Inaminanan neurumeum o recep

isoforms

Thesis submitted for the degree of Doctor of Philosoph University of Leicester

by

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The activation and regulation of mammalian neuromedin U receptors isoforms

Abstract

The neuropeptide, neuromedin U (NmU), shows considerable structural conservation across species. Within the body it is widely distributed and in mammals has been implicated in physiological roles including the regulation of feeding, anxiety, pain, blood flow and smooth muscle contraction. Human NmU-25 (hNmU-25) and other NmU analogs have been identified as ligands for two human orphan G-protein coupled receptors named hNmU-R1 and hNmU-R2. This research characterises signalling mediated by hNmU-R1 and hNmU-R2 expressed as recombinant proteins in HEK293 cells, particularly to define their G-protein coupling and the activation and regulation of signal transduction pathways. These studies show that these receptors couple to both $Ga_{q/11}$ and Ga_i . Activation of either receptor type causes a PTX-insensitive activation of both phospholipase C and mitogen activated-protein kinase and a PTX-sensitive inhibition of adenylyl cyclase with subnanomolar potency for each. Activation of phospholipase C is sustained but despite this capacity for prolonged receptor activation, repetitive application of hNmU-25 does not cause repetitive intracellular Ca^{2+} signalling. Using several strategies this was shown to be a consequence of essentially irreversible binding of NmU to its receptors and that this is followed by ligand internalization. There were no apparent differences in the activation, coupling or regulation of the two hNmU-receptors. Dual coupling and irreversible binding was explored further by using NmU receptors endogenously expressed in cultured rat colonic and fundus smooth muscle cells. Here, receptors were also shown to couple to $Ga_{q/11}$ and Ga_i and the binding of NmU was also shown to be irreversible. This study also demonstrates NmU-mediated contractions of the rat colon in organ bath studies. However, unlike single-cell experiments, repetitive application of NmU did cause repetitive contractions of the rat distal colon. The reasons for this discrepancy are unexplained, although a substantial quantity of NmU was degraded following incubation with homogenates prepared from various rat tissues and this may contribute to differences between single-cells and whole tissues responses.

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Publications

Full, peer-reviewed publications

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Brighton PJ, Szekeres PG, Wise A, Willars GB (2004) Signalling and ligand binding by recombinant neuromedin U receptors: evidence for dual coupling to $G\alpha_{q/11}$ and $G\alpha_i$ and an irreversible ligand-receptor interaction. *Mol. Pharmacol.* 66 1544-1556.

Brighton PJ, Wise A, Willars GB (2004) Neuromedin U signalling in cultured colonic smooth muscle cells. A comparison with contraction of isolated colonic tissue. – *in preparation*.

Book contributions

Tovey SC, **Brighton PJ** and Willars GB (2004) Confocal microscopy: theory and applications for cellular signalling. In: *Calcium Signalling Protocols* 2^{nd} *Edition.* (Lambert D.G. Ed) Humana Press, *in press.*

Peer reviewed abstracts, poster presentations and oral communications

Brighton PJ, Dass N, Wise A, Willars GB (2004) Neuromedin U: a comparison between Ca^{2+} signalling in cultured colonic smooth muscle cells and contraction of isolated colonic tissue. *Proceedings of the 12th International Conference of Second Messengers and Phosphoproteins, Montreal, Canada*. Poster presentation

Brighton PJ, Dass N, Wise A, Willars GB (2004) Re-challenge with neuromedin U contracts isolated rat colon but does not mediate Ca^{2+} responses in colonic smooth muscle cells. *Proceedings of BioScience2004 - from Molecules to Organisms*, *Glasgow, UK.* Poster presentation

Brighton PJ, Wise A, Willars GB (2004) Neuromedin U binding and calcium signalling in cultured rat colonic smooth muscle cells. *Proceedings of the British Pharmacological Society @ pa2online Vol 1(4) 058P*. Oral communication.

Brighton PJ, Szekeres PG, Willars GB (2003) Binding of neuromedin U to cell surface recombinant human receptors is essentially irreversible under physiological conditions. *Proceedings of the British Pharmacological Society* (a) pa2online Vol 1(3) 058P. Poster presentation. Winner of 'Best Poster' prize at the British Pharmacological Society meeting, September 2003.

Brighton PJ, Szekeres PG, Aiyar N, Willars GB (2003) Characterisation of signalling by recombinant human neuromedin U receptors *Brit. J. Pharmacol.* 140: 75P Suppl. Poster presentation.

Abbreviations

¹²⁵ Il-hNmU-25	iodinated human neuromedin U-25
$[^{3}H]$ -InsP.	total inositol phosphates:
$[Ca^{2+}]_{1}$	intracellular calcium concentration
7-TM	7-transmembrane spanning receptor:
ACTH	adenocorticotropic hormone:
ADP.	adenosine di-phosphate:
ATP.	adenosine tri-phosphate:
BSA.	bovine serum albumin;
Ca²⁺ .	calcium:
cAMP.	cvclic adenosine mono-phosphate:
CCh.	carbachol:
CIF.	Ca^{2+} -influx factor:
cGMP.	cvclic guanosine mono-phosphate;
CGRP.	calcitonin gene-related peptide:
CHO.	Chinese hamster ovary:
CNS.	central nervous system;
ConA.	concanavalin A:
CPM.	counts per minute;
CRH.	corticotrophin releasing hormone:
DAG,	diacylglycerol;
DMH.	dorsomedial hypothalamus;
DMSÓ,	dimethyl sulfoxide;
DTT,	dithiothreitol;
ECL cells,	enterochromaffin-like cells;
eGFP,	enhanced green fluorescent protein;
eGFP-PH _{PLC81} ,	enhanced green fluorescent protein-pleckstrin homology
	domain of phospholipase $C_{\delta 1}$;
ERK,	extracellular regulated kinase;
ESCC,	esophageal squamous cell carcinoma;
FALGPA,	furanoyl-acryloyl-leu-gly-pro-ala;
FCS,	fetal calf serum;
FITC,	fluorescein isothiocyanate;
FLIPR,	fluorescence imaging plate reader;
fluo-3-AM,	fluo-3-acetoxymethyl ester;
FSK,	forskolin;
GDP,	guanosine di-phosphate;
GEF,	guanine exchange factor;
GHRP-6,	growth hormone-releasing hexapeptide-6;
GPCR,	G-protein coupled receptor:
GRK.	- F
,	G-protein receptor kinase;
GTP,	G-protein receptor kinase; guanosine tri-phosphate;
GTP, GTPase,	G-protein receptor kinase; guanosine tri-phosphate; guanosine tri-phosphatase;
GTP, GTPase, HEK293 cells,	G-protein receptor kinase; guanosine tri-phosphate; guanosine tri-phosphatase; human embryonic kidney-293 cells;
GTP, GTPase, HEK293 cells, hNmU-25,	G-protein receptor kinase; guanosine tri-phosphate; guanosine tri-phosphatase; human embryonic kidney-293 cells; human neuromedin U-25;
GTP, GTPase, HEK293 cells, hNmU-25, h-NmU-R1,	G-protein receptor kinase; guanosine tri-phosphate; guanosine tri-phosphatase; human embryonic kidney-293 cells; human neuromedin U-25; human neuromedin U receptor 1;

HRP,	horseradish peroxidase;
HUVECs,	human umbilical vein endothelial cells;
IBMX,	isobutylmethylxanthine;
ICC,	immunocytochemistry;
ICV,	intracerebroventricular;
Ins(1,4,5)P ₃ ,	inositol 1,4,5 tri-phosphate;
IV,	intravenous;
JNK,	Jun N-terminal kinase;
KHB,	Krebs'-HEPES buffer;
MAP kinase,	mitogen-activated protein kinase;
MEM,	minimum essential medium;
mNmU-R1,	murine neuromedin U receptor 1;
mNmU-R2,	murine neuromedin U receptor 2;
MPO,	medial preoptic;
NCBI,	National Center for Biotechnology Information;
NEP,	neutral endopeptidase;
NGF1-A,	nerve growth factor 1-A;
NK,	natural killer;
NmU,	neuromedin U;
NSB,	non-specific binding;
PAR,	protease activated receptor;
PBS,	phosphate buffered saline;
PH,	pleckstrin homology;
PKC,	protein kinase C;
PLC,	phospholipase C;
PMSF,	pnenyl metnyl sulfonyl fluoride;
pNmU-8,	porcine neuromedin U-8;
pNmU-8-Cysb,	porcine neuromedin U-8-Cy3B;
$\mathbf{P}\mathbf{r},$	pancreatic polypeptide;
$\mathbf{r}_{101115(4,5)}\mathbf{r}_{2},$	prosphaudylinoshol 4,5 of-phosphate,
IIA, DVN	pertussis toxin,
PCS	regulators of G-protein signalling
NUS, DIA	radio_immunoassav:
rNmII_23	rat neuromedin U-23.
rNmU.R1	rat neuromedin U recentor 1
rNmU-R2	rat neuromedin U receptor 7;
RT	room temperature:
RT-PCR	reverse transcriptase – polymerase chain reaction
s.e.m.	standard error of the mean:
SCN.	suprachiasmatic nucleus:
SCPB.	small cardioactive peptide B;
SDS,	sodium dodecyl sulphate;
SDS-PAGE,	sodium dodecyl sulphate polyacrylamide gel electrophoresis;
SERCA,	sarco/endoplasmic reticulum Ca ²⁺ -ATPase;
siRNA,	small interfering RNA;
SMGS,	smooth muscle growth supplement;
SNP,	single nucleotide polymorphism;
SP,	substance P;

-

SAPK,	stress-activated protein kinase;
TCA,	trichloroacetic acid;
TTX,	tetrodotoxin;
type II GnRH-R,	type II gonadotrophin-release hormone receptor;
UTP,	uridine 5'-tri-phosphate;
VIP,	vasoactive intestinal polypeptide;
VMH,	ventromedial hypothalamus.

Overview of the discovery, structure, function and distribution of neuromedin U, the neuromedin U receptors and G-protein coupled receptors.

Section 1.1 Structure, function and distribution of NmU

1.1.1 Discovery, isolation, structure, and synthesis of NmU

In the 1980s a novel family of peptides, termed "neuromedins" were isolated from porcine spinal cord based on their ability to contract smooth muscle (Kangawa *et al.*, 1983; Minamino *et al.*, 1984a, b and c; 1985a and b). This family of peptides comprises the neuromedins B and C (bombesin-like), K (neourokinin B) and L (neurokinin A, substance K or neurokinin alpha), N (neurotensin-like) and U. These neuromedins are found in many tissues including the central nervous system and have been implicated in a wide variety of physiological functions.

Purification and characterisation of porcine (p) neuromedin U (NmU) identified two peptides with similar biological activity (Minamino *et al.*, 1985a). Both peptides contracted strips of rat uterus *in vitro* (hence the suffix 'U') and intravenous injections caused sustained increases in systemic blood pressure in rats. The two peptides were an icosapentapeptide (pNmU-25) and an octapeptide (pNmU-8) that was identical to the C-terminus of NmU-25 (Fig. 1.1). Indeed pNmU-8 results from the cleavage of NmU-25 on the C-terminal side of two arginine (Arg-Arg) residues immediately preceding the last 8 C-terminal amino acids of NmU-25 (R16 and R17), which form a typical di-basic peptide cleavage signal (Fisher and Scheller, 1988).

Following the initial identification of porcine NmUs, this peptide was purified and sequenced from rat (Conlon *et al.*, 1988; Minamino *et al.*, 1988), frog (Domin *et al.*, 1989), rabbit (Kage *et al.*, 1991), dog (O'Harte *et al.*, 1991a) and chicken (O'Harte *et al.*, 1991b; Domin *et al.*, 1992). The amino acid sequences of these are shown in Fig. 1.1. Most of the NmU analogs that have been isolated are icosapentapeptides with the exceptions of a 23 amino acid NmU in rat (NmU-23) and nonapeptides (NmU-9) from guinea-pig (Murphy *et al.*, 1990) and chicken (O'Harte *et al.*, 1991b). Chicken NmU-9 was the predominant molecular form isolated from the chicken intestines and was indeed the only form isolated from extracts of whole brain (O'Harte *et al.*, 1991b). In addition, as with porcine NmU, an NmU-8 analog has also been identified in dog (O'Harte *et al.*, 1991a) and is identical to the C-terminus of dog NmU-25. Again, the C-terminal NmU-8 is preceded by an Arg-Arg motif (Fig. 1.1), the cleavage of which gives rise to the smaller NmU analog. The source of the nonapeptides from guinea-pig and chicken is less clear. Larger NmU-analogs have not been reported in guinea-pig, suggesting that NmU-9 may arise independently. Similarly, avian NmU-25 lacks the di-basic Arg-Arg cleavage site, suggesting that avian NmU-9 may be produced independently of avian NmU-25 although the possibility of cleavage at a monobasic site cannot be excluded.

The human cDNA encoding the precursor protein of NmU has been sequenced (Austin *et al.*, 1995) revealing that human NmU is also an icosapentapeptide and has no internal dibasic cleavage site for the generation of shorter versions. Comparison with the rat cDNA encoding the precursor protein (Lo *et al.*, 1992) demonstrates approximately 70% homology between the two and indicates that in both instances NmU is synthesized as a 174-amino acid precursor containing the NmU peptide within the C-terminus. In addition to the signal peptide, these NmU precursors have several characteristics associated with the precursors of most other small regulatory peptides. In particular there are a number of paired dibasic amino acids forming putative proteolytic cleavage sites (5 in rat, 4 in human) that, in addition to releasing NmU, may release a series of other peptides. Two of these sites surround a 33 amino acid sequence that differs by only 2 amino acids between rat and human. This high level of conservation suggests a biological role for this peptide although this has not been confirmed.

There is remarkable amino acid homology between the forms of NmU isolated from different species (Fig. 1.1). For example, 15 out of 25 amino acids are conserved between porcine NmU-25 and amphibian NmU-25. Human, rat, rabbit, avian and amphibian NmU lack the di-basic Arg-Arg cleavage sequence in positions 16 and 17, accounting for the absence of shorter analogs in these species (see above). In particular, the C-terminus of NmU displays remarkable homology between species. Thus, human NmU-25, porcine NmU-8 and NmU-25, rabbit NmU-25 and guinea-pig NmU-9 have identical C-terminal octapeptides, whilst dog NmU-8 and NmU-25, rat NmU-23, frog NmU-25, avian NmU-25 and avian NmU-9 have a single amino acid difference. Furthermore, across all species, the C-terminal pentapeptide (Phe-Arg-Pro-Arg-Asn-NH₂) is identical, whilst in mammalian species the Cterminal heptapeptide (Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH₂) is entirely conserved. This high degree of amino acid conservation is not only indicative of the importance of the C-terminus (see Section 1.1.2), but considering that the evolutionary paths of these animals diverged millions of years ago, it also shows strong evolutionary pressure to preserve NmU. The evolution of neuropeptides has been reviewed elsewhere (Holmgren and Jensen, 2001).

Intriguingly, a novel NmU-analog has been isolated and characterised from the defensive skin secretions of the Australian Tree Frog, *Litoria caerulea* (Salmon *et al.*, 2000). This 23 amino acid peptide shows smooth muscle contractile properties comparable to that of pNmU-25 and binds to uterine membrane preparations with an affinity equivalent to that of rat NmU-23. The function of NmU in such skin secretions is unclear but could provide a defensive mechanism by triggering peripheral or neuro-central effects in predators. Alternatively it could act on the frog itself to elicit hypertensive effects following injury, thus reducing blood loss (Salmon *et al.*, 2000). Such skin secretions have long been known as rich sources of biologically active peptides but our understanding of their biological roles is in its infancy.

6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 Position 1 2 3 4 5 Phe- Arg- Val- Asp- Glu- Glu- Phe- Gln- Ser- Pro- Phe- Ala- Ser- Gln- Ser- Arg- Gly- Tyr- Phe- Leu- Phe- Arg- Pro- Arg Human NmU-25 Porcine NmU-25 Phe- Lys- Val- Asp- Glu- Glu- Phe- Gln- Gly- Pro- Ile- Val- Ser- Gln- Asn-Arg- Arg- Tyr- Phe- Leu- Phe- Arg- Pro- Arg Porcine NmU-8 Tyr- Phe- Leu- Phe- Arg- Pro- Arg Rabbit NmU-25 Phe- Pro- Val- Asp- Glu- Glu- Be- Gln- Ser- Pro- Phe- Gly- Ser- Arg- Ser- Arg- Gly- Tyr- Phe- Leu- Phe- Arg- Pro- Arg Dog NmU-25 Phe- Arg- Leu- Asp- Glu- Glu- Bhe- Gly- Pro- Ile- Ala- Ser- Gln- Val- Arg- Arg- Gln- Phe- Leu- Phe- Arg- Pro- Arg Dog NmU-8 pGlu-Phe- Leu- Phe- Arg- Pro- Arg Amphibian NmU-25 Leu- Lys- Pro- Asp- Glu- Glu- Leu- Gln- Gly- Pro- Gly- Val- Leu- Ser- Ang- Gly- Tyr- Phe- Val- Phe- Arg- Pro- Arg Gly- Tyr- Phe- Leu- Phe- Arg- Pro- Arg Guinea-pig NmU-9 Avian NmU-25 Tyr- Lys- Val- Asp- Glu- Asp- Leu- Gln- Gly- Ala- Gly- Gly- Lle- Gln- Ser- Arg- Gly- Tyr- Phe- Phe- Phe- Arg- Pro- Arg Gly- Tyr- Phe- Phe- Phe- Arg- Pro- Arg Avian NmU-9 Position 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 Val- Ala- Pro- Ser- Gly- Gly- Phe- Phe- Leu- Phe- Arg- Pro- Arg Rat NmU-23 Tyr- Lys- Val- Asn-Glu- * Tyr- Gln- Gly- Pro- * Position 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 1 2 3 Mouse NmU-23 Phe-Lvs-Ala- * Glu- * Tyr- Gln- Ser- Pro- Ser- Val- Gly- Gln- Ser- Lys- Gly- Tyr- Phe-Leu- Phe Arg- Pro- Arg 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 Position Ser- Asp- Glu- Glu- Val- Gin- Val- Pro- Gly- Gly- Val- Ile- Ser- Asn- Gly- Tyr- Phe- Leu- Phe- Arg- Pro- Arg Tree frog skin secretion

Fig. 1.1. The structure of NmU isolated from different species. Sequences were obtaine isolated peptides (see Section 1.1) with the exception of human NmU-25 (from NmU precursor 1 see Section 1.1) and mouse NmU-23 (from National Centre for Biotechnology Information (peptide accession number Q9QXH8). Sequences were aligned with Genedoc sequence alignmen (reviewed; Nicholas *et al.*, 1997). The highly conserved C-terminus is shown in bold and the c cleavage recognition site, Arg-Arg, in positions 16 and 17, is shown underlined where present. T more clearly the structural similarities, rat NmU-23, mouse NmU-23 and tree frog skin secretion 23 are elongated with gaps represented by an *asterix* (*). Residues are numbered above the ami sequence. All versions of NmU have an amidated Asn at the C-terminus resulting in -CONH₂ 1 been abbreviated to -NH₂. pGlu, pyroglutamic acid.

NmU appears to be unique in both structure and function and it is difficult to relate these aspects to other peptides with any confidence. However, the C-terminal amidation is a structural feature associated with many peptides that exhibit hormonal or physiological activities and is found in both gastrointestinal hormones and hypothalamic releasing factors (Tatemoto, 1978). Conversely, an amidation linked to asparagine, as observed with NmU, has to date only been identified in vasoactive intestinal polypeptide (VIP). The amino acid sequence of VIP, 28 residues in length (Mutt and Said, 1974; Bodanszky et al., 1973), shows weak but evident structural homology with NmU. Structurally, NmU-25 also shares limited homology with the 36 amino acid peptide, human pancreatic polypeptide (PP) (Boel et al., 1984), with the C-terminal sequence of -Arg-Pro-Arg-X-CONH₂ existing in both. The amino acid sequences of human NmU-25, VIP and PP have been compared with Aplysia small cardioactive peptide B (SCPB), human ghrelin and human neurotensin (NT) (Fujii et al., 2000) and are also detailed in Fig. 1.2. All show low but evident homology. Despite these structural similarities, with the exception of Aplysia SCPB, which shows weak but evident agonism (Fujii et al., 2000), only human NmU-25, and related NmU analogs show any agonist effect at the NmU-receptors (see Section 1.2.4 for details of receptors for NmU).

Fig. 1.2. Comparative amino acid alignment of human neurotensin (NT), vasoactive intestinal po (VIP), *Aplysia* small cardioactive peptide B (SCPB), ghrelin and pancreatic polypeptide (PP), wit NmU-25. Amino acid sequences were obtained through the NCBI protein database with the accession numbers: human NmU-25; NP_006672, VIP; NP-003372, PP; NP_002713; *Aplysia* SCPB; NT; NP_006174; and ghrelin; BAA89371. Residues identical to human NmU-25 are shown in bc denotes spaces to maximize alignment. pGlu, pyroglutamic acid. Similar comparisons have be previously (Fujii *et al.*, 2000).

1.1.2 Structure-activity relationship of NmU

The consistency of NmU structure across species demonstrates a tight structural requirement for function. Further, the very highly conserved C-terminal region of the NmU variants implies much of the activity of the peptide is located here and the absolute conservation of the last 5 amino acids suggests that this is essential for function. Within pNmU-8, replacement of either Phe², Phe⁴, Arg⁵, Pro⁶, Arg⁷ or Asn⁸ with Gly or replacement of either Phe², Phe⁴, Arg⁵, Pro⁶ or Asn⁸ with D-amino acids significantly reduce the ability of NmU to contract chicken crop smooth muscle (Hashimoto *et al.*, 1991).

Studies examining the ability of modified versions of dog NmU-8 to cause contraction of isolated chicken crop smooth muscle have also probed the role of this highly conserved region of the NmU peptides. Thus, the Phe residues at positions 2 and 4 (which are conserved across all NmU peptides) are important for the ability of dog NmU-8 to contract chicken crop smooth muscle (Kurosawa et al., 1996). In particular, the aromatic side chain of Phe at position 2 contributes significantly to activity (Kurosawa et al., 1996) whilst the Arg residue at position 7 (also conserved across all NmU peptides) is indispensable for receptor binding and activation (Sakura et al., 2000). These studies also demonstrate that the carboxylic acid group situated at the NH₂-terminus of dog NmU-8 is a major contributory factor to bioactivity (Hashimoto et al., 1995). Interestingly, dog NmU-8 is considerably more potent than pNmU-8 (Sakura et al., 1995) leading to the suggestion that the pyroglutamic acid residue (pGlu) at position 1 of dog NmU-8 provides resistance to degradation by aminopeptidases. Indeed, modification of the N-terminal of pNmU-8 to give aminopeptidase resistance increased the contractile activity on chicken crop smooth muscle (Sakura et al., 1995).

Despite the importance of the C-terminal, the NH₂ terminal of the longer NmU peptides greatly influences function, particularly in respect of potency. Indeed pNmU-25 is approximately three to five times more potent than pNmU-8 in mediating hypertensive effects in the rat and causing contraction of either rat uterine smooth muscle or chicken crop smooth muscle *in vitro* (Minamino *et al.*, 1985a) (Okimura *et al.*, 1992). Similarly in the rat, intravenous pNmU-25 is a more potent mediator of mesenteric vasoconstriction (see Section 1.3.2.) than pNmU-8 (Gardiner *et al.*, 1990). Furthermore, pNmU-25 causes repeat twitching of isolated uterus and

increases the sensitivity to NmU, whereas pNmU-8 does neither, leading to the suggestion that the NH₂-termini of the longer NmU forms may be involved in strengthening and prolonging stimulant activity (Minamino *et al.*, 1985a).

Using rat NmU-23 and a series of peptides derived from its fragmentation, amino acids 16-23 (the C-terminal octapeptide) were shown to be vital in mediating the contraction of chicken crop smooth muscle and rat uterus, whilst increasing the length of the N-terminus was associated with an increase in activity. In particular, the sequences Tyr-Gln-Gly-Pro corresponding to positions 6-9 and Ser-Gly-Gly corresponding to positions 13-15 of rat NmU-23 were shown to be important for high potency (Sakura et al., 1991). These sequences may act to fold the protein, holding it in a conformation that is more favorable for activity. Similarly, fragmentation of the NH₂-terminal regions of NmU-25 from rabbit, pig and frog demonstrates that the Cterminal eleven-peptide amides retain contractile activity on chicken crop smooth muscle that is at least three times more potent than their C-terminal eight-peptide amide equivalents (Okimura et al., 1992). This suggests a functional role for the three amino acids directly extending from the C-terminal octapeptide, despite considerable heterogeneity between species. However, all are hydrophilic and immediately precede a four amino acid sequence rich in hydrophobic residues, namely Tyr(or Phe)-Phe-Leu(or Val)-Phe. This, in turn precedes a hydrophilic and basic C-terminal Arg-Pro-Arg-Asn-NH₂ sequence in all species, suggesting that an amphiphilic structure could be involved in the enhancement of bioactivity.

Post-translational amide modification of the C-terminal amino acid is common amongst neuropeptides and is essential to their bioactivity (Escher *et al.*, 1982; Bradbury and Smyth, 1987; Eipper and Mains, 1988). Such enzymatic amidation may prevent the carboxy terminus of peptides becoming ionised, therefore making them more hydrophobic and better able to bind to their receptors. Although peptides terminating in amidated versions of all 20 amino acids have been identified, amidated amino acids are generally neutral. Amidation of the neutral amino acid, Asn, in NmU is crucial for activity as synthetic des-amido-NmU-8, which lacks the asparagine-amide structure at the C-terminus, is unable to cause uterine contraction or hypertension (Minamino *et al.*, 1985a). Furthermore, non-amidated NmU-8 does not cause intracellular Ca²⁺ signalling by the recombinant human NmU receptor, GPR66/FM-3 (human (h) NmU receptor 1 (hNmU-R1)) (Hedrick *et al.*, 2000) or

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recombinant murine (m) versions of the two NmU receptors, mNmU-R1 and mNmU-R2 (Funes *et al.*, 2002) (see Section 1.2.4 for details of NmU receptors).

Intracerebroventricular administration of NmU reduces food intake in rats (see Section 1.3.4) and recent studies have demonstrated that the aromatic rings of Phe residues at positions 16, 17 and 19 of rat NmU-23 reduce the effect of NmU on food intake whilst those on Tyr residues at positions 1 and 5 are essential for activity (Abiko and Takamura, 2002 and 2003).

In a structure-activity relationship study based on receptor activation, the individual substitution of each of the 8 amino acids of NmU-8 with alanine resulted in marked decreases in agonist potency on Ca^{2+} signalling by either recombinant mNmU-R1 or mNmU-R2 expressed in HEK293 cells, thereby demonstrating the contribution of the individual amino acids to bioactivity (Funes *et al.*, 2002). Indeed substitution of Arg⁷ resulted in complete loss of activity. Moreover, although all the Ala substituted peptides retained full agonist properties at mNmU-R2, substitution at Phe² Phe⁴ and Arg⁵ resulted in the generation of partial agonists at mNmU-R1 (Funes *et al.*, 2002). The selective effects of substitutions on agonist potencies at the two different receptor sub-types was most apparent with replacement of Arg⁵ which greatly reduced both potency and efficacy at mNmU-R1 compared to mNmU-R2 leading the authors to suggest that this may provide a basis for the development of receptor-specific agonists.

Potency differences between naturally occurring versions of NmU have not, however, been a consistent finding in functional assays with isolated tissues (Benito-Orfila *et al.*, 1991; Westfall *et al.*, 2001) or in studies using the two recently cloned G-protein-coupled receptors (GPCRs) for NmU (see Section 1.2.4). For example, pNmU-8 and pNmU-25 are approximately equipotent at stimulating recombinant versions of these receptors to either elevate intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) (Hedrick *et al.*, 2000; Shan *et al.*, 2000; Szekeres *et al.*, 2000; Funes *et al.*, 2002) or mediate the release of arachidonic acid (Fujii *et al.*, 2000; Hosoya *et al.*, 2000). Alternatively, pNmU-8 has been reported to be 2.5-5 fold more potent than pNmU-25 in Ca²⁺-signalling assays using recombinant receptors (Raddatz *et al.*, 2000). Although, when using rat uterine membranes, rat NmU was shown to have a 60-fold higher affinity than pNmU-8 (Nandha *et al.*, 1993), direct comparison of the affinities of pNmU-8 and -25 using the cloned receptors have not always revealed such

differences. Thus, based on their ability to compete with radiolabelled NmU, the binding affinities of pNmU-8 and -25 are either remarkably similar (Hosoya et al., 2000; Raddatz et al., 2000) or at best NmU-25 has three-fold higher affinity than NmU-8 (Fujii et al., 2000) at the cloned human receptors. In contrast, pNmU-25 has been reported to have a 85-fold higher affinity than pNmU-8 at the cloned canine NmU-R2 expressed in HEK293 cells (Westfall et al., 2001). Thus, the contribution of any differences in affinity to potency differences in functional assays is unclear. The possibility that altered peptide stability may contribute, particularly in tissues or intact organisms, should at least be considered. For example, the longer versions of NmU could be more resistant to proteolytic cleavage of the conserved biologically active C-terminal region. Rat uterine membranes are able to degrade [¹²⁵I]-rat NmU-23 in a temperature-dependent manner (Nandha et al., 1993), demonstrating the susceptibility of such peptides to proteolytic cleavage. Although the issue of ligand degradation has been considered during in vitro and in vivo studies (Westfall et al., 2001; Cao et al., 2003; Yu et al., 2003) the impact of degradation on agonist potency and potential differences between the various forms of NmU has not been fully addressed.

1.1.3 Regional, cellular, and sub-cellular distribution of NmU

Our understanding of the distribution of NmU owes much to the application of chromatographic and immunological techniques, particularly radio-immunoassay (RIA) and immunocytochemistry (ICC) using antibodies, most commonly raised against synthetic pNmU-8, to detect NmU-like immunoreactivity (NmU-LIR). More recently detection of mRNA for NmU or its precursor molecule have also been employed. Such studies have enabled localization of NmU to areas, cell types and sub-cellular structures, thereby providing detailed evidence of expression patterns. This knowledge has provided considerable support to the current ideas about the physiological roles of NmU (see Section 1.3).

NmU-LIR is widely distributed throughout the body. It must be noted that there may well be differences between species and developmental stages but these remain to be thoroughly and systematically explored. In the rat, highest levels are in the anterior pituitary and the gastrointestinal tract (Domin *et al.*, 1987; Steel *et al.*, 1988) whilst significant levels are also found in the brain, spinal cord and both the male and

female genito-urinary tract (Domin *et al.*, 1986). These expression patterns of NmU-LIR have been supported by dot blot analyses or quantitative RT-PCR of mRNA for NmU or its precursor in both rat and human tissues (Fujii *et al.*, 2000; Szekeres *et al.*, 2000).

The distribution of hNmU-25 within the main peripheral (Table 1.5a) and central (Table 1.5b) human tissues is summarized with the distribution of hNmU-R1 and hNmU-R2 in Table 1.5 (see Section 1.2.5).

Circulating NmU-LIR has not been detected (Domin *et al.*, 1987) and this along with its absence from endocrine cells, for example in the stomach (Augood *et al.*, 1988) and small intestine (Domin *et al.*, 1987), suggest that NmU acts more as a neuropeptide or neuromodulator than as a circulating hormone. However, it should be noted that a small number of NmU-LIR-positive endocrine cells in crypts of guinea-pig small intestine have been reported (Furness *et al.*, 1989), and NmU expression has been localised to corticotrophs (all be it without receptor expression (see Section 1.2.5). There is a lack of evidence suggesting an endocrine role for NmU depite its presence in these regions.

1.1.3.1 Central nervous system

RIA of tissue extracts indicates high levels of NmU-LIR in both the spinal cord and brain of rats but with considerable regional differences (Domin *et al.*, 1987). In the spinal cord, levels are greater in the dorsal horn than the ventral horn. This and the high concentrations in the dorsal root ganglia are consistent with a sensory role (Domin *et al.*, 1987), although this has yet to be established.

In the brain, the highest levels of NmU-LIR are within the nucleus accumbens, hypothalamus, septum, amygdala, medulla oblongata and globus pallidus (Domin *et al.*, 1987). Another detailed ICC study demonstrated that within rat brain, distribution of NmU-LIR-positive cell bodies is uneven and largely restricted to a variety of regions associated with distinct functional systems including somatosensory, motor and auditory (Honzawa *et al.*, 1987). Thus, immunoreactivity was present in cranial motor nuclei, reticular nuclei, nucleus vestibularis lateralis, trigeminal nuclei, colliculus superior and inferior, lemniscus lateralis, nucleus pontis, nucleus ruber, zona incerta, substantia innominata, horizontal limb of the diagonal band and the cerebral cortex (Honzawa *et al.*, 1987). Furthermore, nerve fibres

positive for NmU-LIR were observed in other regions including the nucleus nucleus ventralis posteromedialis, reticularis thalami, nucleus ventralis posterolateralis, nucleus tegmentalis dorsalis and ventralis, vertical limb of the diagonal band, nucleus olivaris superior and nucleus pontis. NmU-LIR has also been demonstrated in rat, mouse and human pituitary, particularly the anterior pituitary where it is co-localised in corticotrophs with adrenocorticotropic hormone (ACTH) (see Section 1.3.3) (Ballesta et al., 1988; Domin et al., 1988; Steel et al., 1988). The selective distribution of NmU throughout the human CNS has been supported by dot blot analysis of cDNA libraries (Hedrick et al., 2000), in situ hybridization (Howard et al., 2000) and quantitative RT-PCR of the NmU precursor (Szekeres et al., 2000). This latter study indicates high levels of NmU precursor message occur in the hypothalamus, medulla oblongata, nucleus accumbens, parahippocampal gyrus, substantia nigra, superior frontal gyrus, and thalamus with greatest levels in the cingulate gyrus, locus coruleus, medial frontal gyrus and pituitary (Szekeres et al., 2000). Quantitative RT-PCR has also shown regional expression of NmU mRNA in rat CNS, with by far the highest levels seen in the pituitary gland (Fujii et al., 2000). Similar methodology examining the distribution of NmU precursor mRNA in the mouse CNS demonstrated highest levels in the medulla and spinal cord with lower levels in the hypothalamus and pons (Funes et al., 2002). Using in situ hybridization, the mRNA for NmU has also been shown to be most abundant in the ventromedial hypothalamic (VMH) regions (lateral arcuate nucleus and median eminence) and in the caudal brainstem (nucleus of solitary tract, area postrema, dorsal motor nucleus of the vagus and inferior olive) of the rat (Howard et al., 2000). The presence of NmU mRNA in the arcuate nucleus and median eminence of the rat has been questioned with the authors arguing that expression is localized in the pars tuberalis (Ivanov et al., 2002). Indeed it has been shown that NmU mRNA is abundantly expressed in rat pars tuberalis (Graham et al., 2003), a region that surrounds the hypophyseal stalk, extending along the ventral surface of the median eminence and which may have a photoperiodic function modulating seasonal changes in pituitary activity (prolactin secretion) in some species although its function in non-seasonal animals such as the rat is unknown (Morgan and Williams, A recent study examining the detailed anatomical localization of NmU 1996). mRNA in both the rat and mouse hypothalamus has revealed species differences

(Graham *et al.*, 2003). Thus, NmU is abundantly expressed in the dorsomedial hypothalamus (DMH) and VMH, arcuate nucleus and suprachiasmatic nucleus (SCN) of the mouse but expression is weaker in the rat. In contrast, expression within the pars tuberalis is more pronounced in the rat. These data suggest that NmU has both common and distinct functions in the two species. Given the lack of detailed information about NmU expression in humans, it is currently difficult to identify the most suitable animal model of NmU function in humans (Graham *et al.*, 2003).

