I have been asked if I would like to have a family member or a friend with me while the project is explained to me.

6. I understand that this project follows the guidelines of the National Health and Medical Research Council Statement on Human Experimentation (1992)

7. I understand that this research project has been approved by the Royal Women's Hospital Research and Ethics Committees on behalf of the Women's and Children's Health Care Network Board.

8. I have received a copy of this document.

If you have an inquiry regarding patient rights contact

The Patient Representative **The Royal Women's Hospital** **Phone 9344 2351**

STANDARD INFORMED CONSENT FOR PARTICIPANT TO PARTICIPATE IN A RESEARCH PROJECT

Project No 99/46

Lay Title of Project
Regulation of the effects of nerve mediators on the muscle of the uterus

Principal Investigator(s) Dr Alison Lilley, Mr. Sebastian Ziccone

Brief outline of research project including benefits, possible risks, inconveniences and discomforts (12 lines maximum)
There are still a number of questions about the regulation of uterine function that are not well understood, and this project is an attempt to further understand the processes of regulation of uterine contraction.. To this end we require a very small strip of uterine muscle for our experiments. The project seeks to determine the effects of substances present in uterine nerves on the ability of the uterine muscle to contract and relax. If you consent to being part of this investigation a piece of uterus approximately 2 cm long by 1 cm wide will be obtained from the uterine tissue removed at hysterectomy. There no risks associated with obtaining this specimen of tissue.
All of the information obtained will remain anonymous and your name will not be used anywhere in any reports of this study.
The scientist or research nurse who will collect this consent form will be happy to answer any questions you may have about the procedure. We will be very grateful for your cooperation

I, _____
voluntarily consent to taking part in this research project, which has been explained to me by
Mr / Ms / Dr / Professor _____

I have received a Participant Information Statement to keep and I fully understand the purpose, extent and possible effects of my involvement. I have been asked if I would like to have a family member or friend with me while the project was explained.

I understand that if I refuse to consent, or I withdraw from the study at any time without explanation, this will not affect my access to the best available treatment and care from The Women's and Children's Healthcare Network (The Royal Women's Hospital OR The Royal Children's Hospital.)

SIGNATURE _____ Date _____

WITNESS _____ _____
(not a project investigator) Name Relationship

WITNESS SIGNATURE _____ Date _____

RESEARCHER'S SIGNATURE _____ Date _____

STANDARD INFORMED CONSENT FOR PARTICIPANT TO PARTICIPATE IN A RESEARCH PROJECT

Project No 99/46

Lay Title of Project
Regulation of the effects of nerve mediators on the muscle of the uterus

Principal Investigator(s) Dr Alison Lilley, Mr. Sebastian Ziccone

Brief outline of research project including benefits, possible risks, inconveniences and discomforts (12 lines maximum)
There are still a number of questions about the regulation of uterine contraction that are not well understood, and this project is an attempt to further understand the processes of regulation and control in labour. To this end we require a very small strip of uterine muscle for our experiments. The project seeks to determine the effects of substances present in uterine nerves on the ability of the uterine muscle to contract and relax. If you consent to being part of this investigation a piece of uterus approximately 2 cm long by 1 cm wide will be removed from the incision, which is normally made in your uterus for the delivery of your baby. The removal of such a small amount of tissue will not in any way impair the uterus's ability to function correctly in future pregnancies or affect your quality of life. All of the information obtained will remain anonymous and your name will not be used anywhere in any reports of this study.
The scientist or research nurse who will collect this consent form will be happy to answer any questions you may have about the procedure. We will be very grateful for your cooperation

I, _____
voluntarily consent to taking part in this research project, which has been explained to me by
Mr / Ms / Dr / Professor _____

I have received a Participant Information Statement to keep and I fully understand the purpose, extent and possible effects of my involvement. I have been asked if I would like to have a family member or friend with me while the project was explained.

I understand that if I refuse to consent, or I withdraw from the study at any time without explanation, this will not affect my access to the best available treatment and care from The Women's and Children's Healthcare Network (The Royal Women's Hospital OR The Royal Children's Hospital.)