1.1.3.2 Peripheral distribution

RIA has shown NmU-LIR to be present throughout the gastrointestinal tract of the rat, being particularly high in the small intestine, cecum, colon and rectum with considerably lower levels in the esophagus and stomach (Domin et al., 1987). NmU-LIR is not detectable in the liver or pancreas (Domin et al., 1987). Northern blot analysis of mRNA for the NmU precursor has also demonstrated that in the gastrointestinal tract, highest levels are in the duodenum and jejunum (Austin et al., 1994). Detailed ICC studies have demonstrated that in the gut, NmU-LIR is confined to nerve cell bodies in the submucosal and myenteric plexuses and in fibres in these regions and the mucosa of all areas except the stomach (Augood *et al.*, 1988; Ballesta et al., 1988; Bishop et al., 1988; Honzawa et al., 1990). Ultra structural studies using immunogold labeling demonstrated that NmU-LIR within the cell bodies is localised to large (60-70nm), electron-dense granules characteristic of those found in nerves containing peptides (Ballesta et al., 1988). Many NmU-LIR-positive fibres are present close to the mucosal epithelium whereas the muscularis mucosa has a small number in the small intestine but not the large intestine (Ballesta et al., 1988). The intrinsic nature of the nerves containing NmU-LIR is supported by the observation that extrinsic de-nervation does not affect the number or distribution of NmU-LIR-positive fibres (Ballesta et al., 1988; Bishop et al., 1988). Similarly in the gastrointestinal tract of the guinea-pig, NmU-LIR is within nerve cells of the myenteric and submucous plexuses (Furness et al., 1989). It is also present in nerve fibres of these plexuses (Augood et al., 1988; Furness et al., 1989), around submucous arterioles and in the mucosa (Furness et al., 1989). A study of the distribution of nerve cell bodies containing NmU-LIR in the submucosal nerve

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plexus of the pig demonstrated considerably higher levels in the internal submucosal plexus (Meissner) compared to the external submucosal plexus (Schabadasch), consistent with the projection of the nerve fibres from the submucosal plexus predominantly to the muscularis mucosa and lamina propria of the outer mucosal layer (Timmermans *et al.*, 1989; Timmermans *et al.*, 1990). These studies in the pig also demonstrated that NmU-LIR could coexist with neurons positive for calcitonin gene-related peptide (CGRP) or substance P immunoreactivity. Similarly, in rat small intestine NmU-LIR has been shown to co-exist with CGRP (Ballesta *et al.*, 1988; Bishop *et al.*, 1988) whilst in the submucosal ganglia of guinea-pig small intestine, all cells that were immunoreactive for either VIP, neuropeptide Y or substance P also contained NmU-LIR (Furness *et al.*, 1989). These peptides define specific populations of neurons within the submucosal plexus and this therefore places NmU-LIR in non-cholinergic secretomotor neurons, cholinergic secretomotor neurons and sensory neurons (Furness *et al.*, 1989). This suggests multiple roles for NmU in the gut.

Although levels are not as high as within the gastrointestinal tract, RIA reveals significant amounts of NmU-LIR in most of the genito-urinary tract of the rat, with highest levels in the ureter, vas deferens, prostate, fallopian tubes and urethra, although it is not present in seminal vesicles (Domin *et al.*, 1987). Quantitative RT-PCR of NmU mRNA also supports expression in the testis and ovary of rat (Fujii *et al.*, 2000). NmU mRNA (Fujii *et al.*, 2000) and NmU-LIR (Domin *et al.*, 1990) also exist in the rat thyroid gland where immunoreactivity is localised to a minor population of parafollicular C-cells.

Quantitative RT-PCR of the NmU precursor has also shown levels to be high in human stomach, intestine and bone marrow with some expression in a variety of other tissues including spleen, lymphocytes, adipose tissue, prostate and placenta (Szekeres *et al.*, 2000). Similar studies have also determined distinct patterns of NmU expression amongst different lymphoid cells (Hedrick *et al.*, 2000). There appears to be some developmental regulation of NmU expression given that adult liver and lung express little or no NmU (or precursor) mRNA whereas levels in fetal tissues are high (Hedrick *et al.*, 2000; Szekeres *et al.*, 2000).

Section 1.2 The structure, function, activation and regulation of GPCRs and the NmU-receptors

1.2.1 Structure and function of GPCRs

A cell's ability to sense and respond to diverse extracellular signals and stimuli is fundamental to its continued ability to grow, develop and function. The ability of cells to recognise extracellular signals such as hormones, growth factors, ions, and neurotransmitters and then transduce this stimulus into intracellular responses is most often due to the exsistence of transmembrane receptors. Transmembrane receptors are integral membrane proteins. They mainly reside and operate within the cell's plasma membrane, but may also function and exist within membranes of some subcellular compartments and organelles. The receptors permit the cell to respond specifically to certain extracellular molecules and compounds by only recognising, binding and responding to stimuli intended for that specific receptor. In this respect they can be likened to a 'lock and key', and its this specificity that enables the cell to disect out and react to the enormous amounts of extracellular stimuli efficiently and effectively. Following binding to one or more signalling molecules extracellularly, transmembrane receptors transduce this signal to initiate a response intracellularly, thus playing a unique and important role in communication and signal transduction.

The significance of transmembrane receptors is reflected in the vast and diverse existance of many super-families, families and sub-families of receptors which have been retained through millions of years of evolution. One such example is the <u>G</u>protein coupled receptor (GPCR) or 7-transmembrane spanning receptor (7-TM) super-family. Not only is this the largest family of transmembrane spanning receptors but is also one of the largest protein families in the human genome. Over 720 of these membrane-associated receptors were identified from the human genome (Wise *et al.*, 2004) and can be phylogenetically, functionally and structurally classified into over 100 subfamilies. Although sequence homology between family members is often considerable, there is a huge range of diversity in terms of sequence, function, regulation and ligand interactions. GPCRs are involved in a huge range of physiological processes including sensory physiology (detection of light, gustatory and odorant signals), hormonal physiology and neuronal physiology. They are implicated in a vast array of pathological conditions and are a major therapeutic target.

Our appreciation of the structure, and indeed the function, of GPCRs (see review; Morris and Malbon, 1999; Piece et al., 2002) owes much to the cloning and primary sequence analysis of the β_2 -adrenoceptor (Dixon *et al.*, 1986). Before this, pharmacological analysis, radioligand binding and protein purification had presented a more ambiguous understanding. Studies on the β_2 -adrenoceptor revealed seven hydrophobic regions (hence the name 'seven transmembrane receptors'), providing a model from which our understanding of the structure of all GPCRs developed. These seven regions, each 22-28 amino acids in length, are rich in hydrophobic residues and span the lipid bi-layer of the membrane. An NH2-terminal domain and three hydrophilic loops intervening the membrane spanning regions exist extracellularly and display residues that can potentially be glycosylated. The COOH-terminal domain and a further three hydrophobic loops exist as intracellular regions. The COOH terminus and the intracellular regions between the transmembrane spanning domains exhibit potential sites for phosphorylation by protein kinases, which can act to regulate receptor-mediated signalling (see Section 1.2.3). This region also contains potential sites for protein-protein interactions which are involved in the activation of signalling pathways and receptor regulation. The size of both the extracellular and intracellular loops, the NH₂ terminus and the COOH terminus vary considerably between receptors.

Classically, the most popular method of classifying GPCRs is based on a sixfamily system, termed A through F (Kolakwski., 1994). This system is based on homology and structural features and contains three major families. Here, the largest family is family A (alternate names include family I/class I and rhodopsinlike/related) and includes all the biogenic amine receptors. Typically, family I GPCRs contain short amino-terminal extracellular domains. Family B (or class II) receptors consist of the glucagon, parathyroid hormone, and calcitonin-related receptors. These typically have longer extracellular domains and are activated by large peptides. The final major group consists of the metabotropic glutamate receptors and is termed family C (class III) receptors. These possess large extracellular segments that fold into ligand-binding domains.

1.2.2 Activation of GPCRs

The tertiary structure of GPCRs is commonly organised so the seven transmembrane domains arrange and position residues to permit only specific binding of ligand(s). Binding sites may exist as a small pocket deep within a cleft formed by the receptor for smaller ligands (e.g. amines) or more superficially for larger ligands such as peptides and glycoproteins (e.g. luteinizing hormone (LH)), the receptors for which often posses a larger NH₂ terminus. There is often a positive correlation between the ligand size and the size of the NH₂ terminus domain (Ji *et al.*, 1998). The 25-amino acid NmU peptide is large in comparison to most ligands, and this maybe reflected in both NmU receptors (see Section 1.2.4), where the NH₂ terminus is between 50-60 amino acids in length. Thus, despite no data that map the binding site for NmU, the assumption is that it binds superficially within the NH₂ domain of the NmU-receptors.

The term GPCR is derived from the ability of these receptors to activate heterotrimeric GTP (guanosine tri-phosphate) binding proteins (G-proteins) that are members of a highly conserved super-family of GTPases. These G-proteins consist of an α -subunit and tightly associated $\beta\gamma$ -subunits (the classification of subunits and their effectors is detailed below). Further to ligand binding, (or proteolytic cleavage in the case of protease-activated receptors (PARs)), all GPCRs must activate Gproteins of one or more families. Although our understanding of this propagation is still growing, its known to occur via an agonist-mediated conformational change caused by the disruption of ionic interactions between membrane spanning domains and via protein-protein interactions (Bourne, 1997; Ballesteros et al., 2001; Janetopoulos et al., 2001). Several G-protein contact sites have been documented within the cytosolic domains of the β -adrenoceptors by means of proteolytic cleavage products (e.g., Wong et al., 1994) and by site-directed mutagenesis (e.g., Strader et al., 1994). The cytosolic loop between transmembrane 5 and 6 (the third intracellular loop), for example, is known to be critical in the coupling of receptors to G-proteins, and individual residues have been identified that can alter G-protein specificity. Such work has provided important information about the nature of the G-protein-receptor contact sites that transduce agonist-mediated activation of the receptor to the activation of the G-proteins. No work has been done to characterise the regions within the NmU receptors (see Section 1.2.4) which couple to G-proteins

and only assumptions can be made by extrapolating results from studies on other receptors.

1.2.2.1 GPCR Signalling through G-proteins

Activation of G-proteins results in a transient complex consisting of the agonist, the receptor and the G-protein. This complex acts as a guanine-nucleotide exchange factor (GEF) and permits the release of GDP bound to a guanine nucleotide-binding site within the G-protein α -subunit. The loss of GDP precedes the binding of GTP, and it is this GDP-GTP exchange that activates the G-protein. Once activated, the heterotrimeric G-protein splits into its α -subunit and a $\beta\gamma$ -dimer (reviewed; Neer, 1995). Both can selectively regulate effectors (see below) in a synergistic, independent or antagonistic fashion (Neer and Clapham, 1988). The activation or regulation of effectors can last for several seconds and is terminated only when the terminal phosphate of GTP is hydrolysed to form GDP. This hydrolysis is mediated by the intrinsic GTPase activity of G α subunit. The inactive GDP-bound G-protein will reform with its $\beta\gamma$ -dimer to form an inactive heterotrimeric G-protein, ready for further activation. A simplified schematic representation of the G-protein activation cycle is shown in Fig. 1.3.



Fig. 1.3 Schematic representation of the G-protein cycle. In its inactive state the G-protein exists as a heterotrimer consisting of α and $\beta\gamma$ subunits (a). Upon agonist-mediated activation of a G-protein coupled receptor, the GDP associated with the G α subunit is exchanged for GTP and the α subunit dissociates from the $\beta\gamma$ subunit (b). The separated subunits can now modulate effector enzymes or ion channels (c). On hydrolysis of GTP these will re-associate with each other (d).

G-proteins are generally classified by their α -subunits, but the tightly associated $\beta\gamma$ -dimers also have signalling and regulatory roles (Clapham and Neer, 1997). To date, four major groups of α -subunit have been defined based on amino acid homology and effector activation; $G\alpha_{q/11}$, $G\alpha_s$, $G\alpha_i$ and $G\alpha_{12/13}$. Each group can be divided further into subtypes, and this, along with the structural diversity of both the β (5 sub-types) and γ (11 sub-types) subunits provides a massive combination potential and huge diversity in G-protein constitution. Theoretically, there are approximately 1500 different heterotrimer combinations.

Activation of a $G\alpha_{q/11}$ G-proteins promotes the hydrolysis of a membrane associated phospholipid, phosphatidylinositol-(4,5)-bisphosphate (PtdIns(4,5)P₂) through activation of the enzyme phospholipase C (PLC). The main products of this reaction are inositol trisphosphate (Ins(1,4,5)P₃) and diacylglycerol (DAG), which cause the release of Ca²⁺ from intracellular stores and the activation of protein kinase C (PKC) respectively.

These signalling molecules are involved in a plethora of cellular, tissue and physiological responses. DAG is a second messenger that due to its hydrophobicity is mainly associated with the plasma membrane. DAG directly activates members of the PKC family (PKCs have been extensively reviewed; Newton, 1995). PKC isoforms are serine-threonine kinases consisting of a regulatory domain and a catalytic domain. In the absence of any activating agents, the regulatory domain auto-inhibits any enzymatic activity. Modes of activation differ considerably between various isotypes but may involve the need for Ca²⁺, DAG, and phospholipids such as phosphatidylcholine and/or phophatidylserine for activation. Once activated, PKC is usually recruited to the plasma membrane where it anchors itself so the catalytic domain becomes accessible for substrates. PKC regulates an abundance of different signalling molecules, GPCRs, effector enzymes and tranmembrane channels (reviewed Liu and Heckman, 1998).

The main function of $Ins(1,4,5)P_3$ is to mobilise Ca^{2+} . The initial increase in $[Ca^{2+}]_i$ occurs most often following agonist-mediated release of Ca^{2+} from intracellular stores. Here, $Ins(1,4,5)P_3$ acts on $Ins(1,4,5)P_3$ receptors on the membrane of intracellular organelles, particularly the endoplasmic reticulum. The $Ins(1,4,5)P_3$ receptors have an intrinsic Ca^{2+} channel, which opens upon activation causing a rapid and massive efflux of stored Ca^{2+} into the cytosol (Berridge, 1993;

Taylor, 1998). Ca^{2+} release from intracellular stores is not excluively driven by $Ins(1,4,5)P_3$ and various second messengers are involved. For example, cyclic ADP ribose (Lee, 2001) and sphingosine-1-phosphate (Mao *et al.*, 1996), have all been reported to do similar roles.

Futher changes in $[Ca^{2+}]_i$ occur by a variety of different mechanisms. Ca^{2+} itself can cause further influx of Ca^{2+} into the cell through the plasma membrane (Berridge, 1998; also discussed further in Chapters 3 and 5) and by acting on ryandine receptors on the membrane of endoplasmic reticulum to cause further release of Ca^{2+} into the cytosol (reviewed in Coronado *et al.*, 1994). Furthermore, Ca^{2+} itself can stimulate PLC activity to induce more $Ins(1,4,5)P_3$ and hence more Ca^{2+} release (reviewed in Rhee, 2001). It can also sensitise $Ins(1,4,5)P_3$ receptors (although higher concentrations of Ca^{2+} will desensitise or inhibit the $Ins(1,4,5)P_3$

 Ca^{2+} is a unique and versatile signalling molecule. Ca^{2+} performs certain functions directly or alternatively acts through an interaction with a Ca^{2+} binding protein, calmodulin. Calmodulin is a small acidic protein and can bind either 0, 2 or 4 Ca^{2+} ions and can bind to and regulate different proteins in each state. There are well over 100 different proteins within the cell known to bind to calmodulin. Ca^{2+} is implicated in a multitude of biological processes including muscle-contraction, release of neurotransmitters, vision in retina cells, cell proliferation, endocrine secretion, exocytosis, cytoskeleton management, cell motion, gene expression and metabolism (reviewed in Clapham, 1995). Its not surprising, therefore, that attention has been focussed on elucidating how a simple molecule such as Ca^{2+} can be responsible for such a vast and diverse range of cellular and physiological processes.

The secret of this versitility lies in modulating the concentration, localisation and temporal frequency that Ca^{2+} exists free (and thus active) in the cytosol. Different cell types employ their own unique Ca^{2+} 'toolkit'. By the expression of certain proteins at certain levels, each cell has unique mechanisms to sense, signal through and regulate intracellular Ca^{2+} , thus allowing cells to 'de-code' the Ca^{2+} signal and respond accordingly. The huge repertoire of Ca^{2+} signalling and Ca^{2+} sensing molecules are exploited by different cell types to construct highly adaptable Ca^{2+} signalling networks. (Berridge, 1998; Taylor, 1998; Berridge *et al.*, 2000; Bootman *et al* 2001).

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Activation of G-proteins by the $G\alpha_i$ and $G\alpha_s$ subunits will inhibit or stimulate Adenylyl cyclase activity will increase cellular adenylyl cyclase, respectively. concentrations of the second messenger; cyclic adenosine monophosphate (cAMP). This cyclic nucleotide is synthesized from ATP by, adenylyl cyclase and carries signals from the cell surface to proteins within the cell. Its cellular roles are varied and include the gating of certain ion channels and the activation of protein kinases. cAMP-dependent protein kinases are major intracellular receptors for these nucleotides, and the actions of these enzymes account for much of the cellular responses to increased levels of cAMP. In particular, protein kinase A (PKA), a cAMP-dependent protein kinase phosphorylates an extensive list of target proteins. The roles of cAMP and PKA as well effector targets and their physiological roles have been reviewed extensively elsewhere (Shabb, 2001). Briefly, cAMP has been implemented in numerous signalling pathways. For example, PKA can inhibit cyclic guanosine mono-phosphate (cGMP), Rho (see below) and mitogen-activated protein kinase (MAP kinase) signalling. It can also regulate the phosphoinositide and Ca²⁺ signalling pathways, primarily by interfering with calcium mobilisation or desensitisation of receptors (see below). Further, cAMP-activated PKA phosphorylates transcriptional proteins, thereby exerting longer-term effects on tissues. Other roles include apoptosis and cell survival (depending on cell type), metabolic functions, water homeostasis, activation of membrane transporters and smooth muscle relaxation.

The fourth class of G-proteins is $G\alpha_{12/13}$, (reviewed in Kurose, 2003) the role of which is less well understood. However, they regulate GEFs to activate Rho, a small membrane GTPase protein involved in cytoskeleton changes. Other G α subunits exist and can be linked to one of the major G α groups. For example, $G\alpha_{14}$ $G\alpha_{15}$ and $G\alpha_{16}$ all mediate their effects through PLC activation and as such are linked functionally to the $G\alpha_{q/11}$ family. Furthermore, aside from $G\alpha_i$, inhibition of adenylyl cyclase is also mediated by $G\alpha_o$, $G\alpha_z$ and $G\alpha_t$ G-proteins. The list of effectors described here is in no way definitive, and indeed continues to grow. Many other enzymes and ion channels are targeted by active G-proteins (see Pierce *et al.*, 2002).

1.2.2.2 GPCR-mediated activation of the MAP kinase pathways

In addition to the signalling pathways discussed above, GPCRs can also activate signalling pathways leading to the activation of the MAP kinase cascade (reviewed in Sugden and Clerk, 1997; Belcheva and Coscia, 2002). MAP kinases are evolutionary conserved serine/theronine kinases involved in the regulation of cell growth and proliferation and in reacting to environmental stress. MAP kinase subfamilies include the extracellular-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) (or the stress-activated protein kinases (SAPKs)) and the p38-MAP kinases. Many of the GPCR-mediated effects on cell proliferation and growth are mediated by the ERK cascade, although the JNK-SAPK and p38-MAPK cascades can be also be activated through GPCRs. The ERK subfamily of MAP kinases consists of two main proteins, ERK1 and ERK2. Because the 44 kDa ERK1 and the 42 kDa ERK2 are highly homologous and both function in the same protein kinase cascade, the two proteins are often referred to collectively as ERK1/2 or p44/p42 MAP kinase. The signalling cascade that leads to the eventual activation of ERK1/2 consists of a linear sequence of protein activation. In its simplest form, this consists of a GTPase, Ras, being activated by GEFs. GEFs are coupled to cell surface receptors via adapter molecules such as Shc and on receptor activation cause the exchange of GDP for GTP within Ras proteins. This guanine exchange activates Ras, which subsequently activates the first component of the MAP kinase cascade, namely Raf. This in turn phosphorlyates (and hence activates) mitogen-activated protein kinases 1/2, which phosphorylates ERK1/2. Upon activation, ERK1/2 can phosphorylate a variety of intracellular targets including transcription factors, transcriptional adaptor proteins, membrane and cytoplasmic substrates, and other protein kinases, and can thus alter gene expression and the catalytic activities of many enzymes. Detailed diagrammatic representation of the multiple pathways linking GPCR activation and activation of MAP kinase cascades can be found elsewhere (Marinissen and Gutkind, 2001).

1.2.3 Regulation of GPCR activity

For GPCRs to respond sufficiently to agonists it is fundamental that they have the capacity to become either more, or less, sensitive to stimulation. Physiologically,

this permits receptors to effectively alter responses generated by active receptors, thus either limiting or enhancing their actions. Several mechanisms have been discovered that act to regulate the signalling by receptors, and this can occur at the level of the receptor, or post-receptor (alterations in the amount or activity of downstream signalling components) (see review: Morris and Malbon, 1999). The waning sensitivity of cells in the face of repeated or persistent agonist stimulation is termed desensitisation and ensures the reduction of prolonged, and often detrimental, effects. Desensitisation can be divided along several lines; it can either be agonist-specific (homologous), whereby only active receptors are regulated, or agonist non-specific (heterologous), whereby inactive receptors are regulated by other receptors coupling to the same or different signalling pathways. Furthermore, desensitisation can be rapid (seconds-minutes) or slow (hours to days) and involve loss of receptor signalling function (uncoupling), removal of receptor from the plasma membrane into the sub-cellular compartments (internalisation) or loss of total receptor number (down-regulation). Clinically, the loss of receptor sensitivity and responsiveness has serious implications because the use of GPCRs as therapeutic targets is often subject to the phenomenon of tolerance whereby a greater dose is required to achieve the same response.

1.2.3.1 Covalent modification of receptors

At the level of the receptor, the most rapid desensitisation (seconds-minutes) is caused by the covalent modification of receptors. Three families of regulatory proteins; second messenger kinases (PKA and PKC), G-protein receptor kinases (GRKs) and casein kinases are involved in this method of desensitisation (reviewed in Lefkowitz, 1998).

PKA and PKC are indirectly activated by Ga_s and $Ga_{q/11}$ G-proteins respectively (see Section 1.2.2.1). These protein kinases can either heterologously or homologously phosphorylate GPCRs on cytosolic residues, thus weakening their ability to couple to G-proteins. This process of desensitization is a feedback mechanism in which the second messenger generated by the agonist-stimulated GPCR activates a kinase that decreases the activity of the receptor and ultimately attenuates production of the second messenger.
GRKs (reviewed in Pitcher *et al.*, 1998) are generally involved in mediating rapid, homologous desensitisation. The family of GRKs currently include seven members (termed GRK 1-7), and will specifically phosphorylate intracellular residues on the GPCR following G-protein activation. This event can cause 'uncoupling' proteins such as the arrestins (discussed below) to bind to the GRK-phosphorylated GPCR and will prevent further GPCR-G-protein interactions.

Activity involving casein kinase II-mediated phosphorylation may also be involved in regulation GPCRs. Casein Kinase II is a ubiquitous serine/threonine protein kinase whose only reported roles are in the regulation of signalling by the proto-oncogene Wnt, which binds to the 'frizzled' family of receptors (Song *et al.*, 2000) and in targeting GPCRs to β -arrestin-dependent pathways (Hanyaloglu *et al.*, 2001). Similarly, casein kinase 1 α has also been shown to mediate receptor phosphorylation (Tobin *et al.*, 1997; Budd *et al.*, 2000;) although the role of casein kinase 1 α –mediated phosphorlyation in desensitisation is is poorly understood (Tobin, 2002).

1.2.3.2 Internalisation

A further event in the regulation of GPCRs is receptor internalisation (reviewed; Ferguson, 2001). Several pathways of internalisation exist and the most extensively investigated is the GRK-arrestin-dependent pathway. The arrestins are a family of cytosolic proteins that bind to GRK-phosphorylated GPCRs and in-turn recruit clathrin. Clathrin is a protein that plays a major role in the creation of vesicles (membrane-bound transport packages) in cells, and acts as an adaptor to facilitate the clathrin-mediated endocytosis of the GPCR. This event is usually dynamindependent (dynamin is a GTPase that helps form the clathrin-coated pits). There are several alternative arrestin and clathrin-independent mechanisms of internalisation and these pathways can also be dynamin-independent or -dependent.

The role of internalisation in desensitisation of GPCRs is poorly understood, but is nonetheless an important aspect of GPCR activity and regulation. Generally, receptors are engulfed by membrane invaginations that are pinched away from the surface to exist as intracellular clathrin-coated vesicles (reviewed; Wakeham *et al.*, 2000). It was originally believed that receptor internalisation was the primary mechanism of receptor desensitisation due to the spatial uncoupling of the receptor from its effector system (Sibley and Lefkowitz, 1985). However, desensitisation can appear to precede internalisation and internalisation is now believed to play a role in the resensitisation and responsiveness of GPCRs (Yu *et al.*, 1993; Pippig *et al.*, 1995; Krueger *et al.*, 1997; Zhang *et al.*, 1997).

Regulation of protein expression at the plasma membrane allows cells to control their responses to extracellular stimuli (Marsh and McMahon, 1999), and the majority of the endocytosed plasma membrane proteins are recycled back to the plasma membrane from early endocytic compartments to maintain membrane composition (Mukherjee *et al.*, 1997). Recycled receptors will be dephosphorylated, agonist-free and fully re-sensitised to permit another round of signalling. Endocytosis of cell-surface receptors and subsequent intracellular sorting are therefore critical cellular processes, but the requirements for endocytosis, the endocytic mechanism, the fate of internalised receptor and ligand differ greatly between receptors.

1.2.3.3 Receptor expression

As an alternative to re-sensitisation, movement through endocytic pathways can result in receptor degradation (Tsao *et al* 2000; Tsao *et al.*, 2001). The subsequent loss of receptors from the plasma membrane is certain to limit the responsiveness of cells, but whether or not receptor expression level is maintained by the re-synthesis depends on external and other regulatory factors. Prolonged or repeated activation of receptors often leads to a more gradual attenuation of receptor signalling and long-term desensitisation. Termed down-regulation, this occurs when receptor degradation exceeds receptor synthesis. Regulation of re-synthesis may occur at the translational and transcriptional levels. Transcriptional reduction of GPCR expression can result from hormonal regulation of the transcriptional activities of the genes encoding GPCRs. At the translational stage, mRNA stability can be altered, thus providing a further level of regulation (reviewed in Morris and Malbon, 1999).

It is apparent that GPCRs are subject to a huge variety of regulatory mechanisms and many more exist other than those discussed here (reviewed: Tobin *et al* 1996, Willars *et al.*, 1996; Morris and Malbon, 1999). These include the activity of RGS proteins and some effectors that act to enhance the GTPase activity of G α subunits (reviewed; Siderovski *et al.*, 1996; Helper. 1999; Ross and Wilkie, 2000; Zhong and Neubig, 2001) and palmitylation (covalent lipid modification) of either GPCRs or G-proteins. Further, Ca²⁺ may enhance the activity of PLC, cause heterologous desensitisation by depletion of intracellular Ca²⁺, and can either inhibit or enhance the activity of the Ins(1,4,5)P₃ receptors, which can themselves be the subject of desensitisation and down-regulation.

1.2.4 Discovery and characterisation of receptors for NmU

Binding sites for NmU were characterised well before the molecular nature of the receptor had been identified. Early studies demonstrated that the binding of [125 I]-labelled rat NmU-23 to membranes prepared from rat uterus was saturable, specific, reversible and dependent on time, temperature and *p*H (Nandha *et al.*, 1993). Scatchard analysis suggested a single class of binding site with a K_d of 0.35nM. Furthermore, guanosine 5'-[γ -thio]tri-phosphate (GTP- γ S) (a non-hydrolysable analog of GTP) reduced the binding, thus implying a GPCR. Despite such studies, it was not until molecular biology had established itself alongside classical pharmacology that NmU receptors were cloned and their molecular nature determined. A historical perspective of our understanding of GPCRs has been excellently reviewed (Lefkowitz, 2004).

The amassing of human sequence data in recent years has helped facilitate the discovery of previously unidentified GPCRs, whereby the genetic sequences of established receptors are used as tags to screen genetic libraries. Most of the newly discovered receptors are classified as orphan GPCRs, whereby the endogenous ligand(s) are unknown. It has become possible to express these orphan receptors recombinantly in cell lines and identify the ligand(s) by means of a screening process based on their ability to bind to membrane preparations and/or activate cellular signalling pathways (see Section 1.2.2). It is often possible to predict the structure or classification of the ligand using sequence homology comparisons with other, more familiar, GPCRs. Typically, however, orphan GPCRs cannot be classified with any assurance and the screening process is generally a considerable task.

This approach is termed "reverse-pharmacology", and, in their 1997 review, Stadel, Wilson and Bergsma outlined the process based on its application to rapidly reduce the time between gene identification and clinical development of valuable therapeutic agents. The technology has been applied successfully to identify ligands for several orphan GPCRs. For example, identification of endogenous receptors for cysteinyl leukotrienes (CysLTs) (Sarau *et al.*, 1999), melanin-concentrating hormone (MCH) (Chambers *et al.*, 1999) and uridine 5'-diphosphoglucose (UDP-glucose) (Chambers *et al.*, 2000).

By applying this "reverse pharmacology" approach, several groups reported that NmU is a potent ligand for the orphan GPCR; GPR66 or FM3. This receptor was originally isolated from human and mouse genetic libraries due to its amino acid sequence homology with both the growth hormone secretagogue receptor (subsequently identified as the receptor for ghrelin (Kojima *et al.*, 1999)) and the neurotensin receptor (33 and 29% respectively) which were used as tags to screen a genetic database (Tan *et al.*, 1998). The evidence associating NmU as the endogenous ligand for FM3 is now extensive. Several groups have recombinantly expressed the receptor in a variety of cell lines and shown that NmU binds and activates FM3 at sub-nanomolar concentrations.

Using this receptor as a specific sensor of a screening assay, well over 1500 proposed GPCR ligands, many of which were peptides, were examined for their ability to increase [Ca²⁺]; and phosphoinositide hydrolysis. NmU analogs from various species were the only peptides found to activate recombinant receptors (Fujii et al., 2000; Hendrik et al., 2000; Hosoya et al., 2000; Kojima et al., 2000; Raddatz et al., 2000; Shan et al., 2000; Szekeres et al., 2000). Similar peptides such as neuromedin B, K, C and N, as well as neurotensin, ghrelin, motilin, VIP, and pancreatic polypeptide produced no response. The response to NmU was specific, [¹²⁵I]-labelled pNmU-8 bound robust, concentration-dependent and prolonged. specifically to membrane fractions prepared from CHO cells expressing recombinant human FM3. Unlabelled human-NmU-25 inhibited this binding in a concentrationdependent manner and neither growth hormone-releasing hexapeptide-6 (GHRP-6), human neurotensin, nor pancreatic polypeptide, had any inhibitory effect (Fujji et al., 2000). The FM3/GPR66 orphan receptor is now termed human(h) NmU receptor 1 (hNmU-R1).

Immediately following the identification of hNmU-R1, its sequence was used to search a genetic database and a second NmU receptor was identified (Hosoya *et al.*, 2000; Raddatz *et al.*, in 2000; Shan *et al.*, 2000). The ability to bind and respond to NmU was again specific. The second receptor subtype shows about 45-50% amino acid sequence homology with the first, and is termed hNmU-R2.

The genes for hNmU-R1 and hNmU-R2 have been mapped respectively to SHGC-33253 that is localised to chromosome 2q34-q37 and SHGC-8848 that is localised to chromosome 5q31.1-q31.3 (Raddatz *et al.*, 2000). Their genomic structures differ in that the predicted open reading frame of hNmU-R1 is encoded on two exons, whereas that of hNmU-R2 exists on four (Shan *et al.*, 2000). There is however, a level of homology between the two genes and conservation of certain exon-intron boundaries suggesting that the two receptors may be the products of a duplication event (Shan *et al.*, 2000). Interestingly, several expressed sequence tags from GenBank have been identified that have identity with hNmU-R2 and three of these may represent an alternative transcript (Shan *et al.*, 2000). Although this may represent a sterile transcript, its potential presence should urge caution in the interpretation of receptor expression profiles based on mRNA expression, particularly if it is unclear whether the probes used are able to hybridize to the alternate transcript.

When a new gene is cloned it is not always a simple task to identify conclusively the methionine residue used to initiate translation. Indeed, for both human NmU receptors, more than one N-terminal variant of the protein sequence has been published (e.g., Tan *et al.*, 1998; Raddatz *et al.*, 2000; Shan *et al.*, 2000). Although all variants of the receptors published to date are functionally active, it is informative to consider which form of the receptor is most likely to exist physiologically.

For hNmU-R1, the first group to identify this receptor reported the cloning of a 403 amino acid protein (Tan *et al.*, 1998), whilst a subsequent publication extended the putative N-terminus of the receptor by 23 amino acids, suggesting translation initiation from an in-frame, upstream AUG (Raddatz *et al.*, 2000). Analysis of the nucleotide sequences at the two putative translation initiation sites reveals that the shorter 403 amino acid form seems to have a stronger Kozac sequence (Kozak,

1986). In addition, comparison with the mouse orthologue shows that the murine receptor agrees with the shorter form of the human receptor. Thus, for hNmU-R1 the evidence for the shorter form of the receptor is stronger, although it is not inconceivable that initiation at both human AUG codons may occur, producing two proteins from one mRNA, as reported previously for other proteins (reviewed in Kozak, 1991a and b).

Similarly two forms of hNmU-R2 have been reported which differ in their initiating methionine; a 415 amino acid form (Raddatz *et al.*, 2000; Shan *et al.*, 2000) and a 412 amino acid form (Hosoya *et al.*, 2000; Howard *et al.*, 2000). Again, analysis of the nucleotide sequences surrounding the two putative translation initiation sites and comparison with rodent orthologue sequences provides stronger evidence for the slightly shorter form.

The two receptors are typical GPCRs, possessing 7 putative transmembrane domains and are represented schematically in Fig. 1.4 and a comparative amino acid sequence alignment of both receptors is also shown in Fig. 1.5. The hNmU-R1 consists of 403 amino acids and has a predicted molecular mass of 44979 Da. hNmU-R2 is slightly longer at 412 amino acids and has a predicted molecular mass of 47450 Da. The two receptors show about 45-50% amino acid homology and both show some homology to the human growth hormone secretagogue receptor, the human motilin receptor and human neurotensin receptor, with identities of about 30%. Single nucleotide polymorphisms (SNPs) have been identified, particularly in the non-coding regions of both hNmU-R1 and hNmU-R2 (National Centre for Biotechnology Information (NCBI) Database). Additionally, five SNPs have been reported which result in a change in the amino acid sequence of hNmU-R2. Based on the sequence presented in Fig. 1.5. these are: S295T; F312L; P380L; M385V; and T392A. The frequency and functional significance of these SNPs are, however, unknown.

In comparison with hNmU-R1, the third intracellular loop is shortened in hNmU-R2, as is the N-terminus. Further, the C-terminus of hNmU-R1 is 67 residues in length and is considerably shorter than the 92 residues of hNmU-R2. Conservation is highest within the proposed transmembrane domains whilst the N- and C-terminals show little homology. Both receptor sub-types possess many of the characteristics

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linking them to GPCR family 1, such as the ERY variant of the DRY motif at the boundary of transmembrane domain 3 and the second intracellular loop and the conservation of cysteine residues in extracellular loops 2 and 3 (see Figs. 1.4. and 1.5.). The D/ERY motif is a highly conserved triplet amino acid sequence involved in the modulation of ligand binding (e.g., Rhee et al., 2000), the preservation of inactive receptors (e.g., Barak et al., 2001; Wilbanks et al., 2002) and G-protein coupling (Strader et al., 1994; Scheer et al., 2000). The conserved cysteine residues exist in similar positions to those within other family 1 GPCRs where they form a disulphide bridge (Strader et al., 1994; Perlman et al., 1995) that may be critical for the stability of receptor conformation, protein folding and ligand binding (e.g., Savarese et al., 1992). Further, consensus sites for serine, threonine and tyrosinelinked phosphorylation and asparagine (N)-linked glycosylation exist throughout the receptors, as depicted in Figs. 1.4 and 1.5. Within intracellular domains, consensus recognition sites (Kennelly and Krebs, 1991; Pearson and Kemp, 1991) exist for several kinases including PKA, PKC, casein kinases I and II. These predominate within the C-terminal tail and the third intracellular loop of both receptors, as well as within the second intracellular loop of hNmU-R2. Both receptor sub-types have 2 potential asparagine-linked glycosylation sites within the NH₂-terminus, but in addition, hNmU-R2 possesses a third within the second extracellular loop. Potential sites for phosphorylation by GRKs (Pitcher et al., 1998) are not shown due to lack of information concerning consensus sequences.



hNmU-R2



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Fig. 1.4. Schematic representation of hNmU-R1 and hNmU-R2. Sequences were acquired with NCBI accession numbers; AF272362 (hNmU-R1) and AF272363 (hNmU-R2). Prediction of transmembrane spanning helices was carried out using HMMTOP (v.2.0) (Tusnády and Simon, 2001) which uses the Hidden Markov model (Tusnády and Simon, 1998) for predicting transmembrane helices. Identification of *N*-linked glycosylation sites (*filled-black circles*; •) within extracellular domains and the identification of residues most likely to be phosphorylated (serine residues (*upward triangles*; \blacktriangle), tyrosine residues (*downward triangles*; \triangledown), and threonine residues (*squares*; \blacksquare) within internal domains were accomplished using ExPASy online proteomics tools (Blom *et al.*, 1999). The positions of these residues within the protein that could be subject to post-translational modification are provided. Conserved cystiene residues that may be involved in the formation of disulphide bridges and the position of the ERY motif at the boundary of transmembrane spanning domain 3 and the second intracellular loop are also illustrated. Further details are given in Fig. 1.5.

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NmU-R1 : Q Q E T - - - D P S :403 NmU-R2 : N Y Q S F H F N K T :412 Fig. 1.5. Amino acid sequence alignment of hNmU-R1 and hNmU-R2. Sequence alignment of amino acids for hNmU-R1 (upper) and hNmU-R2 (lower). Full sequences were obtained with accession numbers AF272362 (NmU-R1) and AF272363 (NmU-R2) and aligned with Genedoc sequence alignment editor (reviewed in Nicholas et al., 1997). Spaces, as represented by dashes (-), indicate gaps to maximise alignment, with shaded areas highlighting conserved residues. The putative transmembrane spanning regions were determined on-line using HMMTOP (v.2.0) (Tusnády and Simon, 2001) which uses the Hidden Markov model for predicting transmembrane helices (Tusnády and Simon, 1998). These are indicated by lines above and below the sequence for NmU-R1 and NmU-R2 respectively. Symbols above the sequence are representative of residues with potential for posttranslational modification for NmU-R1, and those below, for NmU-R2. These were determined on-line using ExPASy on-line proteomics tools using NeTNGlyc (Gupta and Brunak, 2002) to identify N-linked glycosylation (filled squares; +) and NetPhos (Blom et al., 1999) to identify potential serine, threonine and tyrosine Only those residues with consensus sequences for phosphorylation sites. phosphorylation by specific kinases (Pearson and Kemp, 1991; Kennelly and Krebs, 1991) are detailed here and include protein kinase C (filled triangle; ▲), protein kinase A (filled circle; ●), casein kinase I (open diamonds; ◊), and casein kinase II (open circles; O). Some residues highlighted in Fig. 1.4 as potential phosphorylation sites do not have consensus sequences for specific kinase activity. These have not been detailed here.

1.2.5 Tissue distribution of hNmU-R1 and hNmU-R2

Despite many studies on the distribution of human NmU receptors, an absolute clear picture has yet to emerge. Different techniques have been applied to determine the pattern of receptor expression, and this could account for some discrepancies. Dot blots, Northern blots, quantitative PCR and *in-situ* hybridisation have been popular, but these address the mRNA signal and may not reflect protein expression. To date, distribution of NmU-Rs has not been investigated at the level of the protein due to the lack of suitable antibodies. Species differences could also contribute to the somewhat ambiguous picture concerning the sites of receptor expression. Despite these difficulties, a pattern has emerged that suggests hNmU-R1 is expressed predominantly in the periphery, especially the gastrointestinal tract, whilst hNmU-R2 expression is predominantly in the CNS.

The mRNA for hNmU-R1 is expressed in a wide variety of tissues but levels are greatest in peripheral tissues, particularly the small intestines and stomach (Hedrick *et al.*, 2000; Howard *et al.*, 2000; Raddatz *et al.*, 2000; Szekeres *et al.*, 2000; Westfall *et al.*, 2001). It is also present in the pancreas, adrenal cortex, heart, lung, trachea, mammary gland, bone marrow, peripheral blood leukocytes (particularly T cells and natural killer (NK) cells, genito-urinary system, placenta, mammary gland, spleen and adipose tissue (Hedrick *et al.*, 2000; Howard *et al.*, 2000; Raddatz *et al.*, 2000; Szekeres *et al.*, 2000; Westfall *et al.*, 2000; Mestfall *et al.*, 2000; Mestfall *et al.*, 2000; NmU-R1 mRNA exists in rat with highest levels in the small intestine, lung and femur (Fujii *et al.*, 2000). NmU-R1 mRNA is also expressed in approximately 25% of the small/medium diameter neurons within the dorsal root ganglia (Yu *et al.*, 2003).

Although several studies examining mRNA expression have failed to demonstrate NmU-R1 in human (Hedrick *et al.*, 2000; Howard *et al.*, 2000) and mouse (Funes *et al.*, 2002) brain, others have shown low levels, but with a widespread distribution within the CNS (Raddatz *et al.*, 2000; Szekeres *et al.*, 2000). Highest levels of hNmU-R1 mRNA expression are found in the cerebellum, dorsal root ganglion, hippocampus and spinal cord, all-be-it at levels 5-25 fold less than the peripheral organs.

In humans, NmU-R2 mRNA is confined predominantly to specific regions within the brain, with greatest expression observed in the substantia nigra (Howard *et al.*, 2000), medulla oblongata, pontine reticular formation, (Raddatz *et al.*, 2000), spinal cord (Shan *et al.*, 2000) and thalamus (Howard *et al.*, 2000; Raddatz *et al.*, 2000). Moderate to high levels are also observed in the hippocampus, hypothalamus and cerebral cortex (Raddatz *et al.*, 2000). The peripheral distribution of hNmU-R2 mRNA is a little unclear. High levels of NmU-R2 mRNA are present in testes (Hosoya *et al.*, 2000; Raddatz *et al.*, 2000; Westfall *et al.*, 2001) whilst lower levels are present in a variety of peripheral tissues including the gastrointestinal tract, genito-urinary tract, liver, pancreas, adrenal gland, thyroid gland, lung, trachea, spleen, thymus and thyroid (Shan *et al.*, 2000; Raddatz *et al.*, 2000; Westfall *et al.*, 2001). Others have failed to detect NmU-R2 mRNA in liver, heart, skeletal muscle, intestines, pancreas, placenta and kidneys (Howard *et al.*, 2000) or bladder (Westfall *et al.*, 2001).

These reports are consistent with NmU-R2 mRNA distribution within mouse CNS (Funes et al., 2002) and rat CNS where expression is detected in the hypothalamus, hippocampus and spinal cord (Hosoya et al., 2000; Howard et al., 2000). More specific localisation in rats suggests that NmU-R2 is expressed within the paraventricular nucleus (PVN), the wall of the third ventricle in the hypothalamus and in the CA1 region of the hippocampus (Howard et al., 2000). A detailed study using in situ hybridization to determine the localization of NmU-R2 mRNA expression in the hypothalamus of mouse and rat revealed some species differences (Graham et al., 2003). Thus, in rats, expression is predominantly in the ependymal layer of the third ventricle and PVN. Using a more sensitive technique some expression was also noted in the arcuate nucleus. In the mouse, distribution is less localised, with stronger expression in the arcuate nucleus, and expression also in clusters of neurons in the dorsomedial hypothalamus (DMH) and surrounding the ventromedial nucleus (Graham et al., 2003). Receptor autoradiographic studies have also localized NmU binding sites in both lamina I and the outer part of laminar II of the spinal cord dorsal horn and the expression profiling of mRNA indicates that binding is most likely attributable to NmU-R2 (Yu et al., 2003). Based on the tissue distribution of mRNA, the greatest level of NmU-R2 expression in the rat is in the uterus (Hosoya et al., 2000), consistent with the role of NmU in uterine contraction in this species. The lower levels or absence of expression in human (Raddatz et al.,

2000; Shan et al., 2000; Westfall et al., 2001) and dog (Westfall et al., 2001) uterus may reflect differences between species and/or physiology.

Although there are differences in the structure of the NmU peptide between species, there is a very high conservation of the C-terminus that is largely responsible for its biological activity (see Section 1.1.2). Further, neither human (Fujii *et al.*, 2000; Hedrick *et al.*, 2000; Hosoya *et al.*, 2000; Howard *et al.*, 2000; Raddatz *et al.*, 2000; Shan *et al.*, 2000; Szekeres *et al.*, 2000) nor murine (Funes *et al.*, 2002) NmU receptors have significant abilities to distinguish between different forms of NmU. Despite this, there are apparent species and tissue selectivity in the biological actions of NmU (see Section 1.3). For example, both forms of porcine NmU stimulate contraction of uterine muscle from the rat but not the guinea-pig (Minamino *et al.*, 1985a). This suggests that differences in the distribution and/or function of NmU receptors between species contributes to functional diversity.

The distribution of hNmU-R1 and hNmU-R2 within the main peripheral (Table 1.5a) and central human tissues (Table 1.5b) is summarized with the distribution of hNmU-25 below.

a) Peripheral tissues

Tissue	NmU	R1	R2
Heart	0	+	+
Lung	+	++	+
Liver	0	+	0
Kidney	+	+	+
Skeletal Muscle	0	+	0
Stomach	+	+	+
Intestines	+++	+++	0
Spleen	+	+++	0
Lympohocytes	+	+++	
Macrophages	0	+	
Adipose	++	+++	
Pancreas	+	++	0
Prostate	+	++	0
Pituitary	++	+	0
Placenta	++	++	
Cartilage	+	+	
Trachea		+	+
Uterus		+	0
Duodenum		+	0
Testes		++	++ '
Mammary gland		+	0
Bone	0	0	
Bone Marrow	++	+	

Tissue	NmU	R1	R2
Amygala	0	+	+
Caudate nucleus	0	+	
Cerebellum	0	÷	+
Globus Pallidus	+	+	
Hippocampus	++	+	++
Hypothalamus	++	+	++
Medial frontal gyrus	++	+	
Medulla oblongata	++	+	+++
Nucleus accumbens	++	+	
Pontine reticulum form			+++
Parahippocampal gyrus	++	+	
Substantia Nigra	++	+	
Superior frontal gyrus	++	+	
Thalamus	++	+	++
Spinal cord	++	+	++
Dorsal root ganglion	++	+	+

Table 1.5a-b. Comparative distribution of hNmU-R1, hNmU-R2 and hNmU-25. The levels of expression of either hNmU-R1, hNmU-R2 and hNmU-25 precursor in human peripheral (a) and central (b) tissues were determined by quantitative PCR (Raddatz *et al.*, 2000, Szekeres *et al.*, 2000). *o* denotes no or trace expression detected, + equals 10-200 copies of the genes mRNA ng mRNA⁻¹. ++ is equivalent to 200-800 and +++ to over 800 copies of mRNA ng mRNA⁻¹. Spaces denote no information available.

1.2.6 The regulation of signalling by hNmU-R1 and hNmU-R2

Observations regarding the intracellular signal transduction pathways regulated by NmU receptors have, to date, been entirely based around the activity of these receptors when expressed as recombinant proteins. It is clear from the functional screening assays that NmU-R1 and NmU-R2 of human and rodent origin are able to mediate intracellular Ca²⁺ signalling with potency in the nM range (Fujii *et al.*, 2000; Hedrick *et al.*, 2000; Hosoya *et al.*, 2000; Howard *et al.*, 2000; Kojima *et al.*, 2000; Raddatz *et al.*, 2000; Shan *et al.*, 2000; Szekeres *et al.*, 2000; Funes *et al.*, 2002). For hNmU-R1 this has been shown to be associated with phosphoinositide hydrolysis (Raddatz *et al.*, 2000; Szekeres *et al.*, 2000). Given the lack of effect of PTX on Ca²⁺ signalling by either hNmU-R1 or hNmU-R2 (Raddatz *et al.*, 2000; Shan *et al.*, 2000; Szekeres *et al.*, 2000), this indicates a G $\alpha_{q/11}$ -mediated activation of PLC and a lack of PLC activation by $\beta\gamma$ -subunits derived from G α_i . Activation of either hNmU-R1 or hNmU-R2 is also able to stimulate the release of arachidonic acid, most likely through a Ca²⁺-dependent activation of phospholipase A₂ (Fujii *et al.*, 2000; Hosoya *et al.*, 2000).

Coupling of these receptors to signal transduction pathways other than Ca^{2+} and phosphoinositide signalling is less clear and could also depend on, for example, the experimental protocol, expression levels of the receptors and the cellular background. Thus, although activation of hNmU-R2 in CHO cells results in a partial inhibition of forskolin (FSK)-stimulated cAMP accumulation (Hosoya *et al.*, 2000), activation of transiently expressed hNmU-R1 in HEK293 cells has no affect on either the basal or FSK-stimulated levels of cAMP (Szekeres *et al.*, 2000).

Using brain slices from rat, it has recently been shown that NmU depolarises a sub-population of hypothalamic paraventricular neurons by enhancing $I_{\rm H}$ channel activity (Qiu *et al.*, 2003). This regulation may be through a Ca²⁺-dependent activation of soluble guanylate cyclase, an increase in cGMP and a subsequent activation of $I_{\rm H}$ through the binding of cGMP to the C-terminus of the channel. NmU-R2 mRNA is expressed within the PVN and the authors suggest that regulation of $I_{\rm H}$ by NmU may regulate the activation of autonomic output from the brain and spinal cord, thereby influencing cardiovascular function (see Section 1.3.2).

Despite the variety of activities that have been reported, the precise physiological roles of NmU have yet to be clearly defined. As well as smooth muscle contraction, a number of other diverse roles have been documented. All evidence to date points to multiple roles, and indeed the wide distribution of NmU (see Section 1.1.3) and the NmU receptors (see Section 1.2.4) supports this.

1.3.1 Smooth muscle contraction

The smooth muscle contractile properties of NmU are well documented in a variety of tissues and from a variety of different species. The contractile properties of both porcine NmU-8 and NmU-25 seen on rat uterine preparations (Minamino et al., 1985a) have, however, not been a consistent finding in other species, for example in the dog (Westfall et al., 2001). Similarly, contractile effects in other tissues are species-specific. For example, a variety of NmU analogs do not mediate contraction of guinea-pig or rat small intestine (Minamino et al., 1985a; Bockman et al., 1989), porcine jejenum (Brown and Quito, 1988), circular smooth muscle of frog stomach; bladder from rat, guinea-pig, ferret, rabbit or mouse (Westfall et al., 2001) or the small and large intestinal longitudinal smooth muscle of rat and frog (Benito-Orfila et al., 1991). NmU mediates a concentration-dependent contraction of turtle small intestine leading to the suggestion that it may regulate gut motility in reptiles but not mammals where other peptides may be more important (Bockman et al., 1989). However, NmU mediates a concentration-dependent contraction of rat circular smooth muscle from the fundus in a manner that is direct, involving neither cholinergic activation nor nervous stimulation (Benito-Orfila et al., 1991). These direct motor actions have been reported elsewhere. For example, NmU contracts human smooth muscle from the ileum, particularly the longitudinal muscle, and this is resistant to both atropine and tetrodotoxin (Maggi et al., 1990). NmU also contracts human urinary bladder in this way (Maggi et al., 1990) whilst NmUmediated contraction of canine bladder (both in vitro and in vivo), stomach, ileum and colon has been shown (Westfall et al., 2001). Despite the relative or absolute lack of NmU in the muscularis externa of the gastrointestinal tract (see Section

1.1.3.2), some of these functional responses suggest a regulatory role for NmU in the gastrointestinal tract.

1.3.2 Blood pressure and regional blood flow

At the time of the identification of NmU, intravenous administration was demonstrated to cause a rapid and sustained increase in arterial blood pressure in the rat (Minamino et al., 1985a). A more recent study has supported an effect on blood pressure but found them to be relatively small and transient (Chu et al., 2002). In anaesthetized dogs, intravenous administration of NmU also caused a very slight and transient increase in blood pressure (Westfall et al., 2001). In contrast, the intracerebroventricular administration of NmU to conscious, freely-moving rats caused rapid, progressive and concentration-dependent increases in mean arterial pressure that were long-lasting at high concentrations (Chu et al., 2002). Furthermore, NmU increased heart rate, whilst a high concentration also increased plasma norepinephrine concentration. These data indicate that NmU is able to increase sympathetic activity consistent with its localisation in the PVN of the hypothalamus (Howard et al., 2000) and the projection of PVN neurons to sympathetic preganglionic neurons in the spinal cord. Note also that NmU depolarizes parvocellular cells of the PVN in hypothalamic slices, most likely through a direct postsynaptic action (Qiu et al., 2003). However, it is unclear if all of the cardiovascular actions of centrally administered NmU are mediated via this route. Indeed intravenous NmU mediates potent constrictor effects on the superior mesenteric vascular bed in conscious rats and this can occur independently of changes in systemic arterial pressure, heart rate and renal or hindquarters blood flow suggesting local rather than central effects (Gardiner et al., 1990). Similarly, intravenous NmU reduces blood flow in the superior mesenteric artery and the portal vein of anaesthetized dogs with little or no effect on axillary artery and pancreatic tissue blood flow or systemic arterial pressure (Sumi et al., 1987). Thus, NmU may be important within the intestines as a regulator of local blood flow, at least in dog and rat. Whether this phenomenon is true of other species remains to be established.

1.3.3 Hypothalamo-pituitary-adrenal axis and the stress response

NmU-R2 mRNA is expressed in the hypothalamic PVN (see Section 1.2.4), which in addition to its role in feeding behaviour (see Section 1.3.4), is responsible particularly for the release of corticotrophin releasing hormone (CRH). CRH is released following stimuli such as stress and subsequently stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. In turn, ACTH stimulates cortisol production by the adrenal cortex and thus the PVN is key in the Subcutaneous injection or control of the hypothalamo-pituitary-adrenal axis. increases intracerebroventricular administration of NmU to rats plasma concentrations of ACTH and corticosterone (Malendowicz et al., 1993; Ozaki et al., 2002). More specifically, administration of rat NmU-23 into the PVN also increases the blood concentrations of both ACTH and corticosterone (Wren et al., 2002). These observations are consistent with a role of NmU in the hypothalamic-pituitary axis although it has been noted that altered organ blood flow (see Section 1.3.2) also has the potential to influence the metabolic clearance of plasma hormones (Malendowicz et al., 1993). In support of a central action of NmU on hypothalamic PVN function, NmU stimulates the release of CRH from hypothalamic explants in vitro (Wren et al., 2002). Activation of central neurons by NmU is also indicated by the enhanced expression of *c-Fos* immunoreactivity or its mRNA in hypothalamic areas of the rat including the PVN (magnocellular and parvocellular divisions), supraoptic nucleus, arcuate nucleus, dorsomedial nucleus and lateral hypothalamic area following intracerebroventricular administration of NmU (Niimi et al., 2001; Ivanov et al., 2002; Ozaki et al., 2002). Taken together, the above observations suggest central effects of NmU on the hypothalamo-pituitary-adrenal axis and hint at other functions for NmU. Indeed arginine vasopressin and oxytocin are also synthesized in the PVN (and supraoptic nucleus) and the intracerebroventricular administration of NmU to rats enhances c-Fos expression in neurons containing these peptides (Niimi et al., 2001) whilst plasma levels of both are increased (Ozaki et al., 2002). Furthermore, NmU stimulates the release of arginine vasopressin from hypothalamic explants (Wren et al., 2002). However, the physiological consequences of these effects remain to be established (see also Section 1.3.4). Based on studies in which NmU was delivered intracerebroventricularly to ovariectomized rats, it has also been argued that NmU inhibits the release of

luteinizing hormone through a CRH-mediated inhibition of pulsatile gonadotropinreleasing hormone release from pituitary gonadotropes (Quan *et al.*, 2003).

In addition to the central effect of NmU on the hypothalamo-pituitary-adrenal axis, *in vitro* studies have demonstrated that NmU can enhance the secretion of steroids from rat adrenal cortex in a manner dependent upon adrenal medullary chromaffin cells (Malendowicz *et al.*, 1994a and b). This is blocked by inhibitors of either ACTH or CRH, leading to the suggestion that a local ACTH/CRH system may be involved (Malendowicz *et al.*, 1994a). Long-term (6 day) treatment of rats with NmU administered subcutaneously also influences the adrenal cortex, with low concentrations directly stimulating its function and growth whilst high concentrations are inhibitory (Malendowicz *et al.*, 1994a).

The direct effects of NmU on the adrenal cortex and its effects within the hypothalamus suggest a role for NmU in the physiological responses to stress. In addition to the impact of NmU on adrenal function in rats, intracerebroventricular administration in rats or mice induces stress-related behaviours (Hanada *et al.*, 2001). These behavioural changes are blocked by either a CRH antagonist or anti-CRH IgG, whilst NmU does not cause locomotor activity in CRH knockout mice (Hanada *et al.*, 2001). CRH neurons involved in the stress response are located in both the central nucleus of the amygdala and the PVN (Wren *et al.*, 2002). However, NmU stimulates the secretion of CRH from hypothalamic explants *in vitro* and the administration of NmU into the PVN has acute effects on stress-related behaviours in rats (Wren *et al.*, 2002). Thus, the actions of NmU on arousal and stress-related behaviours are likely to be via CRH neurons within the PVN (Wren *et al.*, 2002).

1.3.4 Feeding and energy homeostasis

Since the first report that a single intracerebroventricular injection of NmU decreases food intake and feeding-associated behavior in the rat (Howard *et al.*, 2000), the ability of centrally administered NmU to decrease food intake and body weight has been demonstrated in several other studies (Kojima *et al.*, 2000; Nakazato *et al.*, 2000; Niimi *et al.*, 2001; Ivanov *et al.*, 2002; Hanada *et al.*, 2003). Intracerebroventricular injection of NmU antiserum into rats also increases food intake (Kojima *et al.*, 2000) whilst fasting reduces levels of NmU in the ventromedial hypothalamus (Howard *et al.*, 2000). Further, NmU knockout mice (NmU^{-/-}) lacking

the gene that encodes NmU develop increased body weight and adiposity, hyperphagia and a significant decrease in locomotor activity and energy expenditure (Hanada *et al.*, 2004). Thus, effects of NmU play an important role in feeding behavior and energy homeostasis.

More specifically, the effect of NmU has been mapped to particular hypothalamic nuclei. Thus, injection of NmU into either the PVN or arcuate nucleus immediately decreases food intake in rats (Wren *et al.*, 2002). NmU-R2 mRNA is expressed predominantly in the CNS (Section 1.2.5) and indeed has been localized to the PVN and arcuate nucleus in rat and mouse (Howard *et al.*, 2000; Graham *et al.*, 2003). This distribution is consistent with direct effects of NmU via NmU-R2 in these regions. Administration of NmU intracerebroventricularly or into the PVN also increases gross-locomotor activity, body temperature, heat production and oxygen consumption in rats (Hanada *et al.*, 2001 and 2003; Howard *et al.*, 2000; Nakazato *et al.*, 2000; Ivanov *et al.*, 2002; Wren *et al.*, 2002). However, NmU does not induce shivering, suggesting that the effect on body temperature is through chemical means. This generally involves sympathetic stimulation of brown adipose tissues and skeletal muscle. NmU can increase core body temperature in satiated rats in the absence of altered feeding behavior thereby indicating that the effects on feeding and energy expenditure are independent (Ivanov *et al.*, 2002).

The precise way in which the central administration of NmU regulates feeding behavior and energy expenditure is unclear although several mechanisms have been suggested. NmU induces c-Fos expression in a high proportion of oxytocin-immunoreactive neurons within the PVN and supraoptic nucleus (Niimi *et al.*, 2001). Central infusion of oxytocin reduces food intake (Olson *et al.*, 1991a and b) leading the authors to suggest that the activation of brain stem projecting oxytocin-containing neurons is responsible for the inhibitory effects of NmU on feeding. Interestingly, these authors also reported activation of arginine vasopressin-containing neurons in the PVN and supraoptic nucleus leading to the suggestion that the previously reported inhibitory effects of intracerebroventricular NmU on water intake (Howard *et al.*, 2000) are mediated by arginine vasopressin (Niimi *et al.*, 2001). This is consistent with an ability of NmU to stimulate release of arginine vasopressin from hypothalamic explants (Wren *et al.*, 2002) and increase plasma levels following central administration (Ozaki *et al.*, 2002).

Intracerebroventricular administration of CRH also inhibits feeding in rats (Morley and Levine, 1982). The suppression of food intake, the increase in oxygen consumption and the body temperature gain observed following centrally administered NmU are all absent in CRH knock-out mice (Hananda *et al.*, 2003). Furthermore, CRH containing neurons are found within the PVN and NmU stimulates the release of CRH from hypothalamic explants (Wren *et al.*, 2002) (Section 1.3.3). Thus, similar to the effects of centrally administered NmU on stress responses (Section 1.3.3), these data suggest that the effects of NmU on feeding and energy balance are via CRH.

Leptin is a protein hormone with important roles in the regulation of feeding, body weight and metabolism (Considine and Caro, 1997; Friedman and Halaas, 1998). Leptin is secreted from adipocytes and acts in the hypothalamus to mediate its anorectic effects and evidence suggests significant interactions between leptin and NmU in the control of feeding behavior. The expression of NmU mRNA is reduced both within the hypothalamic suprachiasmatic nucleus (SCN) of the leptin-deficient ob/ob strain of mice and also in the ventromedial hypothalamus of fasted rats (Howard et al., 2000). Leptin also stimulates the release of NmU from hypothalamic explants (Wren et al., 2002). Furthermore, in obese Zucker fatty fa/fa rats that lack functional leptin receptors and are hyperphagic, NmU expression is reduced within the nucleus tractus solitarius (but not the inferior olive) of the brain stem. The nucleus tractus solitarius receives information relating to gastric loading and gut nutrients and relays this to the brain stem and forebrain, regions that regulate feeding (Rinaman et al., 1998). Taken together, these data suggest that NmU release is mediated by leptin and that the synthesis of NmU is restricted in the absence of leptin. In contrast to the decrease in NmU mRNA within the SCN of ob/ob mice (Howard et al., 2000) a recent study has reported an increase within the DMH of both ob/ob mice and in fasted normal mice (Graham et al., 2003). Whether this reflects true regional differences within the hypothalamus remains to be established. Effects of leptin on hypothalamic neuropeptide signalling and the subsequent effects on feeding and body weight have been documented previously for several other neuropeptides (Inui, 1999). It is not uncommon, therefore, to associate leptin with neuropeptides that control feeding, and it is unlikely that the reduction in feeding and body weight by NmU occurs independently of leptin.

More recently however, the systemic administration of exogenous leptin to obese NmU knockout mice (NmU^{-/-}) was effective in reducing body weight by ~5%. Data were obtained from wild-type mice suggesting that NmU is not a main target of leptin's action (Hanada *et al.*, 2004). Further evidence using leptin-deficient *ob/ob* animals, leptin receptor mutant *db/db* mice and leptin receptor missense mutant Zucker fatty (*fa/fa*) rats, demonstrates how intracerebroventricular administration of NmU reduces body weight to the same extent as that of controls in these obese models (Hanada *et al.*, 2004). Thus, from this evidence, the effects of NmU and leptin in the development and regulation of obesity appear to be mutually exclusive.

Clearly, these discrepancies offer opportunities for further research, and may provide further insight into the pathophysiological development, regulation and maybe even treatment of obesity.

In contrast to the immediate effects of NmU on feeding when administered into the PVN or arcuate nucleus, it has a very delayed inhibitory effect following administration into the medial preoptic (MPO) area of the hypothalamus (Wren *et al.*, 2002). Although the MPO is involved in reproductive function, c-Fos expression and neuropeptide Y levels increase after feeding. Furthermore, α -melanocytestimulating hormone reduces food intake when administered into the MPO (Kim *et al.*, 2000) leading to the suggestion that NmU may interact with other regulators of feeding behavior to mediate its delayed effect (Wren *et al.*, 2002).

Release of adrenal corticosteroids are enhanced by NmU (Section 1.3.3) and this is associated with the promotion of pro-survival mechanisms whilst limiting other behaviours, such as feeding (Munck and Naray-Fejes-Toth, 1994; Sapolsky *et al.*, 2000). It is therefore possible that some effects of NmU on feeding, particularly longer-term effects are mediated by this loop.

1.3.5 Gastric acid secretion and gastric emptying

Many other hypothalamic peptides that influence feeding behaviour also contribute to the central regulation of gastric acid secretion and gastric emptying (reviewed in Tache *et al.*, 1990; Tache and Yang, 1990). Although intraperitoneal injection of NmU has no effect on gastric acid secretion in conscious rats, intracerebroventricular administration reduces it (Mondal *et al.*, 2003). This effect is independent of vagal innervation of the stomach. Furthermore, the effect is

independent of prostaglandin E_2 , which is known to have both direct and indirect inhibitory effects via an action on parietal cells and the inhibition of gastrin secretion respectively. However, the influence of NmU on gastric acid secretion is blocked by intracerebroventricular administration of anti-CRH IgG suggesting that NmU mediates its effects via CRH (see Section 1.3.3) and an increased sympathetic outflow. This effect is consistent with the hypothalamic expression of NmU and its receptors, particularly NmU-R2 (see Section 1.2.5). Gastric emptying is also delayed by intracerebroventricular administration of NmU and although CRH can reduce gastric emptying via the parasympathetic nervous system, this appears not to be the mechanism through which NmU acts (Mondal et al., 2003). A reduced gastric emptying is known to be associated with reduced food intake and fast gastric emptying is associated with overeating (Duggan and Booth, 1980; Kelly, 1980). Thus, the effects of NmU on gastric acid secretion and gastric emptying are consistent with its effects on food intake and energy balance (Section 1.3.4). NmU and its receptors are located within the gastrointestinal tract, including the stomach (see Sections 1.1.2 and 1.2.5), suggesting that NmU may regulate gastrointestinal function by local effects. Interestingly, compared to other secretagogues, NmU weakly stimulates histamine secretion from enterochromaffin-like (ECL) cells in vitro (Lindström et al., 1997) and in vivo (Norlen et al., 2001). The histamine released by ECL floods adjacent parietal cells to stimulate acid production and histamine release should therefore provide an indication of acid secretion.