SIGNATURE _____ Date _____

WITNESS _____ _____
(not a project investigator) Name Relationship

WITNESS SIGNATURE _____ Date _____

RESEARCHER'S SIGNATURE _____ Date _____

Appendix 2: Details of non-pregnant women undergoing either a total abdominal or vaginal hysterectomy who participated in the present study.

Patient	Age	Indication	Parity/ Gravida	Last pregnancy (yrs ago)	Premed	Anaesthetic details Anaesthetic	Other	Other Medication
1	39	fibroids	P_1G_2	5		G: propofol, midazolam, N_2O_2	rocuronium, metaclopramide, morphine, cephazolin	
2	57	complex ovarian cyst	P_1G_1	29		G: propofol, midazolam	atracurium, voltaren, paracetamol	coversyl
3	49	dysmenorrhoea, menorrhagia	P_1G_1	15		G: propofol, midazolam, isoflurane, N_2O_2	maxalon, fentanyl, ondansetron, rocuronium	
4	49	fibroids	P_5G_5	15		G: propofol, midazolam, isoflurane, N_2O_2	rocuronium, morphine, metoclopramide	
5	38	dysmenorrhoea, menorrhagia, fibroids	P_2G_2	17		G: propofol, midazolam, N_2O_2	rocuronium, morphine, cephazolin metaclopramide	
6	38	dysmenorrhoea, menorrhagia	P_5G_5	5		G: propofol, midazolam, isoflurane	atracurium, fentanyl, morphine, voltaren	
7	49	fibroids	P_0G_0		xanax	G: propofol, midazolam	morphine, cephazolin, atracurium, metoclopramide, paracetamol	losec, xanax, prozac

Patient	Age	Indication	Parity/ Gravida	Last pregnancy (yrs ago)	Premed	Anaesthetic details Anaesthetic	Other	Other Medication
8	57	fibroids	P_3G_3	26		G: propofol, isoflurane	morphine, cephazolin, atracurium, metoclopramide, paracetamol, voltaren	prinivil
9	55	dysmenorrhoea, menorrhagia, fibroids	P_4G_5	20		G: propofol, isoflurane	fentanyl, midazolam, atracurium, cephazolin	nifedipine, irbesarten
10	41	dysmenorrhoea, menorrhagia	P_0G_0	N/A		G: thiopentane, isoflurane	atracurium, morphine, paracetamol, diclofenac	
11	46	menorrhagia	P_2G_2	17		G: propofol, N_2O_2	atracurium, maxalon	
12	65	post-menopausal bleeding	P_4G_6	31		G: propofol, sevoflurane	atracurium, morphine	
13	41	dysmenorrhoea, menorrhagia	P_0G_2	12		G: isoflurane, midazolam, N_2O_2	morphine	
14	33	familial uterine cancer – prophylactic removal	P_3G_3	7		G: isoflurane, midazolam propofol, N_2O_2	morphine, metaclopramide	
15	44	fibroids, menorrhagia	P_3G_5	12		G: isoflurane, midazolam propofol, N_2O_2	atracurium, morphine, paracetamol, cephazolin	avapro, hydrene, ditropan
16	53	fibroids	P_3G_4	20		G: isoflurane, midazolam propofol,	droperidol, morphine, atracurium	