1.3.6 Effects on ion transport

The contraluminal application of NmU-8 increases electrogenic ion transport in isolated porcine jejunal mucosa without contracting the longitudinal muscle overlying the mucosa (Brown and Quito, 1988). This action is dependent upon external chloride and is mediated by non-cholinergic enteric neurons. However, the physiological and patho-physiological roles of NmU in the gastrointestinal tract remain to be fully defined. Expression of hNmU-R1 mRNA in the goblet cells of the ileum (see Section 1.2.5) suggests that NmU also regulates secretory function and may therefore affect mucosal function (Howard *et al.*, 2000).

1.3.7 Cancer

Laser capture microdissection followed by mRNA amplification and the use of high-density oligonucleotide probe arrays has been used for cell-specific profiling of gene expression in normal oral epithelial cells and those from oral tumours. Changes in the mRNA levels of over 600 genes were associated with oral cancer including that for NmU, which was down-regulated in each of the five cases studied (Alevizos *et al.*, 2001). Furthermore, the promoter region of the NmU gene is hypermethylated in esophageal squamous cell carcinoma (ESCC) and this is accompanied by silencing at the mRNA level (Yamashita *et al.*, 2002). Out of approximately 120 genes commonly up-regulated in ESCC cell lines following the unmasking of epigenetic silencing, specific selection criteria identified 3 genes as worthy of further consideration of their tumour suppressing activity. One of these was NmU; and in colony focus assays using an ESCC cell line, the recombinant expression of NmU inhibited growth thereby highlighting its potential role as a tumour suppressor gene (Yamashita *et al.*, 2002). The gene for NmU is localized at 4q12 and this has been reported to show changes associated with cancer (Yamashita *et al.*, 2002).

In complete contrast, a further study has identified NmU as a growth-promotor of human myeloid leukaemia cells (Shetzline et al., 2004). The proto-oncogene c-Myb encodes a transcription factor, Myb, that is expressed predominantly in immature hematopoietic cells and plays a critical role in hematopoiesis (Gewirtz and Calabretta, 1988). c-Myb trans-activates target genes involved in cell proliferation, differentiation and survival. Transcription-profiling in K562 cells (a leukaemia cell line) expressing a dominant-negative Myb protein was used to identify a total of 105 Myb gene targets. Of these, NmU showed the greatest (~5 fold decrease) expression change (Shetzline et al., 2004). Both c-Myb and NmU mRNA expression were also elevated in primary acute myeloid leukaemia cells (AML), which express NmU-R1. Indeed, NmU is shown to induce a small Ca^{2+} influx in K562 cells. The significance of this was demonstrated by two further experiments. Firstly, the application of exogenous NmU 'rescued' the growth of K562-dominant-negative Myb cells, and secondly 'knock-down' of the NmU gene by small interfering RNA (siRNA) inhibited the growth of K562 cells. This clearly demonstrates expression of NmU is related to Myb and that activation of NmU-R1 constitutes a novel growth-promoting autocrine loop in myeloid leukaemia cells.

Our understanding of the role of NmU in cancer is still in its infancy, and clearly there is a long way to go to elucidate the mechanistic and functional consequences of this role. It is clear however, that the role of NmU varies between different cancers and this consideration will inevitably complicate this field of research.

1.3.8 Pro-nociception

Both NmU (see Section 1.1.2) and receptors for NmU (see Section 1.2.5) are expressed in the spinal cord. More specifically, NmU binding sites have been identified in lamina I and in the outer part of lamina II of rat spinal cord and these are associated with the expression of mRNA for NmU-R2 (Yu et al., 2003) (see Section 1.2.5). These areas are essential components in central nociceptive pathways and recent studies indicate that NmU has pro-nociceptive effects (Cao et al., 2003; Yu et al., 2003; Nakahara et al., 2004). Thus, intrathecal administration of NmU to rats resulted in a reduced threshold for mechanical stimulation, thermal hyperalgesia and behavioural responses associated with activation of nociceptive pathways including scratching, licking and biting of the lower body (Yu et al., 2003). Furthermore, NmU administration enhanced flexor α -motorneuron activity in response to touch and noxious pinch. Similarly in mice, intrathecal injection of NmU produced thermal hyperalgesia and morphine-sensitive behavioural responses whilst systemic administration in a perfused hindquarter preparation altered the electrophysiology of nociceptive dorsal horn neurons consistent with an increased basal activity and enhanced responsiveness to noxious mechanical stimulation (Cao et al., 2003). This nociceptive role has also been demonstrated in NmU knockout mice (NmU^{/-}) where nociceptive reflexes were significantly decreased when compared to wild-type mice (Nakahara et al., 2004a). Thus, NmU may be involved in spinal nociceptive transmission and processing although the precise mechanisms through which it mediates these effects remain to be determined. It is also possible that some of the behavioural responses (see particularly Section 1.3.3) are due in part to these pronociceptive effects of NmU.

1.3.9 Immune regulation

There are very specific patterns of expression of the mRNA for both NmU and NmU-R1 in immune cells in humans, strongly suggesting that NmU may play an immunomodulatory role. In particular, NmU is expressed in dendritic cells, monocytes and B cells whereas NmU-R1 is expressed in T cells and NK cells leading to the suggestion that there is an interaction between helper/antigen-presenting cells and effector populations (Hedrick *et al.*, 2000). This has yet to be explored from a functional perspective.

1.3.10 Circadian rhythm

The expression of NmU within the SCN (e.g. Howard et al., 2000, see also Section 1.1.3.1) and the involvement of the SCN in circadian rhythm (Gillette and Tischkau, 1999) led to the investigation of the role of NmU in the circadian oscillator system. Indeed, the expression of NmU-23 mRNA exhibited oscillatory rhythms within the suprachiasmatic nucleus (SCN) of rats kept in constant darkness (Nakahara et al., 2004b). Furthermore, intracerebroventricular injection of NmU caused a non-photic, dose-dependent, phase-dependent shift of the circadian locomotor activity rhythm. It did not however affect the circadian period length. Effects are likely to be via autocrine or paracrine actions as intracerebroventricular (ICV) injection of NmU increased expression of immediate early genes such as c-fos, NGF1-A and JunB within the SCN. Interestingly, the mRNA for the NmU receptors within the SCN also oscillated in a circadian manner (Nakahara et al., 2004b). Different 'peak-time' expression levels between the two receptors were noted, thus indicating that rNmU-R1 and rNmU-R2 are regulated differently. The evidence strongly suggests NmU plays some important role in circadian behaviour and further investigations are required to explore both this and the mechanism(s) underlying this regulation.

Since the discovery of NmU in the mid 1980s, progress toward elucidating the patho-physiological roles of this peptide has been comparatively slow. However, the conservation of structural elements of NmU across species and the widespread distribution of NmU and its receptors throughout the body point to a fundamental role in key physiological processes. Whilst clues have emerged from the patterns of distribution of NmU and its receptors, the complete spectrum of NmU-related activities remains to be defined. Current evidence suggests roles for NmU in pain, cancer, the immune system, the regulation of smooth muscle contraction in the gastrointestinal and genito-urinary tracts, the control of blood flow and blood pressure and the regulation of feeding and energy homeostasis and stress responses. The recent cloning of two NmU receptors from several species should considerably increase the rate of progress in defining NmU function. In addition to allowing the determination of the cellular signalling pathways and cellular functions mediated by NmU, this may also allow the development of small molecule agonists and antagonists, hopefully with receptor subtype-selectivity, that could further help to describe the roles of NmU and the receptor subtypes through which different effects are mediated. Further, the development of mice in which the genes for the receptors have been selectively deleted may greatly improve our understanding of the pathophysiological roles of NmU and may highlight the receptors as important therapeutic targets.

Chapter 2 Materials and methods

Section 2.1 Materials

HEK293 (Human Embryonic Kidney-293) cell culture reagents were from Gibco Life Technologies (Paisley, U.K.) and primary cell culture reagents were supplied by Cascade Biologics (Nottingham, U.K.). Cell culture plastic-ware was from NUNC (Roskilde, Denmark). Male Wistar rats were supplied by Charles Rivers U.K. Ltd.

Fluo-3-acetoxymethyl ester (fluo-3-AM) was supplied by TEF Labs (Austin, TX, U.S.A.) and fluo-4-AM and pluronic F-127 by Molecular Probes Ltd. (Leiden, The Netherlands). myo-[³H]-Inositol (71Ci mmol⁻¹) and [¹²⁵I]-hNmU-25 (2000Ci mmol⁻¹) ¹) were from Amersham Biosciences (Little Chalfont, Bucks., U.K.), [³H]inositol 1,4,5 tri-phosphate ($[^{3}H]$ -Ins(1,4,5)P₃) (22Ci mmol⁻¹) and $[^{3}H]$ -cAMP (34Ci mmol⁻¹) were from NEN (Boston, MA, U.S.A.) and [³⁵S]-GTPyS (1250Ci mmol⁻¹) was from PerkinElmer Life Sciences Inc. (Boston, MA, U.S.A.). Biocoat 384-well blackwalled clear-bottomed microtitre plates were from Becton Dickinson (Bedford, MA, U.S.A.). Costar polypropylene 96-well plates, Unifilter 96-well white microplates with bonded Whatman GF/B filters and Microscint 20 scintillation fluid were all supplied by Packard (Boston, MA, U.S.A.). Emulsifier-safe scintillation fluid was supplied by Packard Bioscience (Groningen, The Netherlands). Protein A Sepharose beads were supplied by Amersham Biosciences (Uppsla, Sweden) and nitrocellulose membrane (Protran) was supplied by Schleicher and Schuell (Keene, NH, U.S.A.). The monoclonal antibody specific for Ga_{0/11} (Bundey and Nahorski, 2001) was generated by Genosys Biotechnologies (Pampisford, U.K.) by inoculation of rabbits with the common C-terminal (positions 344-353) sequence (C)QLNLKEYNLV. Antibodies against Ga_{i(1-3)} (SC-410) and Ga_s (SC-823), ERK (SC-93) and phospho-ERK (SC-7383) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). nucleobond maxiprep kits were supplied by Clonetech Laboratories Inc. (CA, U.S.A.). The monoclonal α -actin and the fluorescein isothiocyanate (FITC)-tagged α mouse antibodies were both supplied by Sigma (Poole, U.K.), and the fluorescence protecting mounting medium was obtained from Dako (CA, U.S.A).

The ECL Western blotting system was from Amersham Biosciences (Little Chalfont, Bucks, U.K.). Genejuice and LipofectAMINE Plus were from Novagen (Madison, WI, U.S.A.) and Life Technologies (Paisley, U.K.) respectively. Protease inhibitor cocktail set 1 and soybean trypsin inhibitor were from Calbiochem (Nottingham, U.K.) and tetrodotoxin (TTX) was from Tocris (Bristol, U.K.).

hNmU-25 and Cy3B-pNmU-8 (see Section 2.2.9) was made 'in-house' at GlaxoSmithKline (Harlow, U.K.), and porcine NmU-8 and rat NmU-23 were supplied by Bachem Ltd. (St. Helens, U.K.).

Other reagents were supplied by either Sigma Aldrich (Poole, U.K.), Fisher Scientific (Loughborough, U.K.), Merck (Darmstadt, Germany) or BDH Laboratory Supplies (Poole, U.K.).

Section 2.2 Methods

2.2.1 Cell culture and creation of stable cell lines expressing either hNmU-R1 or hNmU-R2

hNmU-R1 and hNmU-R2 receptors were cloned by N. Elshourbagy and U. Shabon (Gene Cloning and Expression Proteomics, GlaxoSmithKline, Harlow, U.K.). Stable cells lines expressing either hNmU-R1 or hNmU-R2 were generated by E. Appelbaum and E. Dul (Gene Expression and Protein Biochemistry, Upper Merion, PA, U.S.A.). Briefly, the DNA encoding hNmU-R1 was cloned into EcoR1/EcoRV and hNmU-R2 into Asp718/Bam HI of the expression vector pCDN (Aiyar *et al.*, 1994). Constructs were transfected into wild-type HEK293 cells using LipofectAMINE Plus (as per manufacturer's instructions) and grown under geneticin selection (400µg ml⁻¹) and clonal cell lines were expanded from single foci.

Cells were maintained in Minimum Essential Medium (MEM) with Earl's Salts supplemented with 10% fetal calf serum (FCS), 5% non-essential amino acids, geneticin (400 μ g ml⁻¹) and 50 μ g ml⁻¹ gentamycin. Cells were routinely cultured in 175cm² flasks at 37°C in a 95%/5% air/CO₂ humidified environment.

Geneticin (alternative name G-418) is an aminoglycoside and acts as an elongation inhibitor of 80S ribosomes (Bar-Nun *et al.*, 1983). Incorporation of the

bacterial gene for aminoglycoside-3'-phophotransferase into the vector DNA offer cells resistance to the actions of geneticin by enzymatically digesting it.

Cell lines were passaged every 5-7 days as required, and discarded after passage 20. To passage or split cells for experimentation, the cells were dislodged by incubation (1-2min, RT) with 10ml trypsin. The trypsin was immediately neutralised by addition of 10ml growth media (with supplements) and cells were centrifuged (200g, 2min, RT). Cell pellets were re-suspended in growth media for use in subsequent experiments or cultures. HEK293 cells for experimental use in multi-wells or on coverslips were cultured on poly-D-lysine-coated surfaces. Poly-D-lysine is a synthetic molecule used as a thin coating to enhance the attachment of cells to glass or plastic. To coat coverslips or culture wells, poly-D-lysine (0.1mg ml⁻¹) was added in sufficient volumes to cover the surface of the coverslip or well. This was then aspirated and allowed to dry for over 30mins. For freezing, cells were re-suspended in FCS supplemented with 10% dimethyl sulfoxide (DMSO) and stored at -80° C.

All clonal cell lines were screened by determination of hNmU-25-mediated elevation of $[Ca^{2+}]_i$ in fluo-3-AM-loaded cells using a fluorescence imaging plate reader (FLIPR), accumulation of total inositol phosphates ($[^{3}H]$ -InsP_x), and Ins(1,4,5)P₃ production using both single-cell and population assays (see Chapter 3). Relative expression levels were examined by the binding of $[^{125}I]$ -hNmU-25 to membrane preparations using a concentration of ligand approximating to the K_d (see Chapter 3). Single clones expressing either hNmU-R1 or hNmU-R2 were selected based on both similar expression levels and approximately equivalent functional responses mediated by hNmU-25.

2.2.2 Binding of [¹²⁵I]-hNmU-25

Membrane preparation

Cells expressing either hNmU-R1 or hNmU-R2 were grown to confluency in 175cm² flasks. Cell monolayers were washed once in 5ml phosphate buffered saline (PBS) and then harvested in 10ml PBS by gentle agitation and scraping. Cells were collected by centrifugation (200g, 2min, 4°C) and re-suspended in homogenisation buffer (composition: (mM, unless otherwise stated) EDTA, 1; Tris-HCl, 10; PMSF,

1; and benzamidine 200 μ g ml⁻¹; pH 7.4). After 15min on ice, cells were homogenised (Polytron homogeniser, 20s) and centrifuged (20000g, 4°C, 10min). The pellets were re-suspended in homogenisation buffer and adjusted to 1mg protein ml⁻¹, as determined by the method of Lowry (Lowry *et al.*, 1951).

[¹²⁵I]-hNmU-25 saturation binding

Experiments were performed in assay buffer (composition: (mM, unless otherwise stated) Tris-HCl pH 7.4, 20; MgCl₂, 5; Na-EGTA, 2; and bacitracin, 0.1mg ml⁻¹) in 100 μ l volumes in a 96-well format using 10 μ g of membrane and [¹²⁵I]-hNmU-25 at 0.1–1000pM. Non-specific binding was determined using 1 μ M unlabelled hNmU-25 with a 5min pre-incubation period. After 30min at RT, 100 μ l ice-cold 0.9% NaCl was added and the suspension rapidly filtered through 0.3% polyethylenimine pre-soaked Unifilter 96-well microplates with bonded Whatman GF/B filters. Recovered radioactivity was determined by standard liquid scintillation counting.

2.2.3 Total [³H]-inositol phosphate ([³H]-InsP_x) accumulation

Generation and extraction of $InsP_x$

Cells were seeded into poly-D-lysine-coated 24-well plates in the presence of 3μ Ci ml⁻¹ of *myo*-[³H]-inositol for 48h and, if required, treated with 100ng ml⁻¹ PTX for the last 20-24h. Cells were washed twice with 1ml of Krebs'-HEPES buffer (KHB) (composition: (mM, unless otherwise stated) HEPES, 10; NaHCO₃, 4.2; D-glucose, 11.7; MgSO₄.7H₂O, 1.18; KH₂PO₄, 1.18; KCl, 4.69; NaCl, 118; CaCl₂.2H₂O, 1.29; 0.01% (w:v) bovine serum albumin (BSA); pH 7.4) (for experiments here and elsewhere Ca²⁺-free conditions were obtained by the exclusion of CaCl₂.2H₂O from the KHB) and equilibrated at 37°C for 15min with 250µl KHB containing 10mM lithium chloride (Li⁺). The inclusion of Li⁺ blocks the action of inositol monophosphatases (Berridge *et al.*, 1982), thus preventing the metabolism of inositol-1-monophosphate to inositol and resulting in the accumulation of inositol phosphate species (InsP_x). This method is used to assess the activity of PLC as the activity of PLC. A 250µl volume of KHB/Li⁺ containing hNmU-25 (or other specified agonist) at twice the desired final concentration was added to the 250µl

KHB/Li⁺ in each well and incubated at 37° C for the time period required. The reaction was terminated by the addition of 500µl ice-cold, 1M trichloroacetic acid (TCA) and the cells left on ice for ~15mins. A reproducible volume (700µl) of the contents of each well was transferred to duplicate 5ml polypropylene tubes and 250µl 10mM EDTA was added to each sample followed by 1ml of freshly prepared 1:1 (v:v) mixture of tri-n-octyl-amine and 1,1,2-trichloro-trifluoroethane. Samples were vortexed to ensure thorough mixing and left to stand for approximately 5min to allow the separation of the organic and aqueous layers of the mixture. A reproducible volume (700µl) was removed from the upper aqueous layer and transferred to duplicate 1.5ml microfuge tubes containing 50µl 250mM NaHCO₃. Samples were either processed immediately or stored at -20° C until assay.

Separation of extracts

 $[{}^{3}\text{H}]$ -InsP_x were extracted and separated by anion exchange chromatography (Willars and Nahorski, 1995). Here, samples were processed through gravity-fed columns containing strongly basic Dowex chloride anion exchange media (1X8-200). Following addition of samples, columns were washed with 10ml milli-Q-grade H₂O, and then 12ml 25mM ammonium formate. The flow-through of both was discarded. $[{}^{3}\text{H}]$ -InsP_x were eluted by the addition of 10ml 1M HCl and collected in 25ml scintillation vials. A 3ml aliquot was mixed with 15ml scintillation fluid and the amount of ${}^{3}\text{H}$ -radioactivity determined by standard liquid scintillation counting. Dowex chloride columns were regenerated by washing with 10ml 2M HCl followed by10ml H₂O.

Data analysis

All data points were calculated as a percentage increase from basal levels of $[^{3}H]$ -InsP_x. Here, wells were challenged with agonist-free KHB/Li⁺ for the longest time point of the experiment, and samples were extracted and processed exactly as described above.

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2.2.4 Ins(1,4,5)P₃ mass generation

Generation and extraction of $Ins(1,4,5)P_3$

Ins(1,4,5)P₃ generation in cell populations was achieved using methods described previously (Challiss *et al.*, 1990). Cells were seeded into poly-D-lysine-coated 24-well plates and grown to confluency. Media was aspirated and cells were washed twice with 1ml KHB and incubated at 37°C for 15min with 200µl KHB. A 50µl volume of KHB containing hNmU-25 at five times the desired final concentration was added to the 200µl KHB in each well and incubated at 37°C for the time period required. Reactions were terminated by the addition of 250µl of ice-cold 1M TCA and cells placed on ice for ~15min. A 400µl aliquot from each well was transferred to duplicate 5ml polypropylene tubes where 80µl of 10mM EDTA and 400µl 1:1 (v:v) freshly prepared mixture of tri-n-octyl-amine and 1,1,2-trichloro-trifluoroethane were added. Samples were thoroughly vortexed and left to stand for 5min to allow separation of aqueous and organic layers. A reproducible volume of 200µl from the upper aqueous phase was transferred to duplicate microfuge tubes containing 50µl 60mM NaHCO₃. Samples were either assayed for Ins(1,4,5)P₃ immediately or stored at -20° C until processed.

Preparation of $Ins(1,4,5)P_3$ binding protein

Binding protein used in these studies was kindly provided by Tim Werry (Department of Cell Physiology and Pharmacology, University of Leicester). The binding protein was prepared from bovine adrenal glands using methodology previously described (Challiss *et al.*, 1990). Briefly, the cortical region of bovine adrenal glands were diced by sharp dissection and mixed with buffer (composition (mM): NaHCO₃ 20, dithiothreitol (DTT) 1mM, pH 8.0, 4°C). The resultant tissue pieces were homogenised and centrifuged (3000g, 10mins, 4°C). The supernatant was collected and subject to a further centrifugation (40 000g 20min, 4°C) to pellet the binding protein. The binding protein was re-suspended in buffer at a concentration of 15-25 mg protein ml⁻¹ as determined by the method of Lowry (Lowry *et al.*, 1951) and stored at -20° C.

$Ins(1,4,5)P_3$ radioreceptor assay

 $Ins(1,4,5)P_3$ concentration in samples was determined using a previously described radioreceptor assay (Challiss et al., 1990; Willars and Nahorski, 1995). Here, $Ins(1,4,5)P_3$ competes with [³H]-Ins(1,4,5)P_3 for binding to the binding protein, and is related to standard concentrations. Standard concentrations of $Ins(1,4,5)P_3$ ranging from 0.3nM - 10µM were prepared in KHB buffer. [³H]-Ins(1,4,5)P₃ (34Ci mmol⁻¹) was diluted in water to give a final assay concentration of 3.6nM. Samples were assayed on ice in 1.5ml microfuge tubes by the addition of 30µl sample or standard, 30µl of [³H]-Ins(1,4,5)P₃ and 30µl assay buffer (composition: (mM) Trisbase, 100; EDTA, 4; pH 8.0, 4°C). The reaction was activated by the addition of 30µl binding protein and incubated on ice for 30min. Samples were diluted in 1ml washing buffer (composition (mM); Tris-base, 25; EDTA, 1; NaHCO₃, 5; pH 8.0, 4°C) and rapidly filtered through Whatman GF/B filters that had been pre-soaked in washing buffer. Tubes were rapidly washed a further two times by addition of 5ml washing buffer and filtering. Filters were added to 5ml scintillation fluid and left overnight. ³H-radioactivity was determined by standard liquid scintillation counting methods.

Data analysis

Standard curves were fitted using GraphPad Prism software (GraphPad Software Inc. CA, U.S.A.) and the $Ins(1,4,5)P_3$ content of samples determined by interpolation. All dilutions were taken into consideration and the final concentration of $Ins(1,4,5)P_3$ was related to protein content that was determined in duplicate cell monolayers in 24-well plates. Here, cells were diluted in 1ml 1M NaOH and protein determined by the method of Lowry (Lowry *et al.*, 1951).

2.2.5 Single cell imaging of PLC activation

The vector containing the fusion construct between the enhanced green fluorescent protein (eGFP) and the pleckstrin homology (PH) domain of $PLC_{\delta 1}$ (eGFP-PH_{PLC\delta1}) was generously provided by Professor T. Meyer (Stanford University, CA, U.S.A) and used to monitor PLC activity in single cells as described previously (Nash *et al.*, 2001) (see also Chapter 3).

Transfection of eGFP-PH_{PLC&1} into HEK293 cells

Cells were seeded onto 25mm poly-D-lysine-coated coverslips in 6-well plates at low density and allowed to adhere for 12-20h. The vector containing the eGFP- $PH_{PLC\delta1}$ cDNA was transfected into hNmU-R1, hNmU-R2 and wild-type HEK293 cells using Genejuice transfection reagent as per the manufacturer's instructions. Briefly, 3µl Genejuice was incubated with 100µl HEK293 cell culture media (without any supplements or antibiotics) for 5mins at RT. This was then incubated with 1µg DNA for 15mins at RT. The transfection mix was then added slowly to cells and incubated for a further 48h in normal culture conditions.

Imaging eGFP-PH_{PLC $\delta l}$ to establish PLC activity</sub>

Coverslips were mounted onto the stage of an Ultra*VIEW* confocal microscope (PerkinElmer Life Sciences, Cambridge, U.K.) with a X40 oil emersion objective and excited at 488nm using a Kr/Ar laser. Emitted light was collected above 510nm and images captured at approximately 1 frame sec⁻¹. The chamber volume was maintained at approximately 0.5ml and perfused (5ml min⁻¹) with KHB heated to 37° C with a Peltier unit.

Cells were challenged with 500µl 10nM hNmU-25 following a 30s basal recording. When cells were exposed to hNmU-25, perfusion was stopped and additions made directly to the cell chamber to give a final concentration of 10nM.

Data analysis

Cytosolic fluorescence provides an index of $Ins(1,4,5)P_3$ levels and hence PLC activity (see Chapter 3). Changes in the cytoplasmic fluorescence of 6-10 cells chosen at random from the field of view were recorded and averaged by purpose written Ultra*VIEW* software. This was represented graphically as the fold increase in cytosolic fluorescence relative to basal levels (average of the 30s prior to agonist addition).

2.2.6 [Ca²⁺]_i measurements

The use of confocal microscopy has permitted the investigation of Ca^{2+} signals at the single-cell and sub-cellular level. This is feasible through the use of fluorescent indicators sensitive to changes in $[Ca^{2+}]$ where changes in $[Ca^{2+}]_i$ are determined by

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changes in the fluorescence intensity of the indicator. Here we use the Ca^{2+} indicator fluo-3-AM, an acetoxymethyl ester derivative of fluo-3, with the AM portion making the compound membrane-permeant. Thus it can be loaded into cells via incubation. Because of the relatively low water solubility of the AM ester, pluronic F-127, a mild detergent, is used here as a dispersing agent to facilitate the loading. Fluo-3-AM itself is readily hydrolyzed to fluo-3 by endogenous esterases once the dye is inside the cells. The Ca^{2+} bound form of fluo-3 is ~40 times brighter than the Ca^{2+} -free form (Minta *et al.*, 1989), thus making it suitable for the study of Ca^{2+} changes. Further, fluo-3 has a low level of fluorescence at resting cytoplasmic [Ca^{2+}] ensuring a large dynamic range for detecting Ca^{2+} signals of varying size (Thomas *et al.*, 2000).

Confocal Ca²⁺ imaging

Methods used to determine single-cell changes in $[Ca^{2+}]_i$ was as previously described (Werry *et al.*, 2001). Briefly, cells were seeded onto 25mm poly-D-lysinecoated coverslips and cultured for 24-48h. Cells were washed twice with 1ml KHB before being loaded with fluo-3-AM. Loading conditions were optimised where indicated (see Chapter 4), but the following protocol was used for HEK293 cells. Cells were incubated for 1h at RT in KHB containing 5µM fluo-3-AM and 0.044% (w:v) pluronic F-127. Cells were washed once with 1ml KHB at 37°C and coverslips were mounted in a chamber on the stage of an Ultra*VIEW* confocal microscope (PerkinElmer Life Sciences, Cambridge, U.K.) with a X40 oil emersion objective lens. Cells were excited at 488nm using a Kr/Ar laser and emitted light collected above 510nm. Confocal images were captured at a rate of approximately 1 frame sec⁻¹. The chamber volume was maintained at approximately 0.5ml and perfused (5ml min⁻¹) with KHB heated to 37°C with a Peltier unit.

Addition of agonist and other test reagents was via a bath application in the absence of perfusion and followed a 30s period of basal recording where cells remained unchallenged.

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Data analysis

Changes in cytosolic fluorescence provide an index of changes in $[Ca^{2^+}]_i$ and are expressed as the fold change in cytosolic fluorescence relative to basal levels (average of the 30s prior to agonist addition).

Fluorometric Imaging Plate Reader (FLIPR)

The calcium sensitive dye fluo-4-AM is similar to fluo-3-AM, but designed to have a higher level of fluorescence at resting $[Ca^{2+}]$. The increased sensitivity make the use of fluo-4-AM ideal for the measurement of global or population Ca^{2+} signals.

Cells were seeded into 384-well microtitre plates at a density of 10000 cells well⁻¹ and cultured for 24h. Cell counts were achieved by counting particles of 9.5-30 μ m with a Beckman Coulter Z-Series Cell Counter (Beckman Coulter, Bucks, U.K.). Following loading (1 μ M fluo-4-AM in KHB for 1h at 37°C), cells were washed 3 times and incubated in KHB for 10min at 37°C before assay on FLIPR (Molecular Devices Ltd, U.S.A). The addition of agonist was fully automated by the FLIPR and involved the transfer of 10 μ l from an agonist addition plate into the cell plate 10s after initiation.

Data analysis

The response following agonist addition was taken as the maximum fluorescence intensity units (F.I.U) less the minimum immediately prior to addition.

2.2.7 [³⁵S]-Guanosine 5'-[γ-thio]tri-phosphate determination of G-protein activation

The propagation of intracellular signals following activation of GPCRs begins with the binding of GTP to an activated G-protein (see Introduction, section 1.2.2). The radiolabelled, non-hydrolysable GTP analog [35 S]-guanosine 5'-[γ -thio]triphosphate ([35 S]-GTP γ S), was used to directly assess the G-proteins that couple to the hNmU-receptors. The technique employs anti-sera raised against specific G-proteins and Protein A Sepharose beads. These protein beads bind to the Fc portion (constant fragment portion) of IgG antibodies. The amino acid sequences of Fc portions are identical for all IgG antibodies. The [35 S]-GTP γ S-G-protein, antibody and Protein A Sepharose bead complex is collected by centrifugation and an increase

in [35 S]-GTP γ S binding used to positively identify G-protein coupling. The methods used here are essentially the same as those described elsewhere (Akam *et al.*, 2001).

Membrane preparation

Cells were grown to confluency in 175 cm^2 flasks. Growth media was removed and cells were washed once with 10ml PBS. Cells were harvested by incubation with 10ml PBS (5min, RT), gentle agitation and if necessary scraping. From hereon all subsequent actions were performed at 0-4°C. The resultant suspension was centrifuged (200g, 5min, 4°C) and the pellet re-suspended and homogenised (Polytron homogeniser, ~20s) in 10ml lysis buffer (composition: HEPES, 10mM; EDTA, 10mM; pH 7.4). This suspension was subject to a further centrifugation step (30000g, 15min, 4°C) and the final pellet homogenised in 10ml freezing buffer (composition (mM) HEPES, 10; EDTA, 0.1; pH 7.4). Protein concentration was adjusted to 1mg ml⁻¹ as determined by the method of Lowry (Lowry *et al.*, 1951) and membrane preparations were stored at -20°C.

$[^{35}S]$ -GTPyS binding and precipitation of G α -subunits

All solutions used throughout the binding assay were diluted in assay buffer (composition (mM): HEPES, 10; NaCl, 100; MgCl₂, 10; pH 7.4). Microfuge tubes containing either 1µM (for $G\alpha_{q/11}$), 10µM (for $G\alpha_i$ and $G\alpha_s$), or no ($G\alpha_{12}$ and $G\alpha_{13}$) GDP and 1nM [³⁵S]-GTPyS were pre-warmed to 37°C. The incorporation of GDP in the assay aids in the reaction of the GDP-GTP exchange following receptor activation. The fact that assays relating to different Ga subunits contain different GDP concentrations owes to the differing levels of expression of the Ga-subunits in HEK293 cells. The concentrations used here were optimised to promote GDP-GTP exchange in HEK293 cells (Akam et al., 2001). Where appropriate, the tubes contained 10µM GTPyS to determine non-specific binding and/or 10nM hNmU-25 (or other test agonist). The assay volume of each tube was adjusted to 75μ l by addition of assay buffer, and the contents allowed to equilibrate at 37°C for ~5min. The reaction was initiated by the addition of 25µg (25µl) of the membrane preparation, and allowed to proceed for 2min at 37°C. The reaction was rapidly terminated by addition of 1ml ice-cold assay buffer and incubation on ice. Membranes were separated by centrifugation (~20000g, 6min, 4°C) and all

subsequent experimental steps were performed at $0-4^{\circ}$ C. The supernatant was carefully aspirated and the pellet solubilised by addition of 50μ l ice-cold solubilisation buffer containing sodium dodecyl sulphate (SDS) (composition: (mM, unless otherwise stated) Tris, 100; NaCl, 200; EDTA, 1; 1.25% (v:v) Igepal CA 630; 0.2% (w:v) SDS; pH 7.4) and repeated vortex mixing. Once membranes were in solution a further 50µl solubilisation buffer (less SDS) was added to each sample.

The solubilised membranes were subject to a pre-clearance step. Here, 13μ l of normal rabbit serum (diluted at 1:100), and 30μ l Protein A Sepharose beads (3% (w:v) dilution of Protein A Sepharose beads prepared in TE buffer (composition: Tris, 1mM; EDTA, 1mM)) were added to the solubilised membranes and incubated by rolling for 1h at 4°C. The Protein A Sepharose beads and any insoluble materials were collected by centrifugation (20000g, 6min, 4°C) and a 100µl aliquot of the supernatant was transferred to duplicate tubes containing 5µl of the appropriate Gαspecific antiserum (1:100 dilution for all antibodies) (see Materials, section 2.2 for details on antibody). Samples were vortexed and rolled overnight at 4°C.

The following day, 70µl of Protein A Sepharose beads were added and samples rolled for a further 90min at 4°C. Complexes containing protein A Sepharose, the antibody and the [35 S]-GTPγS-Gα-subunit were colleted by centrifugation (20000g, 6min, 4°C). The supernatant was removed by slow aspiration and the beads washed by repeated (3 times) addition of 1ml solubilisation buffer (less SDS) and centrifugation as before. Following the final wash, the pellet was vortex mixed with 1ml scintillation fluid and radioactivity left to extract overnight. The level of 35 S in each sample was determined by standard liquid scintillation counting methods.

2.2.8 Inhibition of FSK-induced cAMP accumulation

Agonist and FSK stimulation

Determination of cellular levels of cAMP used methods described previously (Challiss *et al.*, 1990). Cell monolayers in poly-D-lysine-coated 24-well plates were washed with 1ml KHB and incubated at 37° C for 10min with 1ml KHB. Buffer was aspirated and replaced by 200µl of buffer containing agonist at the required concentration. Following 10min incubation at 37° C, a further 50µl of buffer containing both agonist at the required concentration and FSK (final concentration 10µM) was added. Following a further 10min incubation at 37° C, buffer was

removed and reactions terminated with 500µl ice-cold 0.5M TCA. This order of addition, as well as the incubation times, was found to be optimum in producing the largest inhibition of FSK-elevated cAMP in hNmU-R1 expressing HEK293 cells based on a maximum stimulation (10nM hNmU-25) (see Chapter 3).

Where appropriate, cells were treated with 100ng ml⁻¹ PTX for 20-24h prior to stimulation.

cAMP extraction

The cAMP was extracted using a method identical to that for the extraction of $Ins(1,4,5)P_3$ (see Section 2.2.4).

Preparation of cAMP binding protein

The cAMP content was determined using a radioreceptor assay with bindingprotein purified from bovine adrenal glands (Brown *et al.*, 1971). Thanks again goes to Tim Werry (Department of Cell Physiology and Pharmacology, University of Leicester) for the production of the cAMP binding protein. Briefly, 10-12 adrenal glands were dissected as described for $Ins(1,4,5)P_3$ binding protein (see Section 2.2.4) and homogenised with ice-cold cAMP buffer (composition: Tris-HCl, 50mM; EDTA, 4mM; pH 7.4). The resultant homogenate was centrifuged (15000g, 4°C, 15min) and the supernatant collected following filtration through muslin. The pellet was re-homogenised and centrifuged as before, and again the supernatant collected following filtration. Pooled supernatant from this final spin was again collected following filtration through muslin. This was stored at -20° C in 500µl aliquots. At the time of assay, the aliquots were diluted to 15ml in assay buffer and repeatedly filtered through a fine syringe needle before use.

cAMP radioreceptor assay

cAMP standards ranging from 0-8 pM were prepared in cAMP assay buffer. The assay mix contained 50 μ l standard or sample, 100 μ l [³H]-cAMP (34Ci mmol⁻¹) (diluted 2 μ l in 8ml cAMP assay buffer) and 150 μ l binding protein (added last to initiate the reaction). These were vortexed and incubated on ice for 90min. Bound [³H]-cAMP was separated from free [³H]-cAMP by a charcoal/BSA buffer

(composition: 50ml cAMP assay buffer, 0.25% (w:v) charcoal powder, 0.1% (w:v) BSA, 4°C). A volume of 250µl was added to each tube, vortexed and incubated on ice for 12min. This was then centrifuged (10000g, 4min, 4°C) and the [³H]-cAMP content of a 400µl aliquot of the supernatant was measured using standard liquid scintillation methods.

Data analysis

Standard curves were fitted using GraphPad Prism software and the cAMP content of samples was interpolated from the standard curve. All dilutions were taken into consideration and the final concentration of cAMP was related to protein content. Here, duplicate cell monolayers in 24-well plates were diluted in 1ml of 1M NaOH and protein determined by the method of Lowry (Lowry *et al.*, 1951).

2.2.9 Generation of fluorescently labelled porcine NmU-8 (Cy3B-pNmU-8) and binding to cells expressing either hNmU-R1 or hNmU-R2

Generation of NmU-8-Cy3B:

Cy3B was attached to the N-terminus of porcine(p) NmU-8 (pNmU-8) using Cy3B-NHS ester (Amersham, U.K.), following standard conditions as recommended by the manufacturer. The product (pNmU-8-Cy3B) was purified by C18 reversephase HPLC, and mass confirmed by MALDI. Special thanks goes to J. Scott and M. Ruediger (GlaxoSmithKline, Harlow, U.K.) for generating and kindly providing pNmU-8-Cy3B.

Imaging of NmU-8-Cy3B

Cells were seeded onto 25mm diameter poly-D-lysine-coated glass coverslips and cultured for 24-48h. Growth media was aspirated and cells were washed with 1ml KHB. The coverslips were mounted onto the stage of an Ultra*VIEW* confocal microscope (PerkinElmer Life Sciences, Cambridge, U.K.) with a X40 oil emersion objective lens. Cells were excited at 568 nm using a Kr/Ar laser and emitted light collected with a broad band RGB emission filter. pNmU-8-Cy3B was added via bath application at a concentration of 10nM (unless otherwise stated) and images were taken at a rate of approximately 1 sec⁻¹. Where appropriate, KHB was perfused over the cells at a rate of 5ml min⁻¹. Temperature was controlled at 37°C with a Peltier unit, or at 12°C with a Peltier unit and perfusion of ice-cold buffer.

2.2.10 Determination of ERK activation

Receptor activation and cell solubilisation

Cells in poly-D-lysine-coated 24-well plates were washed and equilibrated in KHB at 37°C. Cells were stimulated with 10nM hNmU-25 and reactions terminated by aspiration and addition of ice-cold solubilisation buffer (composition: (mM, unless otherwise stated) Tris, 100; EDTA, 10; NaCl, 150; 1% (v:v) NP40; 0.1%; (w:v) SDS; 5mg ml⁻¹ deoxycholic acid; 200µg ml⁻¹ benzamidine; PMSF, 1; and protease inhibitor cocktail; pH 7.4). Cell lysates were pre-cleared by centrifugation (10000g, 10min, 4°C) and supernatant was adjusted to 3mg protein ml⁻¹ as determined by the method of Lowry (Lowry *et al.*, 1951).

Where appropriate, cells were treated with 100ng ml⁻¹ PTX for 20-24h prior to stimulation and solubilisation.

Western blotting

A 30µl aliquot of each sample was mixed with an equal volume of sample buffer (composition; Tris-HCl, 100mM; DTT, 200mM; 4% (w:v) SDS; 0.1% (w:v) bromophenol blue; 20% (v:v) glycerol) and boiled for 10min. A 20µl (20µg protein) volume of boiled sample was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% running gel at 200V for ~45min (running buffer composition: Tris-HCl; pH 7.4, 25mM; glycine, 150mM; 0.1% (w:v) SDS). The molecular mass of proteins was determined by running pre-stained proteins of known molecular mass.

Proteins were transferred from gels to nitrocellulose membranes using semi-dry apparatus at $0.65 \text{ mA} \text{ cm}^2 \text{ gel}^{-1}$, for ~60min in blotting buffer (composition: Tris-HCl; pH 7.4, 48m; glycine, 39mM; 0.037% (w:v) SDS; 20% (v:v) methanol). Transferred proteins were blocked overnight at 4°C in TBS-Tween (composition; Tris-base, 20mM; NaCl, 500mM; 0.05% (v:v) Tween-20) containing 5% (w:v) dried milk. Membranes were then incubated with rabbit polyclonal anti-sera specific for ERK (diluted 1:1000) in 5ml TBS-Tween for 2h at RT with gentle rocking. Membranes were washed 3x 10min in excess TBS-Tween before incubation with horseradish

peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (1:1000 dilution) in 5ml TBS-Tween for 60min at RT with gentle rocking. The membranes were then washed as described before. The ECL reagents kit from Amersham (Bucks, U.K.) was used (according to the manufacturer's instructions) to develop the blot.

Blots were then stripped (see below) and re-probed with mouse polyclonal antisera specific to phosphorylated (activated) ERK (pERK) (1:1000 dilution). The experiment proceeded as described previously with the exception of the use of an anti-mouse HRP-conjugated secondary antibody (diluted 1:1000).

Stripping of blots

Stripping was achieved by incubation at 50°C in stripping buffer (composition; 2mercaptoethanol, 100mM; Tris-HCl, 62.5mM; 2% (w:v) SDS; pH 6.7) for 30min. Blots were then washed with excess TBS-Tween 3x 10min, and re-blocked as described earlier.

Data analysis

Densitometric analysis of the autoradiographs was achieved with a Syngene (Cambridge, U.K.) Bio Imaging System and Genesnap-GeneGnome software (Syngene, Cambridge, UK) using only the density of p38 ERK (ERK 1) against which the antibody was raised.

2.2.11 Smooth muscle cell dissociation and culture

The method of smooth muscle cell dissociation described here is an adaptation of a protocol originally optimised for the dissociation of pig coronary artery smooth muscle cells (Quayle *et al.*, 1996) and has successfully been transferred to the rat mesenteric artery (Lewis *et al.*, 2000) and mouse bladder (Vial *et al.*, 2000). Tissues used here were the distal colon and fundus. Male Wistar rats (<300g) were culled by concussion followed by cervical dislocation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act, 1986. A midline incision was made and 1.5-2cm of appropriate tissue was removed and immediately washed in dissociation buffer (composition (mM): NaCl, 137; KCl, 5.4; HEPES, 10; MgCl₂, 1; Na₂HPO₄, 0.44; NaH₂PO₄, 0.4; NaHCO₃, 4.2; pH 7.4). The tissue was diced into 1-2mm pieces by sharp dissection and subjected to a double enzymatic digestion with DTT and papain (22 units mg⁻¹) (enzyme 1) followed by collagenase (Type F, 2.2 FALGPA units mg⁻¹) and hyaluronidase (330 units mg⁻¹) (enzyme 2) as depicted below.



Smooth muscle cells were dissociated by mechanical sheer via repeated trituration through fire-polished glass Pasteur pipettes and immediately centrifuged (500g, 3min, RT). Cells were re-suspended in medium 231 supplemented with smooth muscle growth supplement (SMGS) and $50\mu g \text{ ml}^{-1}$ gentamycin. Cells were cultured directly onto untreated 25mm glass coverslips in 6-well plates (2ml cell suspension well⁻¹) at $37^{\circ}C/5\%CO_2$ and were never passaged.

2.2.12 Immunocytochemical analysis of a-actin staining

Immunocytochemistry

Smooth muscle cells were cultured on glass coverslips for up to 15 days as described above. Cells were washed 3 times with PBS (without Mg^{2+} or Ca^{2+}), permeabilised and fixed by incubation with 100% methanol (10min, -20°C). Cells were washed a further 3 times as before. The α -actin primary antibody was diluted in 10% goat serum (v:v) in PBS at 1:500, and incubated with cells overnight at 4°C. The following day, cells were washed and incubated with FITC-tagged goat antimouse antibody (diluted 1:200 in PBS/10% goat serum) for 2h at RT in the dark before being washed and fixed onto glass-slides with Dako fluorescence protecting mounting medium. These were left to dry before being sealed with nail varnish and stored at 4°C until imaged.

Imaging

Coverslips were mounted onto the stage of an Ultra*VIEW* confocal microscope with a X40 oil-emersion objective lens. Cells were excited with the 568nm line of a Kr/Ar laser and emitted light collected with a broad band RGB emission filter. Images were captured with purpose written software.

HUVECs

Human umbilical vein endothelial cells (HUVECs) were generously provided by Laura Sampson from the Department of Biochemistry, University of Leicester and were routinely cultured in HUVEC media; Medium 199 with Glutamax, 20% FCS, 2.4 U ml⁻¹ heparin, 50ug ml⁻¹ endothelial cell growth supplement (ECGS) in 75cm² flasks at 37°C in a 5% CO₂/95% O₂ environment. At time of assay (passage 10) HUVECs were cultured onto 1% gelatin-coated 25mm glass coverslips and incubated overnight. HUVECs were stained with α -actin and imaged on the confocal microscope exactly as described for rat cultured SMCs.

2.2.13 Isolated tissue contraction

Isolation of the rat distal-colon

Male Wistar rats (<300g) were culled by CO_2 asphyxiation and cervical dislocation in accordance with Schedule 1 of the U.K. Animals (Scientific Procedures) Act, 1986. A mid-line incision was made and 2-3cm of the distal colon was removed and placed immediately into KHB (composition; (mM) NaCl, 121.5; CaCl₂, 2.5; KH₂PO₄, 1.2; KCl, 4.7; MgSO₄, 1.2; NaHCO₃, 25; glucose, 5.6; pH 7.4; pre-gassed with 5% CO₂/95% O₂ for 15min at RT). The tissue was washed in KHB and opened longitudinally by sharp-dissection. Submucosa and mucosa were removed by gentle peeling and sharp dissection, and full-wall thickness preparations of rat distal-colon were achieved by cutting tissue strips (approximately, 15 x 6mm) parallel to either circular-muscle or longitudinal-muscle as required

Tissue contraction

Methods for the determination of isolated tissue contraction are an adaptation of those described previously (Dass et al., 2003a). Tissues were suspended under 1g tension between two parallel platinum ring recording electrodes in 5ml tissue baths containing KHB continually bubbled with 5% CO₂/95% O₂ at 37°C. Tensioncontraction analysis of the rat distal colon, based on 100µM carbachol (CCh)mediated stimulation, revealed optimum contractions, of both circular and longitudinal muscle, when the tissue was suspended under 1g tension (see Chapter 6). Tissues were allowed to equilibrate for 60min during which time bath solutions were changed once every 15min. NmU and other test-agents were applied by means of a direct tissue-bath addition and tension was measured using a Pioden dynamometer UF1 force-displacement transducer (Pioden Control Ltd, U.K.). Concentration-contraction analysis was achieved non-cumulatively, with individual doses being applied in a random order and separated by a 30min wash whereby the tissue-bath solution was replaced twice (t=0min, and t=15min). Any changes in the washing procedure are indicated throughout the text. Where appropriate, experiments were performed in the presence of 1µM atropine or following 5µM tetrodotoxin (TTX) 15min pre-treatment.

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Data analysis

Isolated tissue contraction data was acquired and analysed using MP100 hardware and AcqKnowledge software (Biopac Systems, Inc., U.S.A.). The effects of NmU on resting muscle tension were expressed as a percentage of the contractile response mediated by 100µM CCh (maximally effective, see Chapter 6).

2.2.14 Generation of cDNA encoding rat NmU receptors and transient expression in HEK293 cells

cDNA encoding either rNmU-R1 or rNmU-R2 was sub-cloned into EcoRI and EcoRI/HindIII restriction sites respectively of the mammalian expression vector pCDN (Aiyar *et al.*, 1994). cDNA was transformed into HEK293 cells that were grown as described previously (see Methods, Section 2.2.1). cDNA was purified using Clontech MaxiPrep kits according to manufacturer's instructions and concentration was adjusted to 1 mg ml⁻¹. cDNA was transiently transfected into wild-type HEK293 cells using LipofectAMINE Plus according to the manufacturer's instructions. Cells were used in FLIPR experiments (see Section, 2.2.6).

2.2.15 Determination of [¹²⁵I]-hNmU-25 degradation.

TCA protein-precipitation is a commonly used method for the determination of the degradation of a variety of proteins (e.g. Vekrellis *et al.*, 2000; Pérez *et al.*, 2000). This method works on the principle that TCA, by interacting with peptides, induces an increase of the hydrophobicity of peptides that can lead to aggregation through hydrophobic interactions (Yvon *et al.*, 1989). Thus, TCA will only precipitate non-degraded proteins.

TCA precipitation was used here to assess the degradation of [¹²⁵I]-hNmU-25 in homogenates prepared from various tissues from rats. Male Wistar rats (<300g) were culled in accordance with the Animals and Scientific Act, 1986, by concussion followed by cervical dislocation. A mid-line incision was made and 4-5cm of the distal colon was removed. A longitudinal incision was made along the entire length of the colon and the tissue was immediately washed in KHB. The majority of fatty and vascular tissue was removed by peeling and sharp dissection under a light microscope. The tissue was placed in 5ml fresh KHB and cut into small (1-2mm)

pieces before being homogenised for 20-30s (or until all large pieces of tissue had disappeared). Tissues were diluted to a stock concentration of 4g ml⁻¹ as determined by the methods of Lowry (Lowry *et al.*, 1951) and diluted as needed. This investigation also involved the use of smooth muscle from the fundus, and hind leg extensor skeletal muscle. These were removed and hogenised exactly as described above.

Tissue homogenate (250µl) was incubated with 150pM [125 I]-hNmU-25 (final concentration) in a final volume of 250µl for 0-30min at 37°C. The experiment was terminated by the addition of 500µl ice-cold 1M TCA, and samples were incubated on ice for 15min. Samples were then centrifuged (10000g, 10min, 4°C) and the amount of 125 I radioactivity in both the pellet and the supernatant were determined by standard liquid scintillation methods.

Where appropriate, the tissue homogenate was 'heat inactivated' by boiling for 10min.

2.2.16 Internalisation of [¹²⁵I]-hNmU-25 in cell populations

Cells expressing hNmU-R1 were seeded into poly-D-lysine coated 6-well plates and cultured for 24h. On the day of assay, cells were washed twice with 1ml KHB and equilibrated in 200µl KHB at 37°C. Cells were challenged with 50µl KHB containing [¹²⁵I]-hNmU-25 to give a final assay concentration of 150pM at 37°C for between 0 and 60min. At the desired time point, extracellular KHB and un-bound [¹²⁵I]-hNmU-25 were aspirated and cells washed immediately with 1ml KHB pH 2.0. A single 1ml wash with this acidified KHB was shown to be sufficient in removing ~95% of bound [¹²⁵I]-hNmU-25 from hNmU-R1 expressing cells (see Chapter 6). The KHB pH 2.0 wash was rapidly aspirated and replaced with 1ml 1M NaOH to solubilise cells. Radioactivity in the solubilised cell extract was determined by standard liquid scintillation methods. All data points were calculated as 100 less the % of cell-surface bound [¹²⁵I]-hNmU-25. This was determined by the addition of 150pM [¹²⁵I]-hNmU-25 and incubation at 4°C for 2h. For this, extracellular KHB and un-bound [¹²⁵I]-hNmU-25 were aspirated and cells immediately solubilised as above. Cells were not washed.

2.2.17 Protein determination

Protein concentrations were determined using the method of Lowry (Lowry *et al.*, 1951). This works on the principle that divalent copper ions complex with peptide bonds and are reduced to monovalent ions. Monovalent copper ions and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that is reduced to molybdenum/tungsten blue.

Briefly, 500µl sample or standard (prepared from BSA and diluted in 0.1M NaOH to give a range of concentration 0-400µg ml⁻¹) were mixed with 1ml of a freshly prepared mixture containing 98% of solution A (2% (w:v) Na₂CO₃ in 0.1M NaOH), 1% solution B (1% (w:v) CuSO₄) and 1% solution C (2% (v:v) Na⁺K⁺tartrate) and incubated at RT for 10min. A further 100µl of Folin reagent was added to each tube, and tubes were vortexed and allowed to stand for 20min at RT. The contents were transferred to cuvettes and the absorbance determined at 750nm.

Protein concentrations in samples were calculated by interpolation of values from a standard curve constructed from the protein standards fitted by a third polynomial equation.

2.2.18 Data analysis

All concentration response curves were fitted using a four-parameter logistic equation analysed in GraphPad Prism software and pEC₅₀ (negative logarithm of the concentration required to bring about half (50%) of the maximal response) were obtained from these graphs. Analysis of saturation binding was achieved using sigmoidal dose-response non-linear regression (GraphPad Prism). The B_{max} (the density of receptors in the membrane preparation, expressed as fmol mg protein⁻¹) and K_d (concentration of radiolabeled drug that produces 50% of the maximum occupancy) were obtained from these graphs.

The data shown are the mean \pm standard error of the mean (s.e.m.) (n=3; unless otherwise stated) of data from separate experiments each with duplicate observations. For representative data, experiments were also performed to an *n* of three or more. Variance in this case is calculated from specific time points and detailed in appendix 1 as mean \pm s.e.m., n=3. Errors for all experiments were determined from the mean of the values from individual experiments.

Chapter 3

Selection of clonal cell lines expressing hNmU-R1 and hNmU-R2 and characterisation of receptor-mediated signalling

Section 3.1 Introduction

The human receptors for NmU were cloned and sequenced in 2000 via reversepharmacology and mass screening. Several groups reported NmU as the cognate ligand for a previously orphan GPCR; FM3 (or GPR66) (Fujii *et al.*, 2000; Hendrick *et al.*, 2000; Hosoya *et al.*, 2000; Raddatz *et al.*, 2000; Shan *et al.*, 2000; Szekeres *et al.*, 2000). This GPCR was originally isolated from human and murine cDNA libraries based on its homology with secretagogue and neurotensin receptors (Tan *et al.*, 1998). At the time of identification of FM3 as an NmU receptor, a second NmU receptor was identified (Hoysoya *et al.*, 2000 and Raddatz *et al.*, 2000). Given the identification of two receptors, the first (FM3) has been designated human NmUreceptor 1) (hNmU-R1) and the second human NmU-receptor 2 (hNmU-R2).

Comparatively, hNmU-R1 and hNmU-R2 share 50% amino-acid homology with conservation highest within the putative transmembrane spanning domains and lower towards both the N- and C- terminals. hNmU-R1 has a larger third intracellular loop and a shorter C-terminal tail in comparison to hNmU-R2 (Raddatz *et al.*, 2000). In humans, the two receptors show differential patterns of distribution. hNmU-R1 mRNA distribution is highest within several peripheral tissues such as the small intestine and stomach but also exists throughout the central nervous system (Hendrick *et al.*, 2000; Howard *et al.*, 2000; Raddatz *et al.*, 2000; Szekeres *et al.*, 2000). hNmU-R2 mRNA, however, is predominantly confined to specific regions within the brain with low, but significant levels present within several peripheral tissues (Howard *et al.*, 2000; Raddatz *et al.*, 2000; Shan *et al.*, 2000). Further details of differences in the structures and tissue distributions of the two receptors are available elsewhere (see Chapter 1, sections 1.2.4 and 1.2.5).

It is clear from the functional screening assays that both hNmU-R1 and hNmU-R2 are linked to phosphoinositide hydrolysis and Ca^{2+} signalling via G-proteins most

likely of the $Ga_{q'11}$ family (Fuji *et al.*, 2000; Hendrik *et al.*, 2000; Hosoya *et al.*, 2000; Howard *et al.*, 2000; Kojima *et al.*, 2000; Raddatz *et al.*, 2000; Shan *et al.*, 2000; Szekeres *et al.*, 2000). Indeed elevation of $[Ca^{2+}]_i$ by activation of hNmU-R1 in HEK293 cells is unaffected by pre-treatment of the cells with PTX, indicating that activation of $Ga_{i/o}$ does not contribute to this response (Szekeres *et al.*, 2000). Responses to hNmU-R1 and hNmU-R2 are concentration-dependent with EC₅₀ values in the sub-nanomolar range and have been observed in a variety of clonal cell lines expressing recombinant receptors. There has been little consideration of the effects of NmU on intracellular cAMP levels, but early indications suggest that both hNmU-R1 and hNmU-R2, attenuate FSK-stimulated cAMP accumulation suggesting they could also couple to Ga_i (Hosoya *et al.*, 2000). To date, our understanding of hNmU receptors (and any differences between the two receptor types) is in its infancy. Here, this has led to an exploration of the intracellular signalling generated by hNmU-R1 and hNmU-R2.

This chapter details the initial characterisation of four HEK293 clonal cell lines expressing recombinant hNmU-R1 (ACC-924, ACC-925, ACC-926 and ACC-927) and two expressing hNmU-R2 (ACC-955 and ACC-958) as well as the parental wild-type HEK293 cell line. Despite the ability of alternative NmU peptides (e.g. porcine NmU-8, rat NmU-23) to activate human forms of the receptors (Fuji *et al.*, 2000; Hosoya *et al.*, 2000; Raddatz *et al.*, 2000; Szekeres *et al.*, 2000) this chapter details activation of hNmU-R1 and hNmU-R2 solely by the human form of NmU, namely hNmU-25. A combination of radio-labeled [¹²⁵I]-hNmU-25 binding and functional assays is employed to aid in the selection of one clonal cell line expressing hNmU-R1 and one expressing hNmU-R2. The signalling properties of these two cell lines are then investigated further, examining the coupling to specific Gα G-proteins and characterising the intracellular signal transduction pathways. Furthermore, this initial work details desensitisation profiles of the receptors and aims to expose any signalling and/or desensitisation differences between the two human NmU receptor subtypes.

Section 3.2 Methods

All methods used are exactly as described in Methods, section 2.2.

i.

Section 3.3 Results

3.3.1 Establishment of HEK293 cells expressing either hNmU-R1 or hNmU-R2

The clonal cell lines expressing either hNmU-R1 or hNmU-R2 used throughout this investigation were kindly provided by GlaxoSmithKline (Harlow, U.K.). I owe thanks to N. Elshourbagy and U. Shabon (Gene Cloning and expression Proteomics, GlaxoSmithKline, Harlow, U.K.) for cloning the receptors and both E. Appelbaum and E. Dul (GEPB, Upper Merion, Philadelphia, PA.) for generating the stable cell lines. The hNmU-receptors were recombinantly expressed in HEK293 cells using methods described previously (see Methods, section 2.2.1). Briefly, the DNA encoding hNmU-R1 was cloned into EcoR1/EcoRV and hNmU-R2 into Asp718/Bam HI of the 'in-house' expression vector; pCDN (Aiyar et al., 1994). Constructs were transfected using LipofectAMINE Plus and grown under geneticin selection. Clonal cell lines were expanded from single foci resulting in the generation of four clonal cell lines expressing hNmU-R1 and two expressing hNmU-R2. Those expressing hNmU-R1 were termed ACC-924 ACC-925, ACC-926 and ACC-927, and those expressing hNmU-R2 were termed ACC-955 and ACC-958. All cell lines were screened at GlaxoSmithKline based on NmU-mediated Ca²⁺ measurements in cell populations and demonstrated viable and functional cell lines.

Here, the clonal cell lines were screened by determination of hNmU-25-mediated $Ins(1,4,5)P_3$ production using both single-cell (see Methods, section 2.2.5) and population assays (see Methods, section 2.2.3) and the accumulation of total inositol phosphates (InsP_x) (see Methods, section 2.2.4). Relative expression levels were examined by the binding of [¹²⁵I]-hNmU-25 to membrane preparations using a concentration of ligand approximating to the K_d (see Methods, section 3.2.2).

The underlying principle behind this initial work was to select one clonal cell line expressing hNmU-R1 and one expressing hNmU-R2 based on both similar expression levels and approximately equivalent functional responses mediated by hNmU-25. Subsequent experiments will be performed predominantly on these cell lines.

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3.3.2 Relative receptor expression levels in clonal cell lines determined by the binding of [¹²⁵I]-hNmU-25 to membranes

Given that radio-labelled hNmU-25 ([¹²⁵I]-hNmU-25) is expensive, rather than performing full saturation analysis on each cell line, the relative receptor expression levels of all seven cell lines was judged on their ability to specifically bind 100pM [¹²⁵I]-hNmU-25. hNmU-R1 and hNmU-R2 are known to have equivalent binding affinities, with K_d values of approximately 140pM (Fujii et al., 2000). From the four cell lines expressing hNmU-R1, ACC-924 showed the highest level of binding followed by ACC-926 (approximately 22% less) (Fig. 3.1). Both hNmU-R2 expressing cell lines showed lower levels of binding (approximately 80% less than ACC-924) with ACC-955 showing the higher of the two. No specific binding could be detected in the hNmU-R1 expressing cell line ACC-927, suggesting low levels of expression or absence of receptor. From these data ACC-925 (hNmU-R1) and ACC-955 (hNmU-R2) showed the most similar levels. However, this will be combined with functional data (see Section 3.3.3) to further aid the selection of clonal cell lines. Non-specific binding represented approximately ~45% of total binding (not shown). No specific binding was observed to membranes prepared from wild-type cells (Fig. 3.1).



Fig. 3.1. Specific binding of 100pM [125 I]-hNmU-25. Membranes were prepared from either wild-type, hNmU-R1 (*grey filled*) or hNmU-R2 (*black filled*) expressing HEK293 cell lines as indicated. Membrane preparations (20µg) were incubated at RT with 100pM [125 I]-hNmU-25 for 30min. Non-specific binding was determined by 5min pre-incubation of 1µM unlabelled hNmU-25 (not shown). Membranes were filtered and radioactivity was ascertained by standard liquid scintillation methods. Non-specific binding represented approximately ~45% of total binding (not shown). Data are from one experiment and representative of three separate experiments.

3.3.3 NmU-mediated signalling in clonal cell lines

To further aid our selection of a single hNmU-R1 and hNmU-R2 cell line, functional assays were performed to assess receptor coupling. These were performed on all clonal cell lines to assess the ability of expressed NmU receptors to couple to $G\alpha_{q/11}$ G-proteins and subsequently activate PLC. Both hNmU-R1 and hNmU-R2 are known to activate PLC (see Section 1.2.6) making the analysis of Ins(1,4,5)P₃ generation and the accumulation of [³H]-InsP_x in cell populations along with single-cell imaging of Ins(1,4,5)P₃ ideal as functional screens.

In all cell lines stimulated with 10nM hNmU-25, $Ins(1,4,5)P_3$ peaked after 5-10 seconds and extended into a lower but sustained plateau phase of approximately 50% of the peak levels (Fig. 3.2). Despite lower receptor expression levels (see Section 3.3.2) both hNmU-R2 cell lines (ACC-955 and ACC-958) showed the highest $Ins(1,4,5)P_3$ peaks, generating between 1000 and 1200 $Ins(1,4,5)P_3$ pmol mg protein⁻¹. Out of the hNmU-R1 expressing cell lines ACC-924 generated the most $Ins(1,4,5)P_3$ with a peak accumulation of approximately 900 pmol mg protein⁻¹. Again, consistent with lower levels of expression in ACC-927, $Ins(1,4,5)P_3$ generation in this cell line was considerably reduced in comparison with the others. Wild-type HEK 293 cells did not respond to application of 10nM hNmU-25.



Fig. 3.2. Time course of $Ins(1,4,5)P_3$ generation in hNmU receptor expressing clonal cell lines. hNmU-R1 expressing cell lines ACC-924 (filled diamonds; \blacklozenge), ACC-925 (downward filled triangles; \blacktriangledown), ACC-926 (upward filled triangles; \blacktriangle) and ACC-927 (filled circles; \bullet) and hNmU-R2 expressing cell lines ACC-955 (open squares; \Box) and ACC-958 (open triangles; \triangle) as well as wild-type HEK293 cells (filled squares; \blacksquare) were seeded onto 24-well plates and stimulated with 10nM hNmU-25 for varying lengths of time between 0-300s. Ins(1,4,5)P₃ was extracted, measured by a radio-receptor assay and related to protein concentration. Results are expressed as pmol Ins(1,4,5)P₃ mg protein⁻¹. Data are mean \pm s.e.m., n=3.

Analysis of total [3 H]-InsP_x accumulation under lithium block of inositol monophosphatase revealed continued activation of PLC by NmU in the majority of cell lines for the 30min duration of the experiment (Figure 3.3). All cell lines had accumulated [3 H]-InsP_x between 400-700% relative to basal levels within 30min. The hNmU-R1 expressing cell line ACC-924 showed an accumulations of [3 H]-InsP_x of approximately 750% above basal levels with the hNmU-R2 cell line ACC-955 also demonstrating a robust response (600% above basal). Two exceptions were noted. Firstly, consistent with low receptor expression levels (see Section 3.3.2) and poor NmU-mediated generation of Ins(1,4,5)P₃ (see Section 3.3.3), the hNmU-R1 expressing cell line ACC-927 demonstrated low [3 H]-InsP_x accumulation (approximately 125% increase relative to basal levels) in response to 10nM hNmU-25. Secondly, wild-type cells did not respond to NmU. The investigation of [3 H]-InsP_x accumulation under lithium block demonstrated good G-protein coupling and functional capabilities of the majority of the clonal cell lines.



Fig. 3.3. Time course of NmU-mediated accumulation of $[{}^{3}H]$ -InsP_x. hNmU-R1 expressing cell lines ACC-924 (*filled diamonds*; \blacklozenge), ACC-925 (*downward filled triangles*; \blacktriangledown), ACC-926 (*upward filled triangles*; \blacktriangle) and ACC-927 (*filled circles*; \bullet) and hNmU-R2 expressing cell lines ACC-955 (*open squares*; \Box) and ACC-958 (*open triangles*; \bigtriangleup) as well as wild-type HEK293 cells (*filled squares*; \Box) were seeded into 24-well plates and loaded with $[{}^{3}H]$ -inositol for 48 hours. Each cell line was stimulated with 10nM hNmU-25 under 10mM lithium block of inositol monophosphatase activity for varying lengths of time between 0–30min. Data are mean ± s.e.m., n=3. $[{}^{3}H]$ -InsP_x accumulation is represented as the % increase relative to basal (un-stimulated) levels that were approximately 3186 ± 686 DPM well⁻¹ (mean ± s.e.m., n= 42 wells). The final functional screen examined the activation of PLC at the single-cell level. The development of a fusion construct with the PH domain of phospholipase $C_{\delta 1}$ (PLC_{$\delta 1$}) with enhanced green fluorescent protein (eGFP) i.e. eGFP-PH_{PLC $\delta 1$} has enabled visualisation of Ins(1,4,5)P₃ generation at the single-cell level and in real time (Nahorski *et al.*, 2003).

Pleckstrin homology (PH) domains are small structurally homologous protein modules, roughly 100-120 amino acids in length, that are found in many proteins involved in cell signalling (reviewed in Rebecchi and Scarlatta, 1998). They have many putative functions but their only confirmed physiological role to date is to bind membrane phosphoinositides (reviewed in Lemmon and Ferguson, 2000). The PH domain from PLC_{δ_1} binds specifically to the head group of PtdIns(4,5)P₂ (Kavran *et al.*, 1998) the main substrate for activated PLC. Under resting conditions eGFP-PH_{PLC δ_1} binds to PtdIns(4,5)P₂ at the plasma membrane. Hydrolysis of PtdIns(4,5)P₂ and or the generation of Ins(1,4,5)P₃ (see below) results in the liberation of eGFP-PH_{PLC δ_1} and its translocation to the cytosol. This translocation can be used as an index of PLC activation (Stauffer *et al.*, 1998; Varnai and Balla, 1998; Hirose *et al.*, 1999; Nash *et al.*, 2001; Nahorski *et al.*, 2003).