Patient	Age	Indication	Parity/ Gravida	Last pregnancy (yrs ago)	Premed	Anaesthetic details Anaesthetic	Other	Other Medication
17	58	ovarian cyst	P_2G_2	27		G: isoflurane	morphine, atracurium	HRT
18	49	menorrhagia, fibroids	P_2G_2	25		G: isoflurane, midazolam propofol, N_2O_2	maxalon, morphine	
19	46	fibroids	P_2G_2	24	ranitidine	G: propofol, sevoflurane, N_2O_2	morphine, droperidol	
20	49	dysmenorrhoea, menorrhagia, fibroids	P_3G_3	18		G: isoflurane, midazolam propofol, N_2O_2	atracurium, morphine	premarin
21	43	dysmenorrhoea, menorrhagia	P_2G_3	17	zantac	G: isoflurane, propofol, N_2O_2	atracurium, morphine, ephedrine, cephazolin, suxamethonium, metaclopramide	cisapride
22	42	fibroids	P_2G_2	8	clonidine, temazepam	G: midazolam propofol, sevoflurane	atracurium, cephazolin,	
23	47	dysmenorrhoea, menorrhagia, fibroids	P_1G_1	25		G: isoflurane, midazolam propofol, N_2O_2	morphine, rocuronium, cephazolin	mersyndol forte
24	42	fibroids	P_2G_2	25		G: bupivicaine, propofol, isoflurane, midazolam	fentanyl, cephazolin, rocuronium	recent blood transfusion

Patient	Age	Indication	Parity/ Gravida	Last pregnancy (yrs ago)	Premed	Anaesthetic details Anaesthetic	Other	Other Medication
25	43	menorrhagia	P_2G_2	20		G: propofol, midazolam, N_2O_2, isoflurane	atracurium, morphine, metaclopramide, cephazolin	
26	42	dysmenorrhoea, menorrhagia	P_1G_3	4		G: propofol, midazolam, isoflurane	metaclopramide, rocuronium	
27	41	fibroids	P_3G_3	10		G : propofol, N_2O_2, isoflurane, midazolam	maxalon, rocuronium, fentanyl, voltaren, cephazolin, paracetamol	primolut, iron injections
28	38	menorrhagia	P_2G_2	5		G : propofol, sevoflurane, droperidol	fentanyl, morphine	
29	43	fibroids, menorrhagia	P_2G_2	9		G : isoflurane, midazolam, propofol, N_2O_2	fentanyl, rocuronium, cephazolin, morphine, flagyl	
30	47	dysmenorrhoea, menorrhagia, fibroids	P_3G_5	7		G: propofol, midazolam, N_2O_2, isoflurane	atracurium, diclofenac, paracetamol, morphine	iron supplement, primolut
31	31	dysmenorrhoea, menorrhagia	P_2G_7	2		G: thiopentane, isoflurane	suxamethonium, rocuronium, morphine, droperidol, cephazolin	

Patient	Age	Indication	Parity/ Gravida	Last pregnancy (yrs ago)	Premed	Anaesthetic details Anaesthetic	Other	Other Medication
32	39	dysmenorrhoea	P_2G_2	13		G: propofol, N_2O_2, midazolam, sevoflurane	metaclopramide, pethidine, atracurium, cephazolin, paracetamol	
33	47	fibroids, menorrhagia	P_4G_4	22		G: propofol, midazolam, N_2O_2	morphine, atracurium, cephazolin, panadol, voltaren	
34	51	fibroids	P_2G_4	29		G: propofol, midazolam, N_2O_2	morphine, atracurium	
35	40	menorrhagia	P_3G_3	16		G – propofol, sevoflurane	fentanyl, rocuronium, atracurium, morphine, propranolol	
36	51	menorrhagia	P_5G_{10}	18	temazepam	G: ,propofol, isoflurane, midazolam, N_2O_2	atracurium, morphine, ephedrine, ketondac	zoloft, primolut, irbesarten, clonazepam
37	46	fibroids, endometriosis	P_1G_1	20	metaclopramide	G: propofol, atracurium, midazolam, sevoflurane, N_2O_2		
38	46	menorrhagia	P_2G_3	13		G: isoflurane, propofol, N_2O	fentanyl, midazolam, morphine	tegretol, dilantin, lamictal

Parity = number of live births, Gravida = number of pregnancies; i.e. P_2G_2 = two children from two pregnancies.
Anaesthetic: G – general

Appendix 3: Details of pregnant women undergoing a caesarean section who participated in the present study.