The use of this fusion construct has been the source of much debate, with arguments raising questions as to the exact nature of its receptor-mediated translocation into the cytosol. One argument states that the eGFP-PH_{PLC51} construct binds to the soluble head group of PtdIns(4,5)P₂ and translocation is therefore indicative of cytosolic increases in $Ins(1,4,5)P_3$. The other argument states that receptor-mediated hydrolysis of PtdIns(4,5)P₂ could be strong enough to lower PtdIns(4,5)P₂ concentrations and thereby induce a dissociation of the eGFP-PH_{PLC51} probe from the plasma membrane (Stauffer *et al.*, 1998).

PH- $_{PLC\delta1}$ was found to have a 20-fold greater affinity for the soluble head group of PtdIns(4,5)P₂ using a surface plasmon assay (Hirose *et al.*, 1999), and evidence suggests that the sensor indicates a cytosolic increase in $Ins(1,4,5)P_3$ (Nash *et al.*, 2001). However, irrespective of the relative contributions of decreasing PtdIns(4,5)P₂ or increasing Ins(1,4,5)P₃ to membrane-to-cytosolic movement, translocation of the fluorescent construct indicates increased activity of cellular PLC and provides a further means of investigating the coupling of the hNmU-receptors to this effector. Each cell line grown on poly-D-lysine coated circular coverslips was transfected with the eGFP-PH_{PLC81} construct as described earlier (see Methods, section 2.2.5). Following transfection (48h), coverslips were mounted onto the stage of a confocal microscope and changes in cytosolic fluorescence were recorded in response to a bath application of 10nM hNmU-25.

Since ACC-924 (hNmU-R1) showed reliable and robust movement of the fusion construct to the plasma membrane this is used here as an example. Application of NmU caused rapid (~5s) and robust (~2-3 fold over basal) translocation of eGFP-PH_{PLC01} (Fig. 3.4a). The initial peak slowly declined into a sustained plateau phase that was approximately 50% of the peak response. In contrast, and as an example of a non-responding cell, wild-type cells showed no translocation (Fig. 3.4b). Α summary of the responses of all the clonal cell lines is represented in Fig. 3.4c. ACC-924, ACC-926 (hNmU-R1), ACC-955 and ACC-958 (hNmU-R2) cell lines showed robust and reproducible translocation of the fusion construct in response to NmU with cytosolic fluorescence increasing up to ~3-fold from basal levels. This initial response gradually decreased into a plateau phase in all responding cells. ACC-925 (hNmU-R1) gave poor reproducibility, whereby responding cells were not representative of all cells in the field of view. ACC-927 (hNmU-R1) and wild-type HEK293 cells failed to respond with a membrane-to-cytosol translocation of eGFP- $PH_{PLC\delta1}$ in response to NmU.



Fig. 3.4a. Single-cell imaging of hNmU-25-mediated PLC activation by ACC-924 (hNmU-R1). Cells expressing recombinant hNmU-R1 (ACC-924) were cultured on glass coverslips and transiently transfected with the eGFP-PH_{PLC61} construct. Cells were subsequently excited at 488nm and imaged by confocal microscopy. Cells were challenged with 10nM hNmU-25 via a bath application at t=15s. The change in cytosolic fluorescence was averaged from 6 cells chosen at random in the field of view using purpose written software. These cells were representative of all cells in the field of view. This is expressed graphically as the fold change in cytosolic fluorescence relative to basal levels (average cytosolic fluorescence in the 15s prior to application of NmU). Images A, B and C were taken at time points indicated on the graph. Data are representative of three separate experiments.

a)



Fig. 3.4b. Single-cell imaging PLC activity in wild-type HEK293 cells during application of NmU. Wild-type HEK293 cells were cultured on glass coverslips and transiently transfected with the eGFP-PH_{PLC81} construct. Cells were subsequently excited at 488nm and imaged by confocal microscopy. Cells were challenged with 10nM NmU via a bath application at t=15s. Changes in cytosolic fluorescence were averaged from 6 cells chosen at random in the field of view using purpose written software. These cells were representative of all cells in the field of view. This is expressed graphically as the fold change in cytosolic fluorescence relative to basal levels (average cytosolic fluorescence in the 15s prior to application of NmU). Images A, B, C were taken at time points indicated. Data are representative of three separate experiments.



c)

Fig 3.4c. Summary data of the peak NmU-mediated PLC activity using singlecell imaging of eGFP-PH_{PLC81}. hNmU-R1 expressing cell lines (*light grey filled*) and hNmU-R2 (*open*) as well as wild-type HEK293 cells (*dark grey filled*) were cultured separately on glass coverslips and transiently transfected with the eGFP-PH_{PLC81} construct. Cells were subsequently excited at 488nm whilst being imaged by confocal microscopy. Cells were challenged with 10nM hNmU-25 via a bath application at t=15s. For each experiment, activation of PLC was determined by averaging the change in cytosolic fluorescence from 6 cells chosen at random in the field of view using purpose written software. This is expressed graphically as the fold change in fluorescence relative to basal levels. The bars represent the peak change in fluorescence. Data are mean + s.e.m., n=3. In summary of the work so far, clonal cell lines expressing either hNmU-R1 or hNmU-R2 have been screened by $[^{125}I]$ -hNmU-25 binding, and the functional analysis of Ins(1,4,5)P₃ and total $[^{3}H]$ -InsP_x accumulation. The aim of this initial work was to enable selection of one cell line expressing hNmU-R1 and one expressing hNmU-R2 that had comparable expression levels of NmU receptors and which responded in functional assays to a similar extent. It will be these cell lines that will be used throughout the majority of the research.

Despite [¹²⁵I]-hNmU-25 binding indicating differences in expression levels, cell lines ACC-924 (hNmU-R1) and ACC-955 (hNmU-R2) showed reliable, robust and reproducible activation of PLC. As such, these two cell lines were chosen for all subsequent experiments (unless otherwise stated).

For continued reference, cell lines ACC-924 and ACC-955 are referred to hNmU-R1 and hNmU-R2 expressing cell lines, respectively.

3.3.4 [¹²⁵I]-hNmU-25 saturation binding

The binding of [¹²⁵I]-hNmU-25 to membranes from the clonal cell lines expressing either hNmU-R1 or hNmU-R2 was saturable with the non-specific component representing approximately 50% of the total at saturating concentrations of [¹²⁵I]-hNmU-25 (Fig. 3.5a(i-ii)). There was no specific binding to membranes prepared from wild-type (non-transfected) HEK293 cells (refer to Fig. 3.1). Analysis of saturation binding curves (Fig. 3.5b(i-ii)) indicated B_{max} values of 4.88±0.33 pmol mg⁻¹ and 1.95±0.16 pmol mg⁻¹ for hNmU-R1 and hNmU-R2 respectively, n=3. These experiments also indicated K_d values of $-9.87\pm0.05 \log_{10} M$ (135pM) and -9.95±0.10 log₁₀ M (112pM) for hNmU-R1 and hNmU-R2 respectively, n=3. A summary of B_{max} and K_d values is shown in Table 3.1. However, it must be noted that given the characteristics of NmU binding that indicate a lack of reversibility (see Chapter 4), these K_d values may be of limited value in describing the binding characteristics.

Table 3.1. B_{max} and K_d values obtained via saturation binding of [¹²⁵I]-hNmU-25 to membranes prepared from cells expressing either hNmU-R1 or hNmU-R2. Data are mean \pm s.e.m., n=3.

	hNmU-R1	hNmU-R2
B _{max} (pmol mg ⁻¹)	4.88±0.33	1.95±0.16
K _d (Log ₁₀ M)	-9.87±0.05	-9.95±0.10

a(i) hNmU-R1



Fig. 3.5a. Saturation binding of [¹²⁵I]-hNmU-25. Membrane preparations of cells expressing either hNmU-R1 (i) or hNmU-R2 (ii) were incubated with varying concentrations of [¹²⁵I]-hNmU-25 for 30min at RT. Non-specific binding was determined by pre-incubation with 1 μ M unlabelled hNmU-25. Membranes were filtered and radioactivity measured by standard counting methods. The specific component represents total binding less non-specific binding. Data are mean \pm s.e.m., n=3.

b(i) hNmU-R1



Fig. 3.5b. Non-linear regression analysis of saturation binding. Saturation binding of [¹²⁵I]-hNmU-25 to cells expressing either hNmU-R1 (Fig. b(i)) or hNmU-R2 (Fig. (b(ii)). Curves were analysed by non-linear regression using GraphPad Prism software (see Methods, section 2.2.17). B_{max} and K_d values (see table 3.1) were obtained from these fits. Data are mean \pm s.e.m., n=3.

3.3.5 G-protein coupling

The development of an immunoprecipitation assay using the non-hydrolysable form of GTP (GTP γ S) and antibodies raised against specific G-proteins (Akam *et al.*, 2002) has enabled us to directly assess the G-protein coupling of the hNmU receptors. Methods are detailed elsewhere (see Methods, section 2.2.7). Briefly, membrane preparations of hNmU-R1- or hNmU-R2-expressing cells were incubated with radiolabelled GTP γ S ([³⁵S]-GTP γ S). Binding of [³⁵S]-GTP γ S was then stimulated by receptor activation using hNmU-25 (10nM, 2min) and specific G α subunits immunoprecipitated using specific G α -subunit anti-sera and Protein A Sepharose beads. Beads were collected by centrifugation and the amount of [³⁵S]-GTP γ S associated used to assess G-protein coupling.

Binding of $[{}^{35}S]$ -GTP γS to immunoprecipitated G $\alpha_{q/11}$ (Fig. 3.6a) or G $\alpha_{i(1-3)}$ (Fig. 3.6c) increased by approximately 3-fold over basal upon activation of either hNmU-R1 or hNmU-R2 with 10nM hNmU-25. The binding of $[{}^{35}S]$ -GTP γS to G α_s did not increase following activation of either receptor type (Fig. 3.6b) although activation of endogenously expressed β_2 -adrenoceptors with 100 μ M noradrenaline resulted in an approximate 1.5-2 fold increase above basal levels (Fig. 3.6d). Non-specific binding using 10 μ M GTP γ S was ~20-50% of basal (un-stimulated) [${}^{35}S$]-GTP γ S binding (Fig.3.6).

Dual coupling is a common phenomenon amongst many G-protein coupled receptors (see Discussion, section 3.4.3). However, it can be a consequence of overexpression (Eason *et al.*, 1992; Zhu *et al.*, 1994; Cordeaux *et al.*, 2000). These studies therefore include an assessment of G-protein coupling to $Ga_{i(1-3)}$ in cell lines that express NmU receptors at lower levels. In additional cell lines expressing either hNmU-R1 (ACC-926) or hNmU-R2 (ACC-958) at 26% and 31% respectively of the level in cell lines used throughout the rest of this study (Fig. 3.7a), 10nM hNmU-25 also increased [³⁵S]-GTP_YS binding to $Ga_{i(1-3)}$ by approximately 2.5-3 fold over basal (Fig. 3.7b).

Binding of $[{}^{35}S]$ -GTP γS to $G\alpha_{12}$ (Fig. 3.8a) and $G\alpha_{13}$ (Fig. 3.8b) did not increase following activation of either hNmU-R1 or hNmU-R2. However, in the absence of a positive control for the coupling of $G\alpha_{12}$ and $G\alpha_{13}$ G-proteins, we cannot conclude the negative coupling of the hNmU-receptors to these G-proteins. To obtain a positive control, attempts were made to activate PAR2 (protease activated receptor-2) with SLIGKV (see below). HEK293 cells express endogenous PAR2 (Kawabata *et al.*, 1999) which are known to couple to $G\alpha_{12}$ and $G\alpha_{13}$ (Offermans *et al.*, 1994, Maragoudakis *et al.*, 2002). Application of SLIGKV (a small peptide that mimics the tethered ligand that activates PAR2) to HEK293 cell membrane preparations did not increase binding of [³⁵S]-GTP_YS to either $G\alpha_{12}$ or $G\alpha_{13}$ under these experimental conditions (Fig. 3.8c).



Fig. 3.6. G-protein coupling of hNmU-R1 and hNmU-R2. Membrane preparations (25µg) from cells expressing either hNmU-R1 or hNmU-R2 (a-c) or wild-type HEK293 cells (d) were incubated in the presence of GDP (1µM for $G\alpha_{q/11}$ and 10µM for $G\alpha_{i(1-3)}$ and $G\alpha_s$), 1nM [³⁵S]-GTPγS and where applicable (stimulated) hNmU-25 (10nM) (a-c) or 100µM noradrenaline (d). Non-specific binding (NSB) was determined using 10µM GTPγS and basal levels were determined by the exclusion of agonist. Immunoprecipitation was carried out using antibodies against specific Gα subunits as indicated and associated [³⁵S] determined. All data are mean + s.e.m., n=4.


Fig. 3.7. $[^{125}I]$ -hNmU-25 specific binding and Ga_i coupling of clonal cell lines ACC-926 (hNmU-R1) and ACC-958 (hNmU-R2). a) Membrane preparations (20µg) from clonal cell-lines expressing either hNmU-R1 (*light grey filled*) or hNmU-R2 (*dark grey filled*) were incubated for 30min at RT with 100pM [^{125}I]-hNmU-25. Non-specific binding was determined by the co-addition of 1µM unlabelled hNmU-25 (not shown). Membranes were filtered and radioactivity was ascertained by standard liquid scintillation methods. Data are mean + s.e.m., n=3. b) Membrane preparations (25µg) from clonal cell lines ACC-926 (hNmU-R1) or ACC-958 (hNmU-R2) were incubated in the presence of 10µM GDP, 1nM [^{35}S]-GTPγS and where applicable hNmU-25 (10nM) (stimulated). Non-specific binding (NSB) was determined using 10µM GTPγS and basal levels were determined by the exclusion of agonist. Immunoprecipitation was carried out using an antibody against Ga_i(1-3) G-proteins and associated [^{35}S] determined. Data are mean + s.e.m., n=4.



Fig. 3.8. hNmU-R1 and hNmU-R2 coupling to $G\alpha_{12}$ and $G\alpha_{13}$ G-proteins. Membrane preparations (25µg) from hNmU-R1 or hNmU-R2 (a-b) and wild-type HEK293 (c-d) cells were incubated in the presence of 1nM [³⁵S]-GTP₇S and where applicable (stimulated) with either 10nM hNmU-25 (a-b) or 10µM SLIGKV (c-d). Non-specific binding (NSB) was determined using 10µM GTP₇S and basal levels were determined by the exclusion of agonist. Immunoprecipitation was carried out using an antibody against either $G\alpha_{12}$ or $G\alpha_{13}$ G-proteins and associated [³⁵S] determined. Data are mean + s.e.m., n=4.

3.3.6 [³H]-InsP_x accumulation

NmU receptor-mediated activation of cells expressing either hNmU-R1 or hNmU-R2 with 10nM hNmU-25 caused marked accumulations of $[^{3}H]$ -InsP_x against a Li⁺-block of inositol monophosphatase activity that continued until the furthest time tested (60min) (Fig. 3.9a). Here, accumulation was approximately either 4000% or 2500% of basal levels for hNmU-R1 and hNmU-R2 respectively. Challenge of wild-type HEK293 cells with 10nM hNmU-25 did not result in accumulation of $[^{3}H]$ -InsP_x (see Section 3.3.3).

Analysis of $[{}^{3}H]$ -InsP_x accumulation during the first three minutes of receptor activation often gives a clearer indication of possible regulation of PLC activity by highlighting changes in the rate of accumulation (Wojcikiewicz *et al.*, 1993). Here, accumulation was biphasic, with a rapid phase (300-350% over basal min⁻¹) that became (at ~20s) slower (50-60% over basal min⁻¹) but sustained (Fig. 3.9b).

The accumulation of $[{}^{3}H]$ -InsP_x was concentration-dependent, with similar pEC₅₀ values of 9.14±0.07 and 8.97±0.18 for hNmU-R1 or hNmU-R2 respectively, n=3 (Figs. 3.9c and 3.9d). PTX had no effect on hNmU-25-mediated accumulation of $[{}^{3}H]$ -InsP_x in either cell line (Figs. 3.9c and 3.9d) indicating a lack of involvement of Ga_{i/o} in hNmU-25-mediated PLC responses. A summary of pEC₅₀ values is shown in Table 3.2.

In cells expressing hNmU-R1, challenge with 10nM hNmU-25 in the absence of extracellular Ca^{2+} had no effect on the bi-phasic profile of the accumulation of [³H]-InsP_x but by 60min had reduced the accumulation to 40±10% (n=3) of that seen in the presence of extracellular Ca^{2+} (Fig. 3.9e and f).



Fig. 3.9a-b. Time course of hNmU-R1 and hNmU-R2-mediated $[{}^{3}H]$ -InsP_x accumulation. Cells expressing either hNmU-R1 (*open squares*; \Box) or hNmU-R2 (*open circles*; O) were seeded into 24-well plates and loaded with $[{}^{3}H]$ -myo-inositol for 48h. a) Cells were challenged with 10nM hNmU-25 for varying lengths of time ranging from 0-3600s (60min) in the presence of a 10mM Li⁺-block of inositol monophosphatase activity. b) Detail from a) showing the accumulation of $[{}^{3}H]$ -InsP_x over the first 180s of agonist stimulation. Data are mean ± s.e.m., n=3. $[{}^{3}H]$ -InsP_x accumulations are presented as the percentage increase relative to basal levels that were 3694 ± 264 and 2946 ± 329 DPM well⁻¹ for hNmU-R1 and hNmU-R2 respectively (data are mean ± s.e.m., n=6 wells).

a)

b)



Fig 3.9c-d. hNmU-R1 and hNmU-R2-mediated accumulation of $[{}^{3}H]$ -InsP_x Concentration-response curves for the accumulation of $[{}^{3}H]$ -InsP_x following activation of either hNmU-R1 (c) or hNmU-R2 (d) by hNmU-25. Cells were challenged under Li⁺-block for 60min. Where applicable, cells were treated with 100ng ml⁻¹ PTX (*filled symbols*) for 24h prior to agonist challenge (filled symbols). $[{}^{3}H]$ -InsP_x accumulations are presented as the percentage increase relative to basal levels. Data are mean ± s.e.m., n=3. $[{}^{3}H]$ -InsP_x accumulations are presented as the percentage increase relative to basal levels which were 5322 ± 1289 and 3648 ± 394 DPM well⁻¹ for hNmU-R1 and hNmU-R2 respectively (data are mean ± s.e.m., n=12 wells). The pEC₅₀ values obtained are shown in Table 3.2.

Table 3.2. pEC₅₀ values obtained for hNmU-25-mediated accumulation of $[^{3}H]$ -InsP_x by hNmU-R1 and hNmU-R2. Data are mean \pm s.e.m., n=3.

	pEC ₅₀	pEC ₅₀ PTX treated cells
hNmU-R1	9.14 ± 0.07	9.15 ± 0.2
hNmU-R2	8.97 ± 0.18	9.14 ± 0.19



Fig. 3.9e-f. [³H]-InsP_x accumulation in the nominal absence of extracellular Ca^{2+} . Cells expressing hNmU-R1 were seeded into 24-well plates and loaded with [³H]-inositol for 48h. Experiments were performed in normal KHB (*open squares;* \Box) or with the exclusion of 1.29mM CaCl₂.2H₂O from KHB to create nominal Ca²⁺ free conditions (*filled squares;* \blacksquare). Cells were challenged with 10nM hNmU-25 for the time points indicated in the presence of a 10mM Li⁺-block of inositol monophosphatase activity. f) Detail from e) showing the accumulation of [³H]-InsP_x accumulations are presented as the percentage increase relative to basal levels that were 3048 ± 345 and 3456 ± 247 DPM well⁻¹ for the presence and absence of extracellular Ca²⁺ respectively (data are mean ± s.e.m., n=6 wells).

e)

3.3.7 Ins(1,4,5)P₃ accumulation

Challenge of either hNmU-R1 and hNmU-R2 expressing cell lines with 10nM hNmU-25 resulted in a rapid and marked increase in $Ins(1,4,5)P_3$ mass that peaked at 10s and declined to a lower but sustained phase (Fig. 3.10). Basal levels of $Ins(1,4,5)P_3$ were 100 and 300 pmol mg protein⁻¹ for hNmU-R1 and hNmU-R2 respectively, and peaked at ~900 pmol mg protein⁻¹ $Ins(1,4,5)P_3$ for hNmU-R1 and ~1200 pmol mg protein⁻¹ for hNmU-R2. For both receptors this declined into sustained generation of approximately 50% of the peak. Wild-type HEK293 cells did not respond to hNmU-25 challenge.

Through the use of eGFP conjugated to the PH domain of PLC_{$\delta1$} (eGFP-PH_{PLC $\delta1$}) the generation of Ins(1,4,5)P₃ at the single-cell level can be analysed (see Section 3.3.3). Transfection of cells expressing either hNmU-R1 or hNmU-R2 with eGFP-PH_{PLC $\delta1}$ resulted in the expression of the construct and localization predominantly to the plasma membrane (Fig. 3.11(a and b), image A). This is due to the high affinity of the PH domain for PtdIns(4,5)P₂ (Hirose *et al.*, 1999). Activation of either hNmU-R1 or hNmU-R2 with 10nM hNmU-25 resulted in an immediate translocation of eGFP-PH_{PLC $\delta1$} to the cytosol (Fig. 3.11(a and b), image B) that was reflected by an increase in cytosolic fluorescence of approximately 2-3 fold relative to basal levels. For both receptor subtypes, the initial peak slowly declined over the following 60s to approximately 40-60% of the peak response and this partial relocalization of the eGFP-PH_{PLC $\delta1} construct (Fig. 3.11(a and b) image C) to the plasma membrane was sustained for the 5min duration of the expression of the specific term of the 5min duration of the expression of the specific term.</sub></sub>$



Fig. 3.10. hNmU-R1- and hNmU-R2-mediated accumulation of $Ins(1,4,5)P_3$. Data as from Fig 3.2. Wild-type HEK293 cells (*upward filled triangles*; \blacktriangle) or cells expressing either hNmU-R1 (*open squares*; \Box) or hNmU-R2 (*open circles*; o) were cultured on 24-well plates and challenged with 10nM hNmU-25 for the time shown before extraction and determination of $Ins(1,4,5)P_3$ using a radio-receptor assay. Data are mean \pm s.e.m., n=3.





b) hNmU-R2





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3.3.8 Single-cell [Ca²⁺]_i changes

Single-cell imaging of fluorescence changes in fluo-3-loaded cells as an index of changes in $[Ca^{2+}]_i$ in cells expressing either hNmU receptor type revealed robust (2-3 fold over basal), rapid (5s) peaks followed by lower (1.2-1.4 fold over basal) sustained phases in response to 10nM hNmU-25 (Fig. 3.12a and 3.12b). Removal of extracellular Ca²⁺ had little effect on the peak elevation but abolished the sustained phase (Fig. 3.12c and 3.12d). Removal of extracellular Ca²⁺ during the hNmU-25-mediated sustained elevation of $[Ca^{2+}]_i$ caused a reduction in $[Ca^{2+}]_i$ back to basal levels in hNmU-R1 and hNmU-R2 cell lines (Fig. 3.12e and 3.12f). Pre-treatment of cells for 10min with the sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) inhibitor thapsigargin (1µM) abolished the Ca²⁺ responses in both hNmU-R1 and hNmU-R2 expressing cells (Fig. 3.12g and 3.12h).

a) hNmU-R1



Fig. 3.12a-b. hNmU-R1- and hNmU-R2-mediated changes in $[Ca^{2+}]_{i}$. hNmU-R1 (a) or hNmU-R2 (b) expressing cells were cultured on glass coverslips, loaded with fluo-3-AM and cytosolic fluorescence determined by confocal microscopy as an index of $[Ca^{2+}]_{i}$. Temperatures were maintained at 37°C with a Peltier unit. Cells were challenged with 10nM hNmU-25 at t=30s. Traces show the average change in cytosolic fluorescence of 6-10 cells in the field of view chosen at random. Image panels A, B and C were taken at the time points indicated on the traces. Data are representative of at least 4 separate experiments. Variance is detailed in appendix 1.

c) hNmU-R1





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e) hNmU-R1



Fig. 3.12e-f. hNmU-R1- and hNmU-R2-mediated changes in $[Ca^{2+}]_i$ following removal of extracellular Ca^{2+} during the sustained phase of the hNmU-25medited Ca^{2+} signal. hNmU-R1 (e) or hNmU-R2 (f) expressing cells were loaded with fluo-3-AM and imaged by confocal microscopy. Temperatures were maintained at 37°C with a Peltier unit. Changes in cytosolic fluorescence were measured as an index of $[Ca^{2+}]_i$. Cells were incubated in KHB and challenged with 10nM hNmU-25 at t=30s. At t=180s, Ca^{2+} -free KHB (containing 10nM hNmU-25) was perfused at a rate of 5ml min⁻¹. Traces show the average change in cytosolic fluorescence of 6-10 cells in the field of view chosen at random. Image panels A, B and C were taken at the time points indicated on the traces. Data are representative of at least 3 separate experiments. Variance is detailed in appendix 1.





Fig. 3.12g-h. hNmU-R1- and hNmU-R2-mediated changes in $[Ca^{2+}]_i$ following depletion of intracellular Ca^{2+} stores with thapsigargin. hNmU-R1 (g) or hNmU-R2 (h) expressing cells were cultured on glass coverslips, loaded with fluo-3-AM and mounted on the stage of a confocal microscope. Temperatures were maintained at 37°C with a Peltier unit. Cells were treated with 1µM thapsigargin for 10min and washed by KHB perfusion (5ml min⁻¹) for 1-2min prior to assay. Cells were challenged with 10nM hNmU-25 at t=30s and changes in cytosolic fluorescence were measured as an index of $[Ca^{2+}]_i$. Traces show the average change in cytosolic fluorescence of 6-10 cells in the field of view chosen at random. Image panels A, B and C were taken at the time points indicated on the traces. Data are representative of at least 3 separate experiments. Variance is detailed in appendix 1.

3.3.9 FLIPR analysis of [Ca²⁺]_i changes

Analysis of Ca^{2+} signalling by FLIPR demonstrated hNmU-25-mediated elevation of $[Ca^{2+}]_i$ in populations of cells (Fig. 3.13a-b) consistent with those in single-cells (see above). Here, an initial $[Ca^{2+}]_i$ peak following hNmU-25 challenge slowly declined into a sustained plateau signal approximately 30-35% for hNmU-R1 (Fig. 3.13a) and 20-25% for hNmU-R2 (Fig. 3.13b) of the peak response.

Concentration-response curves generated using this methodology revealed pEC_{50} values for the hNmU-25-mediated peak elevation of $[Ca^{2+}]_i$ in hNmU-R1 (Fig. 3.13c) and hNmU-R2 (Fig. 3.13d) cells of 9.41±0.09 and 9.37±0.06 respectively, n=3.

a) hNmU-R1



Fig. 3.13a-b. $[Ca^{2+}]_i$ changes in populations of hNmU-R1- and hNmU-R2-cells. Cells were cultured in 384-well plates at a density of 10000 cells well⁻¹. $[Ca^{2+}]_i$ in cell populations was determined using fluo-4-loaded cells and a FLIPR. The time-course of 10nM hNmU-25-mediated changes in fluorescence intensity units (F.I.U.) as an index of $[Ca^{2+}]_i$ is shown for cells expressing either hNmU-R1 (a) or hNmU-R2 (b). Traces are total changes in F.I.U. from one individual well, and are representative of 9 different wells from 3 separate FLIPR experiments.

a) hNmU-R1



Fig. 3.13c-d. Concentration-response curves showing $[Ca^{2+}]_i$ changes in populations of hNmU-R1- and hNmU-R2-cells. Cells expressing either hNmU-R1 (c) or hNmU-R2 (d) were cultured in 384-well plates at a density of 10000 cells well⁻¹. $[Ca^{2+}]_i$ in cell populations was determined using fluo-4-loaded cells and a FLIPR. Changes in fluorescence intensity units (F.I.U.) were used as an index of changes in $[Ca^{2+}]_i$. The concentration-response relationships for hNmU-25-mediated peak elevations of $[Ca^{2+}]_i$ in cells expressing either hNmU-R1 (c) or hNmU-R2 (d) gave pEC₅₀ values of 9.41±0.09 and 9.37±0.06 respectively Data are mean ± s.e.m., n=3.

3.3.10 Regulation of cAMP accumulation

Methods for the inhibition of FSK-elevated cAMP are detailed in Methods (Section 2.2.8). Briefly, hNmU-R1 and hNmU-R2 cells in 24-well plates were stimulated with 10 μ M FSK for 10min prior to being stimulated with 10nM hNmU-25 for a further 10min. The cAMP in cell extracts was then determined by radio-receptor assay and related to cell protein. The order and timing of FSK and hNmU-25 addition was determined in preliminary experiments to be optimal for NmU-mediated inhibition of FSK-stimulated cAMP accumulation. Some examples are shown in Fig. 3.14a. The differences between the various protocols were not great, however stimulation with FSK for 10min followed by stimulation by hNmU-25 for 10min gave the largest (60±5%, mean ± s.e.m., n=3) decrease in cAMP.

Activation of either hNmU-R1 or hNmU-R2 with hNmU-25 resulted in the inhibition of FSK (10μ M) stimulated cAMP accumulation (Fig. 3.14b) with pEC₅₀ values of 10.10 ± 0.16 and 10.06 ± 0.17 in cells expressing hNmU-R1 or hNmU-R2 respectively. PTX treatment (20h, 100ng ml⁻¹) abolished this inhibition (Fig. 3.14b). In this assay system, PTX-treatment has no effect on either basal or FSK-stimulated levels of cAMP (Challiss *et al*, unpublished observations, University of Leicester, U.K.). Basal levels, therefore, are likely to come from the basal activity of adenylate cyclase.

Addition of 10nM hNmU-25 did not increase cAMP in cells expressing either NmU receptor type even in the presence of IBMX (Fig. 3.14c). In contrast, challenge of endogenously expressed $G\alpha_s$ -coupled β_2 -adrenoceptors with 100 μ M noradrenaline caused a 5-fold increase in cAMP above basal levels in the presence of IBMX (Fig. 3.14c).



Fig. 3.14a. Optimisation of NmU-mediated inhibition of FSK-stimulated cAMP accumulation. Cells expressing hNmU-R1 were cultured in poly-D-lysine-coated 24-well plates. Shown on the left of the panel (*dark grey filled*) are basal and FSK-stimulated levels of cAMP. Cells were either stimulated first with FSK (10 μ M, 10min) (*grey filled*) followed by 10nM hNmU-25 for the time period indicated, or alternatively were stimulated initially by 10nM hNmU-25 (*light grey filled*) for the time period indicated followed by FSK (10 μ M, 10min). cAMP was extracted and measured by radio-receptor assay. Data are mean + s.e.m., n=3.



hNmU-R1 PTX-treated

b)

• hNmU-R2 PTX-treated

hNmU-R-mediated inhibition of FSK-stimulated 3.14b. **cAMP** Fig. accumulation. Cells expressing either hNmU-R1 or hNmU-R2 were cultured in poly-D-lysine-coated 24-well plates. hNmU-25 was added for 10min prior to addition of 10µM FSK. Following a further 10min incubation, cAMP was extracted and measured by radio-receptor assay. Shown on the left of the panel are basal and FSK-stimulated levels of cAMP in cells expressing either hNmU-R1 or hNmU-R2. On the right of the panel are curves showing the concentration-dependence of the hNmU-25-mediated inhibition of FSK-stimulated cAMP accumulation. The pEC₅₀ values for inhibition were 10.10±0.16 for hNmU-R1 (open squares;) and 10.06±0.17 for hNmU-R2 (open circles; o). Following PTX-treatment of cells (20h, 100ng ml⁻¹), 10nM hNmU-25 failed to inhibit FSK-elevated cAMP accumulation in cells expressing either hNmU-R1 (filled square;) or hNmU-R2 (filled circle;). All data are mean \pm s.e.m., n=3.



Fig. 3.14c. Accumulation of cAMP. Wild-type HEK293 cells (*filled triangles*; \lor), or cells expressing either hNmU-R1 (*open circles*; \circ) or hNmU-R2 (*open squares*; \Box) were cultured in poly-D-lysine-coated 24-well plates. Assays with involved the inclusion of 500µM isobutylmethylxanthine (IBMX). hNmU-R cells were challenged with 10nM hNmU-25 and wild-type cells with 100µM noradrenaline. cAMP in the samples was extracted and measured using a radio-receptor assay. Data are mean \pm s.e.m., n=3.

3.3.11 Activation of ERK

Challenge of either hNmU-R1 (Fig. 3.15a(i)) or hNmU-R2 (Fig. 3.15b(i)) expressing cells with 10nM hNmU-25 did not alter cellular levels of ERK. However, hNmU-25 increased the level of phospho- (p) ERK, as an index of ERK activation, which peaked after 5-10min of stimulation and then slowly declined (Fig. 3.15a(ii), 3.15b(ii); 3.15c). ERK phosphorylation following activation of either receptor subtype was unaffected by pre-treatment of cells with PTX (24h, 100ng ml⁻¹) (Fig. 3.15d).



Figure 3.15a-d. Activation of ERK in hNmU-R1 or hNmU-R2 expressing cells. Cells expressing either hNmU-R1 (a) or hNmU-R2 (b) were cultured into poly-Dlysine-coated 24-well plates and stimulated with 10nM hNmU-25 for up to 60min. Levels of ERK were determined by Western blotting (a(i); hNmU-R1 and b(i); hNmU-R2) before being stripped and re-probed for phosphorylated (activated) ERK (pERK) (a(ii); hNmU-R1 and b(ii); hNmU-R2). Data are representative of 4 separate experiments. The density of pERK following stimulation of hNmU-R1 (*open* squares; \Box) or hNmU-R2 (*open circles*; \circ) was then related to the corresponding ERK density using a Syngene Genegenius Bioimaging System (c). d) Densitometric analysis of pERK following stimulation of hNmU-R1 (*filled squares*; \blacksquare) or hNmU-R2 (*filled circles*; \bullet) in cells pre-treated with 100ng ml⁻¹ PTX prior to stimulation and assay. All data are mean ± s.e.m., n=4.

3.4.1 Summary

In summary, these data show that activation of either human NmU receptor type expressed recombinantly in HEK293 cells results in the activation of both PLC and inhibition of adenylyl cyclase. This was demonstrated by increases in $[Ca^{2+}]_i$, Ins(1,4,5)P₃ and $[^{3}H]$ -InsP_x accumulation and by a reduction in FSK-elevated cAMP respectively. Furthermore, by directly assessing the coupling of G-proteins these studies demonstrate that both receptors are able to couple to both Ga_{q/11} and Ga_i Gproteins, whilst, consistent with a lack of increase in levels of cAMP upon receptor activation, no coupling to Ga_s was observed. Both hNmU-R1 and hNmU-R2 also activate ERK1/2. Signalling through PLC, and activation of ERK1/2 was insensitive to PTX, whereas the inhibition of FSK-stimulated cAMP was PTX-sensitive. Despite structural differences between the two hNmU-receptor subtypes these studies have not revealed differences in their signalling properties.

3.4.2 hNmU-receptor-mediated signalling through PLC

It is clear from the functional screening assays that NmU-R1 and NmU-R2 of human and rodent origin are able to mediate intracellular Ca²⁺ signalling with potency in the nM range (Fujii et al., 2000; Hedrick et al., 2000; Hosoya et al., 2000; Howard et al., 2000; Kojima et al., 2000; Raddatz et al., 2000; Shan et al., 2000; Szekeres et al., 2000; Funes et al., 2002). For hNmU-R1 this has been shown to be associated with phosphoinositide hydrolysis (Raddatz et al., 2000; Szekeres et al., 2000). The current data show that agonist activation of either hNmU-R1 or hNmU-R2 with hNmU-25 cause accumulations of $[^{3}H]$ -InsP_x for at least 1h against a Li⁺block of inositol monophosphatase activity. Furthermore, studies on cell populations demonstrate rapid, transient elevations of [Ca²⁺], that quickly subsided to small but hNmU-25-mediated accumulations of $[^{3}H]$ -InsP_x and sustained elevations. elevations of $[Ca^{2+}]_i$ were potent, each with EC₅₀ values of approximately 1nM for both receptor subtypes. The sustained accumulation of $[^{3}H]$ -InsP_x over at least 1h of agonist stimulation indicates that neither hNmU-R1 nor hNmU-R2 are subject to rapid and full desensitisation. However, closer examination over the first few minutes of stimulation reveal a bi-phasic accumulation consisting of an initial rapid

but transient accumulation followed by a slower but sustained accumulation. This early switch from rapid to slower accumulation indicates a reduction in PLC activity (Wojcikiewicz *et al.*, 1993) consistent with a rapid but partial desensitisation of signalling. This pattern is also consistent with a variety of other PLC coupled receptors (Wojcikiewicz *et al.*, 1993; Willars and Nahorski, 1995). Whilst the mechanism of desensitisation is unclear an obvious candidate is receptor-G-protein uncoupling following agonist-dependent receptor phosphorylation by G-protein receptor kinases (GRKs) or second messenger-dependent kinases. Although the level of $Ins(1,4,5)P_3$ is determined by both its generation and metabolism, the peak and plateau of hNmU-25-mediated increases in this second messenger is also consistent with a rapid but partial desensitisation of signalling.

The similarity of the EC₅₀ values for both $[^{3}H]$ -InsP_x accumulation and elevation of $[Ca^{2+}]_{i}$ are consistent with a tight coupling between these two events (reviewed; Putney and Bird, 1993). Further, the single-cell imaging of $[Ca^{2+}]_{i}$ in fluo-3-AM loaded cells and Ins(1,4,5)P₃ using the eGFP-PH_{PLC81} biosensor demonstrate that these events are temporally similar and reflective of the average signals generated by the study of cell populations.

Generally, a receptor activated Ca^{2+} mobilization via the $Ins(1,4,5)P_3$ signalling pathway involves two distinct stages. In the first stage, Ca^{2+} is released from an intracellular store primarily via the actions of $Ins(1,4,5)P_3$ acting on $Ins(1,4,5)P_3$ receptors (Berridge and Irvine, 1984). With the hNmU receptors, this is evident from a failure to elicit Ca^{2+} responses following depletion of Ca^{2+} stores with thapsigargin. Thapsigargin discharges intracellular Ca^{2+} stores as a consequence of basal 'leak' following by specific inhibition of the sarco/endoplasmic ATPdependent Ca^{2+} pump of endoplasmic reticulum (Thastrup *et al.*, 1990; Treiman *et al.*, 1998). The second phase of $[Ca^{2+}]_i$ elevation is a sustained or prolonged signal and the current data show sustained Ca^{2+} signalling by both hNmU receptor types. The two phases of the Ca^{2+} response have been dissected by a comparison of extracellular Ca^{2+} , both hNmU-R1 and hNmU-R2 generate a transient increase in $[Ca^{2+}]_i$ on activation with no evidence of a sustained signal. Furthermore, removal of extracellular Ca^{2+} following the initial Ca^{2+} response caused a rapid loss of the

sustained Ca²⁺ signal. Thus, it is clear that the second phase of NmU receptormediate $[Ca^{2+}]_i$ elevation relies on influx of Ca^{2+} across the plasma membrane most likely reflecting capacitative Ca^{2+} entry. The mechanism(s) for the maintenance of the sustained signal is, for a while, a source of much debate (reviewed Putney and Bird, 1993). It is now, however believed to involve a multitude of mechanisms (Putney et al., 1999, 2001) and may involve some receptor or cell specificity. The two most popular mechanisms for explaining capacitative Ca²⁺ entry are the conformational coupling mechanism and those involving a soluble second messenger (reviewed in Putney et al., 2001). The conformational coupling theory involves direct protein-protein interaction between $Ins(1,4,5)P_3$ receptors and Ca^{2+} channels at the plasma membrane. Here, discharge of Ca^{2+} from intracellular stores leads to a conformational change in the $Ins(1,4,5)P_3$ receptor which acts to physically couple the receptor to Ca^{2+} channels at the plasma membrane. This interaction opens Ca^{2+} channels and leads to an influx of Ca^{2+} . An alternative theory of capacitative Ca^{2+} entry involves a diffusible messenger. Depletion of Ca^{2+} from intracellular stores causes the release of a Ca²⁺-influx factor (CIF) from endoplasmic reticulum. The CIF will diffuse to the plasma membrane where it activates plasma membrane Ca^{2+} channels, thus causing the influx of Ca^{2+} .

The hNmU-25-mediated accumulation of $[{}^{3}H]$ -InsP_x by either hNmU-R1 or hNmU-R2 was also insensitive to PTX demonstrating a lack of involvement of $Ga_{i/o}$ in this response. This is consistent with the PTX-insensitive Ca²⁺ signalling by recombinant hNmU-R1 and hNmU-R2 reported elsewhere (Raddatz *et al.*, 2000; Shan *et al.*, 2000; Szekeres *et al.*, 2000) and indicates a $Ga_{q/11}$ -mediated activation of PLC. The direct coupling of both receptors to $Ga_{q/11}$ was confirmed by showing an hNmU-25-dependent increase in binding of $[{}^{35}S]$ -GTP_YS to these G-proteins.

3.4.3 Dual coupling of both hNmU-receptors to Gaq/11 and Gai

These studies also demonstrated activation of Ga_i by both receptors. Potential differences in the ability of antibodies to immunoprecipitate the different G-protein α -subunits means that we are unable to directly compare the levels of $Ga_{q/11}$ and Ga_i activation. However, both receptor subtypes were able to inhibit FSK-stimulated cAMP accumulation thereby demonstrating functional relevance of Ga_i activation.

The coupling of GPCRs to multiple G-proteins has, of course, been reported previously (for review see Hermans, 2003). Dual coupling to many different Ga subunit combinations has been reported. For example, dual coupling to $G\alpha_{\alpha/1}$ and Ga_s (Allgeiger et al., 1994, 1997; Jin et al., 2001), and Ga_i and Ga_s (Fraser et al., 1989; Kilts et al., 2000; Xiao, 2000). As with both hNmU-receptors, dual coupling to $G\alpha_{\alpha/11}$ and $G\alpha_i$ has been reported for the human MeIIa melatonin receptor (Brydon et al., 1999), type 1a metabotropic glutamate receptor (Hermans et al., 2000), muscarinic receptors (Offermanns et al., 1994), rat A3 adenosine receptor (Palmer et al., 1995) and the rat pancreastatin receptor (Santos-Álvarez and Sánchec-Margalet, 1999) amongst many others. Coupling to more than two G-proteins has also been the corticotropin-releasing hormone receptor reported. For example, (Grammatopoulos et al., 2001) and the luteinizing hormone receptor (Herrlich et al., 1996; Kuhn and Gudermann, 1999) couple to $G\alpha_i$, $G\alpha_s$ and $G\alpha_{\alpha/11}$. Further, the thyrotropin receptor has been reported to couple to four unrelated G-proteins ($G\alpha_i$, $G\alpha_s \ G\alpha_{o/11}$ and $G\alpha_{12}$) (Lauguitz et al., 1996). In the current study there was no evidence of the coupling of either NmU receptor type to $G\alpha_s$, $G\alpha_{12}$ or $G\alpha_{13}$. A positive control for $G\alpha_s$ (activation of β_2 -adrenoceptor) but not $G\alpha_{12}$ or $G\alpha_{13}$ was obtained. The coupling of NmU receptors to Ga_{12} , Ga_{13} and other Ga-subunits that were not explored here is still, therefore, a possibility.

The multiplicity of G-protein coupling by a single type of receptor could be influenced by numerous physiological and experimental parameters (Tuček *et al.*, 2002) giving rise to the possibility of the evidence for promiscuity being artifactual. One major cause for concern is the level of receptor expression, with higher levels favouring dual coupling (Eason *et al.*, 1992 and Zhu *et al.*, 1994; Cordeaux *et al.*, 2000). This study also show the activation of Ga_i using the immunoprecipitation protocol in membranes from additional hNmU receptor clonal cell lines that express lower levels of the NmU receptors, suggesting that dual coupling by hNmU receptors may not be a consequence of over-expression. However, the lower expressing clones may not represent true physiological expression levels, and the dual coupling of endogenous NmU receptors is considered further in Chapter 5.

Other factors effecting G-protein promiscuity include the agonist used (both in terms of efficacy and affinity), the agonist concentration, the availability of G-proteins within the cell, competition for G-proteins between receptors and receptor

desensitisation (Tuček *et al.*, 2002). However, promiscuity appears to be a physiological reality for a number of receptors (Hermans, 2003). Indeed, this research demonstrates that both hNmU-R1 and hNmU-R2 inhibited FSK-stimulated cAMP accumulation more potently than the elevation of Ca^{2+} or accumulation of $[^{3}H]$ -InsP_x, suggesting that this coupling may not be simply an expression artifact. Previously hNmU-25 has been reported to partially inhibit FSK-stimulated cAMP accumulation in CHO cells with stable expression of hNmU-R2 (Hosoya *et al.*, 2000), whilst in contrast activation of transiently expressed hNmU-R1 in HEK293 cells had no affect on either the basal or FSK-stimulated levels of cAMP (Szekeres *et al.*, 2000). The physiological and therapeutic relevance of any dual coupling by hNmU receptors remains to be established.

3.4.4 Activation of ERK

These studies demonstrate that hNmU-25-mediated activation of ERK is PTXinsensitive suggesting that $G\alpha_{q/11}$ coupling to phosphoinositide and Ca^{2+} signalling may be responsible. This is consistent with a variety of other receptors (e.g. Anderson *et al.*, 1991; Luttrell *et al.*, 1999; Vanbiesen *et al.*, 1996; Gutkind, 1998; Belcheva and Coscia, 2002). The activation of ERK by both recombinant and endogenous GPCRs is therefore well documented. However, it is mechanistically complex and may depend on the receptor and cell-type (Sugden and Clerk, 1997; Belcheva and Coscia, 2002).

The MAP kinase cascade is discussed previously (see Introduction, section 1.2.2.2). The activation of mitogenic signals by GPCRs coupling to $Ga_{q/11}$ may be mediated by a multitude of signalling cascades. Evidently, inhibition of G $\beta\gamma$ has no effect on MAP kinase activation (Hawes *et al.*, 1995) suggesting signals are mediated predominantly through the α -subunit of $G_{q/11}$ G-proteins. It has been proposed that this may involve one of two distinct signalling pathways (reviewed in Biesen *et al.*, 1996). Firstly, activation of $Ga_{q/11}$ will activate PLC thus converting PtdIns(4,5)P₂ into Ins(1,4,5)P₃ and DAG. DAG will then activate PKC (see Introduction, section 1.2.2). PKC will then activate Raf kinase within the sequence of the MAP kinase cascade leading to the eventual activation of ERK1/2. This mechanism essentially 'by-passes' Ras and is often termed the Ras-independent pathway. The second mechanism is Ras-dependent. In this scenario, activation of

 $G\alpha_{q/11}$ causes subsequent activation of second messengers (e.g. PKC, Ca^{2+} , calmodulin) that cause the phosphorylation of the Shc adapter molecule. Shc will then activate Ras and thus the MAP kinase cascade leading to the eventual activation of ERK1/2. It is clear that further investigations are required to fully understand the exact mechanisms of ERK phosphorylation by hNmU-receptor activation.

As an additional note, for some GPCRs (Daaka *et al.*, 1998) but not all (Budd *et al.*, 1999), internalisation appears to be a requirement for activation of MAP kinase. Although these data indicate rapid internalisation of both hNmU-R1 and hNmU-R2 within 4-5min of addition (see Chapter 4), the consequence of this internalisation in the activation and regulation of signalling pathways, including the MAP kinase pathway remain to be established.

Chapter 4

NmU binding to recombinant hNmU-R1 and hNmU-R2 expressed in HEK293 cells is irreversible under physiological conditions.

Section 4.1 Introduction

Initial work using recombinantly expressed hNmU-R1 and hNmU-R2 in HEK293 cells explored NmU-mediated Ca^{2+} signalling (see Chapter 1). During the course of these studies it became apparent that a second challenge with NmU does not elevate $[Ca^{2+}]_i$ despite extensive washing of the cells following the initial challenge. These investigations relied on a re-stimulation protocol whereby HEK293 cells expressing either hNmU-R1 or hNmU-R2 were loaded with a Ca^{2+} sensitive dye and challenged with a maximal concentration of NmU to initiate a $[Ca^{2+}]_i$ elevation. The cells were then washed with KHB pH 7.4 and were re-challenged with the same maximal concentration of NmU. The magnitude of the second Ca^{2+} response was used to assess the extent of NmU-receptor desensitisation. In all cases, the second NmU challenge failed to elicit a Ca^{2+} response.

This failure to elevate $[Ca^{2+}]_i$ to the second NmU challenge could be explained by one of two phenomenon. Firstly, the receptors are subject to rapid, full and prolonged desensitisation after agonist addition and have not re-sensitised by the time they are re-challenged with NmU. However, this is inconsistent with sustained increases in $[Ca^{2+}]_i$, $Ins(1,4,5)P_3$ and accumulation of $[^3H]$ -InsP_x seen following activation of either hNmU-R1 or hNmU-R2 (see Chapter 1). A second explanation is that the KHB wash is unable to remove receptor-bound NmU.

In this latter scenario, the receptors are presumably still active and are possibly depleting Ca^{2+} stores. Additionally, due to the receptors already being occupied by NmU from the first challenge, the second challenge simply cannot bind, and as such cannot activate receptors to cause a second Ca^{2+} response. The re-challenge method of characterising receptor desensitisation relies on the ability to remove the ligand from the receptor between agonist challenges. Failure to do so will result in receptors appearing to desensitise rapidly, completely and for an extensive duration.

This chapter explores repetitive applications of NmU in HEK293 cells recombinantly expressing hNmU-R1 or hNmU-R2. To further investigate the reversibility of binding, four main approaches were employed to assess the ability of the KHB pH 7.4 wash to remove bound hNmU-25 from its receptors. These were i) the influence of washing on the accumulation of $[^{3}H]$ -InsP_x, ii) receptor cross-talk, iii) the visualization of NmU binding using a fluorescently-labelled NmU analog and iv) the ability of excess cold NmU to displace pre-bound $[^{125}I]$ -NmU-25 from membrane preparations. As an additional approach, the internalisation of hNmU receptors was explored using either confocal microscopy and the fluorescent NmU analog or by using $[^{125}I]$ -hNmU-25 in cell populations.

Section 4.2 Methods

All methods are exactly as described previously (see Methods, section 2.2).

4.3.1 Ca²⁺ signalling by repetitive challenges of NmU

In the initial attempt to examine the potential desensitisation of hNmU-25mediated Ca²⁺ signalling using classical re-challenge protocols, single-cell Ca²⁺ imaging demonstrated that following the stimulation of either hNmU-R1 (Fig. 4.1a) or hNmU-R2 (Fig. 4.1b) expressing HEK293 cells with 10nM (maximal) hNmU-25, perfusion (5ml min⁻¹) with agonist-free KHB did not return [Ca²⁺]_i to basal levels. Furthermore, re-application of 10nM hNmU-25 following this perfusion had no effect on [Ca²⁺]_i (Fig. 4.1a-b. In contrast, application of 100µM CCh to activate endogenous $G\alpha_{q/11}$ -coupled muscarinic M₃ receptors also evoked a peak and plateau [Ca²⁺]_i elevation that was similar to that evoked by 10nM hNmU-25 (Fig. 4.1c). Perfusion of agonist-free buffer reduced [Ca²⁺]_i to basal levels and re-application of 100µM CCh resulted in a Ca²⁺ response that was 40±10% (n=34 cells) of the original (Fig. 4.1c).

Further, neither hNmU-R1 (Fig. 4.2a) nor hNmU-R2 (Fig. 4.2b) generated increases in $[Ca^{2+}]_i$ to a second NmU-challenge even when the KHB wash period that separates the successive agonist applications was extended to 12min.







b) hNmU-R2



Fig. 4.1a-b. $[Ca^{2+}]_i$ responses to repeated application of hNmU-25 in cells expressing either hNmU-R1 or hNmU-R2. Cells were cultured on glass coverslips, loaded with fluo-3-AM and cytosolic fluorescence was measured as an index of $[Ca^{2+}]_i$ using confocal microscopy. Cells expressing either hNmU-R1 (a) or hNmU-R2 (b) were challenged with 10nM hNmU-25 at t=30s. At t=60s, cells were perfused with agonist-free buffer at a rate of 5ml min⁻¹ for 120s. At 180s, 10nM hNmU-25 was re-applied. Changes in cytosolic fluorescence of all cells in the field of view were averaged and expressed relative to basal levels. Images A, B, C and D, were taken at the time points indicated. All data are representative of 3 separate experiments. Variance is detailed in appendix 1.

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Fig. 4.1c. $[Ca^{2+}]_i$ responses to repeated application of CCh in HEK293 cells. hNmU-R1 expressing HEK293 cells were cultured on glass coverslips, loaded with fluo-3-AM and cytosolic fluorescence was measured as an index of $[Ca^{2+}]_i$ using confocal microscopy. Cells were challenged with 100µM CCh at t=30s. At t=60s, cells were perfused with agonist-free buffer at a rate of 5ml min⁻¹ for 120s. At 180s, 100µM CCh was re-applied. Changes in cytosolic fluorescence of all cells in the field of view were averaged and expressed relative to basal levels. Images A, B, C and D, were taken at the time points indicated. All data are representative of 3 separate experiments. Variance is detailed in appendix 1.

The second secon

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c)

a) hNmU-R1



Fig. 4.2a-b. $[Ca^{2+}]_i$ responses to repeated application of hNmU-25 in cells expressing either hNmU-R1 or hNmU-R2. Effects of extended washing between applications. Cells were loaded as per Fig. 4.1a). Cells expressing either hNmU-R1 (a) or hNmU-R2 (b) were challenged with 10nM hNmU-25 at t=30s. At t=60s, cells were perfused with agonist-free KHB at a rate of 5ml min⁻¹ for 720s (12min). At 780s 10nM hNmU-25 was re-applied. Changes in cytosolic fluorescence of all cells in the field of view were averaged and expressed relative to basal levels. Images A, B, C and D, were taken at the time points indicated. All data are representative of 3 separate experiments. Variance is detailed in appendix 1.
As a further point for investigating repetitive agonist challenges in HEK293 cells, the effects of washing with KHB were observed on either NmU- or CCh-mediated Ca^{2+} responses following initial challenge with the alternative (CCh or NmU) ligand.

In hNmU-R1 expressing HEK293 cells, the addition of either 10nM hNmU-25 or 100 μ M CCh provoked a Ca²⁺ response of approximately 3-4 fold over basal (Fig. 4.3). Addition of 10nM hNmU-25 at 150s following 100 μ M CCh resulted in a Ca²⁺ response of approximately 25 ± 10% of the CCh-mediated response (n=26 cells) (Fig. 4.3a). If cells had been washed (120s) with agonist-free buffer, Ca²⁺ responses to 10nM hNmU-25 were approximately 100 ± 20% of CCh, (n=45 cells) (Fig. 4.3b). In contrast, application of 100 μ M CCh at 150s following hNmU-25 evoked [Ca²⁺]_i elevation of approximately 25 ± 15% (n=37 cells) that of the initial hNmU-25 response (Fig. 4.3c) irrespective of whether there had been a KHB wash (120s) or not following hNmU-25 application (Fig. 4.3d).



Fig. 4.3a-b. $[Ca^{2+}]_i$ responses to application of hNmU-25 following CCh challenge. Cells were prepared as per Fig. 4.1a. Cells were challenged with 100µM CCh at t=30s (a and b), and were either not washed (a), or washed (KHB, 5ml min⁻¹, 120s) (b) before application of 10nM hNmU-25 at t=180s. Changes in cytosolic fluorescence of all cells in the field of view were averaged and expressed relative to basal levels. Images A, B, C and D, were taken at the time points indicated. All data are representative of 3 separate experiments. Variance is detailed in appendix 1.

b)



Fig. 4.3c-d. $[Ca^{2+}]_i$ responses to application of CCh following hNmU-25 challenge. Cells were prepared as per Fig. 4.1a. Cells were challenged with 10nM hNmU-25 at t=30s (c and d), and were either not washed (c), or washed (KHB, 5ml min⁻¹, 120s) (d) before application of 100µM CCh at t=180s. Changes in cytosolic fluorescence of all cells in the field of view were averaged and expressed relative to basal levels. Images A, B, C and D, were taken at the time points indicated. All data are representative of 3 further experiments. Variance is detailed in appendix 1.

c)

d)

4.3.2 Effects of washing on functional responses

To further highlight the lack of effects of KHB washing of the sustained Ca^{2+} response, hNmU-R1 (Fig. 4.4a) or hNmU-R2 (Fig. 4.4b) expressing cells were challenged with 10nM hNmU-25 and washed with KHB (5ml min⁻¹). With both receptor sub-types, hNmU-25 challenge resulted in peak and plateau Ca^{2+} responses as reported previously (for internally controlled experiments without the wash refer to Fig 3.3a-b), and KHB washing had no effect on the sustained signal.

To explore the ability to remove receptor-bound hNmU-25, the impact of extensively washing cells during the linear phase of accumulation of $[^{3}H]$ -InsP_x under a Li⁺-block of inositol monophosphatase was examined. Cells expressing hNmU-R1 were challenged with 10nM hNmU-25 and after 10min were either untreated (i) or alternatively, the buffer removed and the cells washed (three times with 1ml buffer) before replacement of buffer without (ii) or with (iii) 10nM hNmU-25. Irrespective of the manipulation, the rate and extent of accumulation of $[^{3}H]$ -InsP_x was similar (Fig. 4.5a). Identical data were obtained using cells expressing hNmU-R2 (Fig. 4.5b). This is in contrast to similar manipulations using 100µM CCh to stimulate endogenous muscarinic M₃ receptors in wild-type HEK293 cells, where removal of CCh abolished further accumulation of $[^{3}H]$ -InsP_x (Fig. 4.5c).



Fig. 4.4a-b. Effects of washing with KHB on sustained Ca^{2+} signalling by hNmU-R1 and hNmU-R2. Cells expressing hNmU-R1 (a) or hNmU-R2 (b) were cultured on glass coverslips, loaded with fluo-3-AM and cytosolic fluorescence measured as an index of $[Ca^{2+}]_i$ using confocal microscopy. Cells were challenged with 10nM hNmU-25 at t=30s and were washed with KHB (5ml min⁻¹) at t=60s. For internally controlled experiments without the wash refer to Fig 3.3a-b. Changes in cytosolic fluorescence of all cells in the field of view were averaged and expressed relative to basal levels. Images A, B, and C were taken at the time points indicated. All data are representative of 3 separate experiments. Variance is detailed in appendix 1.



Fig. 4.5a-c. hNmU-25-mediated accumulation of $[{}^{3}H]$ -InsP_x is unaffected by washing to remove extracellular hNmU-25. Cells expressing hNmU-R1 (a) or hNmU-R2 (b), or wild-type HEK293 cells (c) were cultured in 24-well plates and loaded with *myo*- $[{}^{3}H]$ -inositol for 48h before challenge in the presence of a 10mM Li⁺-block of inositol monophosphatase activity. Cells were challenged with 10nM hNmU-25 (a-b) or 100 μ M CCh (c) and after 10min were either untreated (*filled circles*; •) or the buffer removed and the cells washed (three times with 1ml buffer) before replacement of buffer either without (*open circles*; •) or with (*downward filled triangles*; ∇) agonist. Data are mean ± s.e.m., n=3.

4.3.3 Receptor cross-talk

By making use of cross-talk between receptors coupled to $G\alpha_{q/11}$ and those coupled to either $G\alpha_s$ or $G\alpha_i$ an assay was developed to assess receptor activation and provide an indication of ligand-removal.

As a consequence of such cross-talk, following activation of a $G\alpha_{q/11}$ -coupled receptor, activation of either a $G\alpha_s$ - or $G\alpha_i$ -coupled receptor can, in some instances, result in the appearance or potentiation of Ca^{2+} signalling (Werry *et al.*, 2003). Often the ongoing activation of $G\alpha_{a/11}$ -coupled receptors is required for the cross-talk and this has the potential to reveal whether these receptors are active at the time of a subsequent challenge of $G\alpha_s$ - or $G\alpha_i$ -coupled receptors. In HEK293 cells, challenge of an endogenous β_2 -adrenoceptor with 10µM noradrenaline did not elevate $[Ca^{2+}]_i$ (Fig. 4.6a). However, following and in the continued presence of CCh-mediated activation of the $G\alpha_{a/11}$ -coupled muscarinic M₃ receptor, application of 10µM noradrenaline resulted in a robust elevation of $[Ca^{2+}]_i$ (Fig. 4.6b). Removal of CCh by a 2min wash with KHB abolished the $[Ca^{2+}]_i$ response to a subsequent application of noradrenaline (Fig. 4.6c) confirming the need for ongoing activation of the $G\alpha_{q/11}$ coupled receptor to mediate receptor cross-talk. Challenge of cells with 10µM noradrenaline following and in the continued presence of 10nM hNmU-25 also provoked a robust elevation of $[Ca^{2+}]_i$ in cells expressing hNmU-R1 (Fig. 4.6d). Washing the cells with KHB for 3min following challenge with 10nM hNmU-25 did not abolish the subsequent $[Ca^{2+}]_i$ response to noradrenaline (Fig. 4.6e) suggesting that hNmU-R1 was still active. Data obtained using cells expressing hNmU-R2 were identical to those obtained using cells expressing hNmU-R1 (Fig. 4.6f-g).



Fig. 4.6a. Ca^{2+} signalling following activation of endogenously expressed β_2 adrenoceptors in HEK293 cells. Wild-type HEK293 cells were cultured on glass coverslips, loaded with fluo-3-AM and cytosolic fluorescence was measured as an index of $[Ca^{2+}]_i$ using confocal microscopy. Cells were challenged with 10µM noradrenaline at t=30s. Changes in cytosolic fluorescence of all cells in the field of view were averaged and expressed relative to basal levels. Images A, B and C were taken at the time points indicated on the individual traces. All data are representative of 3 separate experiments. Variance is detailed in appendix 1.

a)



c)



Fig. 4.6b-c. Cross-talk between mucarinic M₃-receptors and β_2 -adrenoceptors. Cells were prepared as per Fig. 4.6a. Cells were challenged with 100µM CCh at t=30s to activate endogenous Ga_q/11-coupled muscarinic M₃ receptors. This was followed by 10µM noradrenaline at 150s to activate endogenous Ga_s-coupled β_2 -adrenoceptors. Noradrenaline was applied either in the continued presence of CCh (b) or following a 120s wash with buffer (c). Changes in cytosolic fluorescence of all cells in the field of view were averaged and expressed relative to basal levels. Images A, B and C were taken at the time points indicated on the individual traces. All data are representative of 3 separate experiments. Variance is detailed in appendix 1.

b)

d) hNmU-R1

e) hNmU-R2



10nM 10µM hNmU-25 noradrenaline wash 3 Cytosolic fluorescence (relative to basal) P 2 1 0 0 60 120 180 240 Time (s)

Fig. 4.6d-e. Cross-talk between hNmU-R1 and β_2 -adrenoceptors. hNmU-R1expressing HEK293 cells were cultured on glass coverslips, loaded with fluo-3-AM and cytosolic fluorescence was measured as an index of $[Ca^{2+}]_i$ using confocal microscopy. Cells were challenged with 10nM hNmU-25 at t=30s. This was followed by 10µM noradrenaline at 150s to activate endogenous Ga_s-coupled β_2 adrenoceptors. Noradrenaline was applied either in the continued presence of hNmU-25 (d) or following a 120s wash with buffer (perfused at 5ml min⁻¹) (e). Changes in cytosolic fluorescence of all cells in the field of view were averaged and expressed relative to basal levels. Images A, B and C were taken at the time points indicated on the individual traces. All data are representative of 3 separate experiments. Variance is detailed in appendix 1. f) hNmU-R2







Fig. 4.6f-g. Cross-talk between hNmU-R2 and β_2 -adrenoceptors. hNmU-R2 expressing HEK293 cells were cultured on glass coverslips, loaded with fluo-3-AM and cytosolic fluorescence measured as an index of $[Ca^{2+}]_i$ using confocal microscopy. Cells were challenged with 10nM hNmU-25 at t=30s. This was followed by 10µM noradrenaline at 150s to activate endogenous $G\alpha_s$ -coupled β_2 -adrenoceptors. Noradrenaline was applied either in the continued presence of hNmU-25 (d) or following a 120s wash with buffer (perfused at 5ml min⁻¹) (e). Changes in cytosolic fluorescence of all cells in the field of view were averaged and expressed relative to basal levels. Images A, B and C were taken at the time points indicated on the individual traces. All data are representative of 3 separate experiments. Variance is detailed in appendix 1.

4.3.4 Binding of fluorescent NmU

CyDye fluorophores are commercially available fluorescent conjugates that were developed from a dye class called the cyanines. These pH insensitive fluorophores can be conjugated to a variety of proteins. As a final approach to determine whether a wash with KHB is sufficient to remove receptor-bound NmU, pNmU-8 was conjugated with a CyDye fluorophore termed Cy3B via an amine group within the N-terminus of pNmU-8 as described in Methods, section 2.2.9. The resultant NmU-analog (pNmU-8-Cy3B) was then visualised on hNmU-R1 and hNmU-R2 expressing cells via confocal microscopy.

In studies based on $[{}^{3}H]$ -InsP_x accumulation in HEK293 cells expressing either hNmU-R1 (Fig. 4.7a) or hNmU-R2 (Fig. 4.7b), pNmU-8-Cy3B was equipotent with both unlabelled hNmU-25, rNmU-23 and porcine NmU-8. A summary of pEC₅₀ values obtained is shown in Table 4.1.







Fig. 4.7a-b. A comparison of concentration-response relationships for [³H]-InsP_x accumulation mediated by hNmU-25, rNmU-23, pNmU-8 and pNmU-8-Cy3B. Cells expressing either hNmU-R1 (a) or hNmU-R2 (b) were seeded into 24well plates and loaded with [³H]-*myo*-inositol for 48h before challenge in the presence of a 10mM Li⁺-block of inositol monophosphatase activity. Concentrationresponse curves for the accumulation of [³H]-InsP_x were generated by challenge of cells with varying concentrations of hNmU-25 (*filled squares*; **•**), rNmU-23 (*upwards filled triangles*; **•**), pNmU-8 (*open diamonds*; \diamond) or pNmU-8-Cy3B (*open circles*; \circ) for 60min. The pEC₅₀ values obtained are summarised in Table. 4.1. Data are mean ± s.e.m., n=3. [³H]-InsP_x accumulations are presented as the percentage increase relative to basal levels that were 3549 ± 463 and 2984 ± 384 DPM well⁻¹ for hNmU-R1 and hNmU-R2 respectively (data are mean ± s.e.m., n=8 wells).

Table. 4.1. pEC₅₀ values of NmU analogs based on the stimulation of $[^{3}H]$ -InsP_x accumulation under Li⁺-block of monophosphatase activity in HEK293 cells expressing either hNmU-R1 or hNmU-R2. Data are mean \pm s.e.m., n=3.

Agonist	hNmU-R1	hNmU-R2
hNmU-25	8.93 ± 0.05	8.87 ± 0.09
rNmU-23	9.01 ± 0.16	8.80 ± 0.08
pNmU-8	8.81 ± 0.09	8.70 ± 0.05
pNmU-8-Cy3B	8.88 ± 0.09	8.79 ± 0.06

The inability of hNmU receptors to distinguish between different forms of NmU as well as the comparable potency of fluorescently labelled and unlabelled pNmU-8 at hNmU-R1 and hNmU-R2, make the pNmU-8-Cy3B fluorescent analog ideal for use in confocal imaging to visualise ligand-receptor binding.

Addition of 10nM pNmU-8-Cy3B to cells expressing hNmU-R1 resulted in an immediate appearance of intense fluorescence localized to the plasma membrane (Fig. 4.8a). These confocal images were confirmed as receptor-ligand binding by further experiments that demonstrated the specificity of this interaction. Here, addition of 1 μ M hNmU-25 prior to the addition of pNmU-8-Cy3B abolished the appearance of plasma membrane fluorescence in hNmU-R1 expressing cells (Fig. 4.8b(ii)). Similar experiments were repeated with hNmU-R2 expressing cells, with identical results (Fig. 4.8c-d)

As a negative control, no fluorescence was observed following an identical addition to wild-type HEK293 cells (Fig. 4.8e) suggesting a lack of non-specific binding.