Patient	Age	Gestation (weeks)	Indication	Parity/Gravida	Premed	Anaesthetic details		Other medication
						Anaesthetic	Other	
1	31	39	2nd LUSCS	P_2G_3	ranitidine	S: bupivicaine	fentanyl	
2	36	39	3rd LUSCS	P_3G_5	ranitidine	S: bupivicaine	fentanyl, ephedrine, atropine	
3	33	38	diabetic, 2nd LUSCS	P_1G_2	ranitidine	S: marcain, fentanyl		
4	30	40	4th LUSCS	P_2G_4	ranitidine	G: midazolam, isoflurane	syntocin, suxamethonium, atracurium, morphine	
5	27	37	2nd LUSCS	P_1G_2	ranitidine	S: bupivicaine	fentanyl, atropine, ephedrine, metaclopramide, cephazolin	
6	29	39	1st LUSCS	P_1G_4	ranitidine	S: bupivicaine	fentanyl, syntocin, maxalon, ephedrine	
7	29	38	1st LUSCS	P_0G_2	ranitidine	S: bupivicaine	fentanyl, ephedrine	
8	36	38	gestational diabetic, 2nd LUSCS	P_2G_7	ranitidine	S: marcain	fentanyl, ephedrine	
9	29	38	2nd LUSCS	P_2G_4	ranitidine	S: bupivicaine	fentanyl, midazolam	
10	32	38	2nd LUSCS	P_1G_2	ranitidine	S: bupivicaine	fentanyl, ephedrine	
11	28	38	2nd LUSCS	P_1G_9	ranitidine	S: bupivicaine, marcain	fentanyl, ephedrine	aspirin (factor 5 deficiency)

Patient	Age	Gestation (weeks)	Indication	Parity/Gravida	Premed	Anaesthetic details		Other medication
						Anaesthetic	Other	
12	30	37	2nd LUSCS	P_3G_{10}	ranitidine	S: bupivicaine	fentanyl, ephedrine	
13	32	39	breech	P_0G_2	ranitidine	S: marcain	fentanyl, ephedrine, cephazolin, syntocin	naloxone
14	37	38	breech, 2nd LUSCS	P_1G_2	ranitidine	S: bupivicaine	ephedrine	cortisone nasal spray
15	28	36	placenta praevia	P_0G_1	ranitidine	G: thiopentane, isoflurane	suxamethonium, atracurium, syntocin morphine, cephazolin	Ventolin, aldomet
16	31	38	2nd LUSCS	P_2G_4	ranitidine	S: marcain,	fentanyl, ephedrine, syntocin, cephazolin	
17	33	38	2nd LUSCS	P_1G_2	ranitidine	S: marcain	fentanyl, ephedrine, syntocin, cephazolin	aldomet
18	23	38	breech	P_0G_1	ranitidine	S: bupivicaine	fentanyl, ephedrine, syntocin, cephazolin	
19	35	39	2nd LUSCS	P_4G_7	ranitidine	S: bupivicaine	fentanyl, ephedrine, atropine, syntocin	
20	30	38	2nd LUSCS	P_2G_4	ranitidine	S: bupivicaine	cephazolin fentanyl	

Patient	Age	Gestation (weeks)	Indication	Parity/Gravida	Premed	Anaesthetic details		Other medication
						Anaesthetic	Other	
21	31	39	2nd LUSCS	P_1G_2	ranitidine	S: bupivicaine	fentanyl, maxalon, syntocin	
22	29	38	2nd LUSCS	P_1G_4	ranitidine	S: marcain	fentanyl, ephedrine	
23	31	39	2nd LUSCS	P_1G_2	ranitidine	S: bupivicaine	ephedrine, syntocin	
24	34	41	pelvic injury	P_5G_7	ranitidine	G: isoflurane, thiopentane	fentanyl, cephazolin, suxamethonium, atracurium, lignocaine, morphine, syntocin	dexamphetamine, tegretol
25	37	39	2nd LUSCS	P_2G_5	ranitidine	S: marcain	fentanyl, ephedrine	
26	33	39	3rd LUSCS	P_2G_4	ranitidine	S: marcain	fentanyl, ephedrine, syntocin, cephazolin	
27	38	38	7th LUSCS	P_6G_{10}	ranitidine	S: marcain	fentanyl, ephedrine, syntocin	ventalin
28	36	39	4th LUSCS	P_3G_5	ranitidine	S/E: marcain	fentanyl, ephedrine, syntocin	
29	37	34	triplets	P_0G_1	ranitidine	S:lignocaine, adrenaline	fentanyl, ephedrine, syntocin, cephazolin	
30	24	38	2nd LUSCS	P_1G_2	ranitidine	S: marcain	fentanyl	
31	26	39	3rd LUSCS	P_2G_3	ranitidine	S: marcain	fentanyl	

Patient	Age	Gestation (weeks)	Indication	Parity/Gravida	Premed	Anaesthetic details Anaesthetic	Other	Other medication
32	36	38	3rd LUSCS high blood pressure	P_2G_3	ranitidine	S: marcain	fentanyl, ephedrine, syntocin, cephazolin	aldomet
33	38	38	4th LUSCS	P_3G_5	ranitidine	S: marcain	fentanyl, ephedrine	
34	35	37	twins	P_0G_2	ranitidine	S: marcain	fentanyl, ephedrine	
35	36	38	breech	P_1G_2	ranitidine	S: mepivacaine	fentanyl, ephedrine	
36	38	39	2nd LUSCS	P_1G_7	ranitidine	S: bupivicaine	fentanyl, ephedrine, aramine, maxalon	
37	32	39	1st LUSCS	P_2G_3	ranitidine, Na citrate	S: marcain	fentanyl, ephedrine, cephazolin	
38	33	39	baby changing positions	P_0G_1	ranitidine	S: marcain	fentanyl, ephedrine, syntocin	
39	35	39	3rd LUSCS	P_2G_4	ranitidine	S: marcain	fentanyl, ephedrine	
40	21	39	breech	P_1G_3	ranitidine	S: marcain	fentanyl, ephedrine	
41	34	38	2nd LUSCS	P_1G_2	ranitidine	S: bupivicaine	fentanyl, syntocin, ephedrine,	
42	30	38	3rd LUSCS	P_2G_3		S: bupivicaine	fentanyl, aramine, syntocin	
43	32	38	placenta praevia	P_1G_4	ranitidine	S: bupivicaine	fentanyl, syntocin, ephedrine, cephazolin	
44	33	39	2nd LUSCS	P_1G_4	ranitidine, Na citrate	S: marcain	fentanyl, syntocin	
45	35	33	twins	P_1G_3		S: marcain	fentanyl	
46	23	41	cephalopelvic disproportion	P_0G_1		S: marcain	fentanyl	
47	34	38	placenta praevia	P_0G_2	ranitidine	S: marcain	fentanyl, ephedrine	
48	30	38	gestational diabetic, 2nd LUSCS	P_1G_2	ranitidine	S: marcain	fentanyl, ephedrine, maxalon	
49	29	36	LuCTR in 2nd twin	P_0G_1	ranitidine	S: marcain	fentanyl	total colectomy - steroids
50	38	39	breech	P_3G_4	ranitidine	S: marcain	fentanyl, ephedrine	
51	35	38	3rd LUSCS	P_5G_7	ranitidine	S: bupivicaine	fentanyl, atropine, ephedrine	
52	32	39	1st LUSCS	P_1G_3	ranitidine	S: bupivicaine	fentanyl	
53	26	40	breech	P_0G_1	ranitidine	S: marcain	fentanyl, ephedrine, maxalon	
54	26	39	breech	P_0G_3		S: marcain	fentanyl, ephedrine, syntocin	
55	28	38	3rd LUSCS	P_2G_4	ranitidine	S: marcain	fentanyl, maxalon, ephedrine, aramine, atropine, oxytocin	

Parity = number of live births, Gravida = number of pregnancies; i.e. P_1G_2 = one child from two pregnancies (does not include present pregnancy)
Anaesthetic: S – spinal, E – epidural, G - general

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