In attempts to remove bound pNmU-8-Cy3B from NmU receptors, at 1min following the addition of 10nM pNmU-8-Cy3B, the addition of 1µM hNmU-25 did not result in a loss of plasma membrane fluorescence in hNmU-R1 (Fig. 4.9a(i-ii)) or hNmU-R2 (Fig. 4.9b(i-ii)) expressing cells. Furthermore, following addition of 10nM pNmU-8-Cy3B to hNmU-R1 (Fig. 4.9c) or hNmU-R2 (Fig. 4.9d) expressing cells at 12°C (to block receptor internalisation), continuous perfusion of cells with KHB (5ml min⁻¹) for 12min did not diminish plasma membrane fluorescence.



Fig. 4.8a-d. Binding of fluorescently-labelled pNmU-8 (pNmU-8-Cy3B) to cells expressing hNmU-R1 or hNmU-R2. Cells expressing hNmU-R1 (a-b) or hNmU-R2 (c-d) were cultured on glass coverslips, excited at 568nm and imaged using confocal microscopy. Phase image (a(i)) and fluorescence image (a(ii)) of hNmU-R1 cells following addition of 10nM pNmU-8-Cy3B. Phase image (c(i)) and fluorescence image (c(i)) of hNmU-R2 cells following addition of 10nM pNmU-8-Cy3B. Phase image (c(i)) and fluorescence image (b(i)) and fluorescence image (b(ii)) of hNmU-R2 cells following addition of 10nM pNmU-8-Cy3B. Phase image (b(i)) and fluorescence image (b(ii)) of hNmU-R1 cells that were exposed to 10nM pNmU-8-Cy3B following addition of (and in the continued presence of) 1μ M hNmU-25. These experiments were repeated using hNmU-R2 expressing cells (d(i-ii)). All images are representative of 3 separate experiments.

e) Wild-type HEK293 cells



Fig. 4.8e. fluorescently-labelled pNmU-8 (pNmU-8-Cy3B) to wild-type HEK293 cells. Wild-type HEK293 cells were cultured on glass coverslips, excited at 568nm and imaged using confocal microscopy. Phase image (e(i)) and fluorescence image (e(ii)) of cells following addition of 10nM pNmU-8-Cy3B. All images are representative of 3 separate experiments



Fig. 4.9(a-d). Attempted removal of fluorescently-labelled pNmU-8 (pNmU-8-Cy3B) from cells expressing hNmU-R1 or hNmU-R2. HEK293 cells expressing hNmU-R1 (a and c) or hNmU-R2 (b and d) were cultured on glass coverslips, excited at 568nm and imaged using confocal microscopy. Fluorescence image of cells following addition of 10nM NmU-8-Cy3B (a(i) and b(i)) and the same cells following subsequent addition of 1 μ M hNmU-25 (a(ii) and b(ii)). c and d) pNmU-8-Cy3B (10nM) was added to cells at t=0s (c(i) and d(i)) and the cells were then perfused with KHB (5ml min⁻¹) for 720s. Images were collected at 300 (c(ii) and d(ii)) and 720s (c(iii) and d(iii)) at 12°C. All images are representative of 3 separate experiments

4.3.4 Displacement of [¹²⁵I]-hNmU-25 from membranes prepared from hNmU-R1 or hNmU-R2 expressing cells

As a final approach to examine the possible irreversible binding of NmU, [¹²⁵I]hNmU-25 was pre-bound to membranes prepared from cells expressing either hNmU-R1 (Fig. 4.10a) and hNmU-R2 (Fig. 4.10b). Membranes (10µg) were incubated for 1h at room temperature with 150pM [¹²⁵I]-hNmU-25 to label approximately 50% of the receptors (see Chapter 3). An excess of unlabelled hNmU-25 (1µM) was then added and the amount of [¹²⁵I]-hNmU-25 that remained bound over the next hour was determined. The pre-binding of [¹²⁵I]-hNmU-25 resulted in the specific binding of approximately 2,600 d.p.m.. Unlabelled hNmU-25 did not reduce the amount of bound [¹²⁵I]-hNmU-25 and 100±10% remained bound after 1h (Fig. 4.10) (data are mean ± s.e.m., n=3). a) hNmU-R1



Fig. 4.10a-b. Attempted displacement of pre-bound [^{125}I]-hNmU-25 from membranes prepared from hNmU-R1- and hNmU-R2-expressing cells by unlabelled hNmU-25. Membranes (10µg) from preparations of hNmU-R1 (a) or hNmU-R2 (b) cells were incubated with 150pM [^{125}I]-hNmU-25 for 10min at RT. Incubation proceeded at RT in the absence (control) or presence of 1µM unlabelled hNmU-25. Membranes were filtered and radioactivity was ascertained by standard liquid scintillation counting methods. Data are mean + s.e.m., n=3.

4.3.5 Removal of bound pNmU-8-Cy3B using alternate wash protocols

In light of the conclusions made from both the $Ga_{q/11}/Ga_s$ cross-talk experiments and from the pNmU-8-Cy3B wash-off experiments, it is clear that NmU cannot be removed from the binding site of either NmU-R1 or NmU-R2 with a KHB pH 7.4 wash. Several alternative wash-protocols were used in an attempt to remove bound pNmU-8-Cy3B. Here, confocal visualisation of pNmU-3-Cy3B binding to HEK293 cells expressing hNmU-R1 was used as a screen to assess a variety of alternatative washes. The inclusion of either 150mM (Fig. 4.11a) or 200mM (Fig. 4.11b) sodium chloride, or 10mM (Fig. 4.11c) or 50mM (Fig. 4.11d) acetic acid in KHB had no effect on removal of bound pNmU-8-Cy3B. The addition of hydrochloric acid (HCl) to KHB to reduce pH also had no effect at pH 5.0 (Fig. 4.11e), pH 4.0 (Fig. 4.11f) or pH 3.0 (Fig. 4.11g). Only when the pH of KHB was reduced to 2.0 was there any loss of plasma membrane fluorescence. Here, the loss of membrane fluorescence was immediate and full (Fig. 4.11h).

Once removed by acid wash, pNmU-8-Cy3B rebound to hNmU-R1 (Fig. 4.12a) and hNmU-R2 (Fig. 4.12b) cells following a return to pH 7.4 and re-addition. The return to pH 7.4 conditions alone had no effect on fluorescence (Fig. 4.12a(iii) and b(iii)) and demonstrates that loss of fluorescence was not representative of the effect of acidic conditions on the fluorescence of the fluorophore. Re-application of pNmU-8-Cy3B produced fluorescent images identical to those following the original application (Fig. 4.12a(iv) and b(iv)) and confirms the receptor's integrity in such that it retains the ability to bind NmU after being subject to these acidic conditions. This re-bound NmU can subsequently be removed with the pH 2.0 KHB acidic wash (Fig. 4.12a(v) and b(v)). As further confirmation, in cell populations, a single 1ml wash of KHB pH 2.0 was sufficient in removing ~100% of bound [125 I]-hNmU-25 (see below, Section 4.3.7).

Even in the absence of any agonist pre-stimulation, the pH 2.0 wash resulted in a marked disorganisation of CCh-mediated $[Ca^{2+}]_i$ responses (Fig. 4.13a-b) and a ~65% reduction in 100µM CCh-mediated $[^3H]$ -InsP_x accumulation after 30min stimulation (Fig. 4.13b). Similar data was obtained following stimulation of hNmU-R1 and hNmU-R2 expressing cells with 10nM hNmU-25 (data not shown). Following the acid wash, cells appeared more granular with a thickened plasma

membrane (Fig. 4.13c). Thus, it is difficult to use such protocols for classic rechallenge protocols with NmU. The detrimental effects of acidic conditions therefore means any use of acidic washing in experimental protocols must be as an end-point.



Fig. 4.11a-h. Alteration of wash protocol to remove bound pNmU-8-Cy3b from hNmU-R1 expressing cells. Cells expressing hNmU-R1 were cultured on glass coverslips, excited at 568nm and imaged using confocal microscopy. pNmU-8-Cy3B (10nM) was applied to cells and images captured before (i) and following (ii) perfusion (5ml min⁻¹) with KHB containing 150mM (a) and 200mM (b) sodium chloride, 10mM (c) and 50mM (d) acetic acid, KHB pH 5.0 (e), pH 4.0 (f), pH 3.0 (g) and pH 2.0 (h). Images are representative of 3 experiments.

a) hNmU-R1



Fig. 4.12a-b. Effects of KHB pH 2.0 on binding of pNmU-8-Cy3B to hNmU-R1 and hNmU-R2 cells. Cells expressing either hNmU-R1 (a) or hNmU-R2 (b) were cultured on glass coverslips, excited at 568nm and imaged using confocal microscopy. pNmU-8-Cy3B (10nM) was applied to cells and images captured before (i) and following (ii) perfusion wash (5ml min⁻¹) with KHB pH 2.0. Cells were then returned to KHB pH 7.4 conditions (iii) and 10nM pNmU-8-Cy3B was reapplied (iv). Cells were then washed again with KHB pH 2.0 (v). Experiments are representative of 3 experiments.



Fig. 4.13a-b. Effects of KHB pH 2.0 on CCh-mediated Ca^{2+} responses in HEK293 cells. a-b) Wild-type HEK293 cells were cultured on glass coverslips, loaded with fluo-3-AM and cytosolic fluorescence measured as an index of $[Ca^{2+}]_i$ using confocal microscopy. a) Cells were challenged with 100µM CCh at t=30s and washed (KHB pH 7.4, 120s, 5ml min⁻¹) before re-addition of 100µM CCh at t=180s. The experiment shown in b was identical with the exception that the wash was with KHB at pH 2.0. Traces are representative of 3 experiments.

b)



Fig. 4.13c. Effects of KHB pH 2.0 on CCh-mediated $[{}^{3}H]$ -InsP_x accumulation in HEK293 cells. Wild-type HEK293 cells were cultured in 24-well plates and loaded with *myo*- $[{}^{3}H]$ -inositol for 48h before challenge in the presence of a 10mM Li⁺-block of inositol monophosphatase activity. Cells were either pre-treated (1ml, 10min) with KHB pH 2.0 (*filled squares*; \blacksquare) or KHB pH 7.4 (*upward filled triangles*; \blacktriangle) prior to challenge with 100µM CCh for times varying between 0-30min. ³H in cell extracts was determined by standard liquid scintillation methods. Data are mean ± s.e.m., n=3.



d)

Fig. 4.13d. Morphological appearance of HEK293 cells following pH 2.0 treatment. Wild-type HEK293 cells were cultured on glass coverslips and imaged via confocal microscopy. Images were taken prior to (i) or following (ii) perfusion with KHB pH 2.0 (2min, 5ml min⁻¹). Images are representative of 3 experiments.

4.3.6 Receptor internalisation

All previous experiments using pNmU-8-Cy3B were performed at $\leq 12^{\circ}$ C to prevent receptor internalisation. At 37°C, addition of pNmU-8-Cy3B to hNmU-R1 (Fig. 4.14a) or hNmU-R2 (Fig. 4.14b) cells also resulted in plasma membraneassociated fluorescence (Fig. 4.14a(i) and b(i)) that could not be removed using KHB. Furthermore, after approximately 5min (300s) (Fig. 4.14a(ii) and b(ii)), membrane fluorescence began to reduce coincident with the appearance of punctuate fluorescence within the cell cytosol indicating internalisation of the ligand. By approximately 10min (600s), cellular fluorescence was almost exclusively punctate and cytosolic (Fig. 4.14a(ii) and b(iii)). Based on the assumption that the fluorophore was intact and was internalised following binding to the NmU-receptors, the internalisation of this fluorophore may present an opportunity to examine ligand and receptor internalisation.

To confirm the images seen were a true reflection of internalisation, the experiments were repeated in conditions where internalisation would be expected to be essentially blocked. In previous experiments receptor internalisation was prevented at temperatures below 12°C (see Section 4.3.4 and 4.3.6). To confirm this, internalisation of pNmU-8-Cy3B following application to hNmU-R1 was blocked up to 1h (3600s) at temperatures below 12°C (Fig. 4.15a). In addition, using cells expressing hNmU-R1, pre-treatment (50µg ml⁻¹, 30min) of cells with the internalisation inhibitor concanavalin A (ConA) blocked internalisation of 10nM pNmU-8-Cy3B at 37°C (Fig. 4.15b). Concanavalin A is a plant sugar-binding protein (lectin) and has been shown previously to inhibit the internalisation of numerous receptors (e.g. Wlado *et al.*, 1983; Toews *et al.*, 1984; Lohse *et al.*, 1990; Pippig *et al.*, 1995; Kramer and Ejmu, 2000; Trincavelli *et al.*, 2000). Pre-treatment of cells with this compound has been shown to inhibit clathrin-mediated GPCR internalisation without affecting signal transduction (DeGraff *et al.*, 1999).

a) hNmU-R1



b) hNmU-R2



Fig. 4.14a-b. Internalisation of pNmU-8-Cy3B at 37°C. Cells expressing either hNmU-R1 (a) or hNmU-R2 (b) were cultured on glass coverslips, excited at 568nm and imaged using confocal microscopy following addition of 10nM Cy3b-NmU. Temperatures were maintained at 37°C by a Peltier unit. Images (i-iii) were taken at the time points indicated and are representative of 3 experiments.

The internalisation of [¹²⁵I]-hNmU-25 could be studied in cell populations using the KHB pH 2.0 wash as an end point. Here, hNmU-R1 cells were challenged with 150pM [¹²⁵I]-hNmU-25 for various times and cell membrane-associated radioactivity was stripped with KHB pH 2.0. The amount of cell-associated radioactivity was determined following solubilisation of cells. Preliminary experiments revealed that a single 1ml wash with KHB at pH 2.0 was sufficient in removing ~90% of membrane-associated [¹²⁵I]-hNmU-25 (Fig. 4.15a). This is entirely consistent with the confocal imaging of pNmU-8-Cy3B binding to single-cells (see Fig. 4.11). KHB washes of pH 6.0 and 7.4 were unable to remove receptor-bound [¹²⁵I]-hNmU-25 in cell populations (Fig. 4.15a) whereas washing with KHB pH 4.0 removed ~30% of [¹²⁵I]-hNmU-25. To confirm the acid wash was sufficient in removing bound [¹²⁵I]hNmU-25, the extracellular washes were collected and pooled and the radioactivity contained within them was determined. Radioactivity in the extracellular washes confirms that KHB pH 2.0 removes ~90% of bound [¹²⁵I]-hNmU-25 (Fig. 4.15b).

The study of internalisation in cell populations revealed that, at 37° C, [¹²⁵I]hNmU-25 rapidly internalised on application to hNmU-R1 expressing cells. At ~10min following application internalisation was approximately 50% and this was maintained until the furthest time point tested (60min) (Fig. 4.15b). Further, consistent with pNmU-8-Cy3B internalisation in single-cells, this internalisation was blocked by pre-treatment of cells with conA (50µg ml⁻¹, 30min) (Fig. 4.15c).

a) Cell-associated radioactivity



b) Collection of extracellular radioactivity



Fig. 4.15a-b. Acid stripping of [125 I]-hNmU-25 from cell populations of hNmU-R1 expressing cells. Cells expressing hNmU-R1 were seeded into poly-D-lysine coated 24-well plates and cultured for 24h. Cells were washed in KHB pH 7.4 and 150pM [125 I]-hNmU-25 was applied at 4°C for 30min. Extracellular buffer/[125 I]hNmU-25 was aspirated and cells were washed with 1ml KHB buffer at pH 7.4, 6, 4 or 2 as indicated. Cells were washed either once, twice or three times as indicated in separate experiments and extracellular collections were pooled. Extracellular fluid was collected (a) or cells were solubilised with 1ml 1M NaOH (b) and 125 I in each was determined by standard liquid scintillation methods. 125 I radioactivity was calculated as a percentage of 150pM [125 I]-hNmU-25 total binding which was 1560±314 dpm well⁻¹, n=6 wells (not shown). Data are representative of 3 experiments.



Fig. 4.15c. Internalisation of hNmU-R1 in cell populations and the effects of ConA treatment. Cells expressing hNmU-R1 were seeded into poly-D-lysine coated 24-well plates and cultured for 24h. Cells were washed and equilibrated at 37°C for 15min in KHB. Where applicable (*open squares*; \Box) cells were pre-treated (50µg ml⁻¹, 30min) with ConA. Cells were challenged with 150pM [¹²⁵I]-hNmU-25 at 37°C for between 0 and 60min. Reactions were terminated by aspiration of extracellular buffer and cells were immediately solubilised in 1ml NaOH. ¹²⁵I in the cell solute was determined by standard liquid scintillation methods. Data were calculated as 100 less the percentage of cell associated [¹²⁵I]-hNmU-25 following 2h incubation at 4°C, aspiration of extracellular fluid, and solubilisation of cells as before. Cell associated radioactivity was 1346±231 and 1547±410 dpm well⁻¹ for untreated and conA-treated cells respectively, n=6 wells (not shown). Data are mean ± s.e.m., n=3.

Section 4.4 Discussion

4.4.1 Summary

Following an exploration of single-cell Ca^{2+} signalling by hNmU-R1 and hNmU-R2, it became apparent that repetitive applications of hNmU-25 do not elicit Ca^{2+} signals to the second challenge when the two applications were separated by extensive washing with KHB. One possible explanation for this is full and rapid receptor desensitisation, but this profile is inconsistent with sustained PLC signalling (see Chapter 3) and sustained Ca^{2+} signals during washing. Furthermore, NmU-mediated Ca^{2+} signals following challenge with CCh were markedly increased if cells were first washed to remove CCh. However, washing following NmU challenge had no effect on the magnitude of the signal generated by CCh. These data suggest that the KHB wash was unable to remove bound NmU from its receptors.

To further explore this we employed four complimentary approaches: i) the influence of washing on the accumulation of $[^{3}H]$ -InsP_x; ii) receptor cross-talk; iii) the visualization of NmU binding using a fluorescently-labelled NmU analog and; iv) the ability of excess cold NmU to displace pre-bound $[^{125}I]$ -NmU-25 from membrane preparations.

Washing had no effect on $[{}^{3}H]$ -InsP_x accumulation, receptor cross-talk or the binding of fluorescently-labelled NmU. Further, in membrane preparations, $[{}^{125}I]$ -hNmU-25 could not be displaced by maximal concentrations of unlabelled hNmU-25. In all cases, the evidence suggested an irreversible ligand-receptor interaction. Indeed, only using washing buffers with an acidity of pH 2.0 could $[{}^{125}I]$ -hNmU-25 and fluorescently-labelled NmU be removed from either receptor type, albeit at great detriment to the function of the cells.

At 37°C, fluorescently-labelled NmU, when bound to either hNmU-R1 or hNmU-R2, moved from the plasma membrane to exist almost entirely within the cytosol, suggesting ligand internalisation and most probably receptor internalisation as well. Indeed, internalisation of $[^{125}I]$ -hNmU-25 in cell populations proceeded until a steady state existed whereby ~50% of total cellular-associated activity was internal (i.e. resistant to acid-stripping). Internalisation was blocked by temperatures below 12°C and by pre-treatment of cells with conA, an inhibitor of clathrin-

mediated internalisation. These studies did not reveal any differences between hNmU-R1 and hNmU-R2.

4.4.2 Irreversible ligand binding

This study demonstrates that the standard wash protocol (perfusion (5ml min⁻¹) of KHB for up to 3min) was unable to remove hNmU-25 from its receptors. This resistance to washing presumably reflects high-affinity binding, consistent with data from binding studies (despite possible difficulties in interpretation of such data (see below)). This was confirmed for recombinant hNmU-R1 and hNmU-R2 using a variety of approaches, namely: the sustained accumulation of [³H]-InsP_x despite attempts to remove the ligand; the phenomenon of cross-talk between $Ga_{q/11}$ and Ga_s -coupled receptors even following washing and the irreversible binding of fluorescently labelled NmU.

Although slightly acidic washes (pH 4-5) are often used to remove peptide ligands these, as with the endothelin-A receptor (Hilal-Dandan *et al.*, 1996), proved ineffective in the removal of pNmU-8-Cy3B from either hNmU-R1 or hNmU-R2. Indeed, only highly acidic washes (\leq pH 2) were able to remove pNmU-8-Cy3B and although re-binding was possible, such acidity alone, not surprisingly, was damaging to cells based on accumulation of [³H]-InsP_x and Ca²⁺ signalling.

Following hNmU-25 challenge, the inability of a KHB wash to allow Ca^{2+} responses to subsequent additions of either hNmU-25 or CCh is suggestive of homologous and partial heterologous desensitisation that either persists despite agonist removal or alternatively is a consequence of continued signalling by NmU receptors. The latter is consistent with the sustained accumulation of [³H]-InsP_x under a Li⁺-block (see Chapter 3), suggesting that at least heterologous desensitisation could occur simply through, for example, depletion of a shared intracellular Ca²⁺ store. Taken together, these data also indicate that the wash protocol was insufficient to remove receptor-bound hNmU-25.

The inability of maximal concentrations of hNmU-25 to displace pre-bound [¹²⁵I]-hNmU-25 from membrane preparations of either hNmU-R1- or hNmU-R2- expressing HEK293 cells further confirmed the tight association between NmU and

its receptors. Many previous reports have demonstrated an inhibitory effect of various unlabelled NmU forms on the binding of [125 I]-labelled rNmU-23 (Howard *et al.*, 2000; Kojima *et al.*, 2000) and [125 I]-pNmU-8 (Hosoya *et al.*, 2000). In all cases, labelled and unlabelled NmU peptides were added simultaneously to the assay, and thus represent competition for receptors.

Concentration-response curves for single-cell Ca^{2+} imaging following activation of hNmU-R2 transiently expressed in HEK293 cells have been published previously (Shan *et al.*, 2000). However, individual data points were obtained from separate experiments and no evidence of the effects of washing are shown. Similarly, cell population analysis with recombinant NmU receptors demonstrate concentrationdependent Ca^{2+} signals, with individual data points being obtained from individual wells containing cells not previously exposed to NmU (Funes *et al.*, 2002; Hedrick *et al.*, 2000; Hosoya *et al.*, 2000; Howard *et al.*, 2000; Szekeres *et al.*, 2000).

There is therefore, a distinct lack of evidence from the literature to support the findings reported in this chapter and the conclusions drawn detail novel characteristics of recombinant human NmU receptors.

Concerns that the addition of Cy3B to pNmU-8 would either have adverse effects on the association of NmU with its receptors or allow non-specific interactions with cells (for example, Cy3B may be hydrophobic and simply anchor itself into the membrane) were not supported as there was minimal non-specific binding.

One further point of concern was the possibility that the washing procedure was ineffective simply because the complexes of NmU and receptor were subject to immediate internalisation or compartmentalisation and were inaccessible to dissociation or competition. In this case, confocal images of pNmU-8-Cy3B that appeared to be membrane-associated could in fact exist just below the plasma membrane. However, this is very unlikely due to the fact that a KHB pH 2.0 wash effectively removes virtually all pNmU-8-Cy3B from the plasma membrane, which can be replaced by re-addition in buffer at pH 7.4.

The true affinity of an irreversible agonist cannot be determined reliably in a conventional competition-binding assay. K_d values are dissociation constants, whereby the association between the agonist [A] and the receptor [R] to form

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[A+R] is matched by an equal and opposite dissociation $([A+R] \rightarrow [A] + [R])$ as determined by the laws of mass action. The rate at which receptor-ligand complexes form is proportional to the radio-ligand concentration and the number of receptors still unoccupied. The rate of dissociation is proportional to the concentration of receptor-ligand complexes. Thus, binding increases over time until it reaches equilibrium. Considering binding of NmU is irreversible, equilibrium dissociation is not a useful description and the K_d values obtained from saturation binding analysis of [¹²⁵I]-hNmU-25 (see Chapter 3) may be of limited value as a means of interpreting agonist affinity.

It has been demonstrated that endothelin binding to the endothelin A receptor is irreversible, even after acid stripping (Hilial-Dandan *et al.*, 1996). Furthermore, ligand is still bound to the substance P receptor after washing at pH 7.4 (Schmidlin *et al.*, 2001). Like NmU, both endothelin and substance P are peptides. Human substance P has limited but evident homology with human NmU and NmU can be classed alongside these ligands that show essentially irreversible binding to their receptors.

The physiological consequences of this irreversible interaction are unclear, but as with endothelin A receptors (Leite *et al.*, 1994) is certain to limit the responsiveness of cells to repeated agonist challenge. In the absence of intracellular machinery to remove bound-NmU and re-sensitise the receptors (see below) the irreversible nature of activation could mean NmU acts as a 'one-shot' receptor. If this was the case, then NmU receptors would be like a family of GPCRs that are activated by N-terminal proteolytic cleavage by thrombin, namely; the protease-activated receptors (PARs). Thus, upon activation, PARs are rapidly internalised and degraded (Trejo and Coughlin, 1999). Of note is that the responsiveness of cells to continued exposure to thrombin is critically dependent on the constant supply of new receptors to the plasma membrane. Furthermore, hormones such as progestins have been shown to substantially up-regulate PAR expression thereby increasing cellular responsiveness to thrombin (Herkert *et al.*, 2001). Clearly, the fate of internalised NmU and NmU receptors cannot be elucidated from the current studies and several possibilities are discussed below (see Section 4.4.4).

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4.4.3 Receptor internalisation

Interestingly, at 37°C there was a substantial internalisation of the fluorescentlylabelled NmU over relatively short time-frames (<5min). Considering the high affinity and apparent irreversible nature of NmU-binding, it is likely that confocal images showing pNmU-8-Cy3B internalisation are also representative of NmU-R1 and NmU-R2 internalisation. Further, in cell populations, internalisation advanced rapidly on application of [125I]-hNmU-25 and ~50% of total cellular-associated activity was internal (resistant to extracellular acid-stripping) after 10min. However, substantial receptor internalisation is somewhat in contrast to the sustained linear accumulation of [³H]-InsP_x between 1 and 60min even following removal of free hNmU-25 by washing. This suggests that the recycling of receptors and binding of additional hNmU-25 is unlikely to be required for sustained signalling and that sufficient active receptors either remain at the cell surface or are returned (with or Further studies are required to distinguish these possibilities. without ligand). Another possibility is that internalised receptors continue signalling and although it has been demonstrated that internalised muscarinic receptors cannot contribute to phosphoinositide turnover (Sorenson et al., 1997), whether this is true of all receptors in all circumstances is essentially unknown.

Confocal imaging of pNmU-Cy3B suggets that NmU is substantially endocytosed at approximately 4-5min after agonist addition at 37°C. The method of NmU-receptor internalisation is unclear, and several potential pathways and mechanisms exist (see Introduction, section 1.2.3.2). hNmU-25 internalisation was sensitive to both temperature and conA. ConA works by inhibiting clathrin-mediated GPCR internalisation (DeGraff *et al.*, 1999). Thus, internalisation for hNmU-R1 and/or hNmU-25 is likely to involve clathrin. Whether this is phosphorylation and arrestin dependent is unknown. Phosphorylation of many GPCRs follows agonistbinding and aids in the recruitment of β -arrestin which aids to target the receptor for internalisation (Ferguson, 2001). We can only speculate as to the pathways and mechanisms of NmU-receptor internalisation, but this work will hopefully lead to further investigations.
4.4.4 Possible fate of internalised receptor and ligand

Following budding from the plasma membrane, vesicles can rapidly lose their clathrin coating and become very unstable. This in turn will assist fusion with early endosomes, which exist as a network of tubules and vesicles throughout the cytoplasm and function to sort received material. From these early endosomes the receptors can be efficiently recycled back to the plasma membrane, have their ligands removed (which are subsequently degraded or recycled) or the receptors themselves can be degraded (Mukherjee et al., 1997). The possible fate of internalised NmU-receptors and NmU are therefore numerous. It is possible that they could be internalised and not recycled back to the membrane. This could be the consequence of the high affinity of NmU with its receptors, whereby the only means of separation of the receptor-ligand complex is degradation. This is true for the endothelin B receptor, which is predominantly targeted to lysosomes for degradation with its ligand (Bremnes et al., 2000) and for many PARs, where activation is effectively irreversible (Trejo and Coughlin, 1999). Subsequent receptor expression at the plasma membrane in these cases will be reliant on receptor synthesis. Delivery of endocytosed material from endosomes to lysosomes is thought to occur by endosomal fusion with lysosomes, resulting in the digestion of contained material within the lysosomes by low pH and lysosomal enzymes (Gruenberg et al., 1989). The resultant degradation products are transferred out of the lysosomes to the cytosol, where they can be either utilized, or transported out of the cell (Mellman, 1996).

In some cases, such as the endothelin A receptor, internalised GPCRs are retained within endosomal compartments (Bremnes *et al.*, 2000). This can have implications for prolonged signalling due to the vast array of signalling proteins found in endosomal compartments (review; Sorkin and Zastrow, 2002). For example, MAP kinases, such as ERK1 and ERK2, are activated by a wide variety of GPCRs and can therefore activate mitogenic responses (reviewed; Luttrell *et al.*, 1999). Although it is clear that GPCRs use multiple pathways that eventually converge upon MAP kinase activity, it has been demonstrated that for some GPCRs activation of these pathways requires GPCR internalisation (Luttrell *et al.*, 1997, Daaka *et al.*, 1998 and Gutkind, 1998) due to signalling complexes associated with

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endosomes. Chapter 3 details NmU-receptor mediated activation of ERK1, however, the role of internalisation in this process is a subject for further investigation.

It may also be possible that internalised NmU-receptors continue to signal through the phosphoinositide pathway. $[^{3}H]$ -InsP_x still accumulate 60 minutes after agonist addition, yet receptors are seen to internalise after 4-5 minutes. Whether only part of the receptor population is internalised and those remaining at the cell surface account for the continued $[^{3}H]$ -InsP_x accumulation remains to be seen, but following a decline in the initial rate of PLC activity after 20s, accumulation from there on remains linear with no apparent changes around times when receptors are seen to be markedly internalised. These studies demonstrate that removal of extracellular-hNmU-25 10min after addition has no effects of [³H]-InsP_x accumulation up to 30min when compared to accumulation in the continued presence of NmU. This suggests that receptor recycling and re-binding of NmU at the plasma membrane do not account for continued $[^{3}H]$ -InsP_x accumulation and generates questions as to the source of inositol phosphates. Its been demonstrated that internalised muscarinic cholinergic receptors cannot contribute to phosphoinositide turnover (Sorenson et al., 1997) with the availability of substrate for PLC $(PtdIns(4,5)P_2)$ being one factor that limits the activity of muscarinic receptors in this sub-cellular compartment. Whether this true of the NmU-receptor is unclear at present but is certainly worth pursuing.

The fate of internalised ligand could also be an important aspect of NmUreceptor signalling. Although no experiments performed here address the concept of ligand recycling it has been shown for the somatostatin sst2 receptor that there is dynamic independent cycling of both agonist and receptor between internal compartments and the cell surface (Koenig *et al.*, 1998). The concentration of recycled agonist accumulating in the extracellular environment was sufficient to reactivate the receptor, and has also been proposed for the endothelin A receptor (Schmidlin, 2001). This is one possible explanation for the sustained InsPx accumulation in the absence of extracellular NmU and throws up intriguing questions about NmU-receptor and NmU recycling and whether they recycle as a complex or independently from each other.

Chapter 5

Signalling by endogenously expressed NmU receptors in cultured rat smooth muscle cells.

Section 5.1 Introduction

The majority of investigations into the signalling properties of NmU-receptor isoforms have involved the expression of human receptors as recombinant proteins (Fujii *et al.*, 2000, Hendrik *et al.*, 2000, Hosoya *et al.*, 2000, Kojima *et al.*, 2000, Raddatz *et al.*, 2000, Shan *et al.*, 2000). Indeed, work presented in Chapters 3 and 4 details the signalling, ligand-binding and internalisation properties of human NmU receptors as recombinant proteins in HEK293 cells. The signalling properties of endogenously expressed NmU receptors have been surprisingly under-explored. Only in a recent paper studying the putative cancer-preventing properties of NmU (Shetzline *et al.*, 2004) did the first evidence NmU-mediated Ca²⁺ signalling appear by endogenous receptors expressed in K562 cells (a human erythro-leukemic cell line).

To date therefore, much of what we know about the signalling and G-protein coupling of NmU-receptors in physiological systems has been based on the interpolation of data from recombinant receptors. However, unlike endogenous expression systems, data from recombinant receptor systems may not truly reflect the physiological importance of complex protein-protein interactions, ligand-binding and stoichiometry (the relevant expression levels of cell protein) (reviewed; Kenakin, 1997). Amongst many others, data concerning G-protein coupling (especially G-protein promiscuity) is foremost in the doubts about recombinant expression systems. Given that the conclusions drawn from recombinant data presented previously (Chapters 3 and 4) concern both these phenomenon, it is imperative that evidence from endogenous systems is sought to support these findings. To this end, this chapter characterises aspects of NmU-receptor signalling and ligand-binding by endogenously expressed NmU receptors in cells isolated from a number of tissue sources of the rat, particularly colonic smooth muscle cells.

As well as human forms, rodent NmU receptors have also been cloned and characterised. Two forms from mice(m) (mNmU-R1 and mNmU-R2) (Funes *et al.*, 2002) and two from rat(r) (unpublished, NCBI accession numbers: rNmU-R1; AAF82754 and rNmU-R2; NP_071611) have been identified. The distribution patterns of rodent NmU-receptors (Fujii *et al.*, 2000, Funes *et al.*, 2002, Hosoya *et al.*, 2000; Graham *et al.*, 2003) are consistent with those reported for human receptors (Hedrick *et al.*, 2000; Hosoya *et al.*, 2000; Howard *et al.*, 2000; Raddatz *et al.*, 2000; Shan *et al.*, 2000; Szekeres *et al.*, 2000; Westfall *et al.*, 2000). In summary, NmU-R1 exists largely in peripheral organs, whilst NmU-R2 is predominantly located in the CNS (a detailed description of NmU receptor distribution can be found in Introduction, section 1.2.5).

Neither human (Fujii *et al.*, 2000; Hendrik *et al.*, 2000; Hosoya *et al.*, 2000; Raddatz *et al.*, 2000; Shan *et al.*, 2000; Szekeres *et al.*, 2000; Shan *et al.*, 2000) nor murine (Funes *et al.*, 2002) NmU receptors have significant abilities to distinguish between different forms of NmU. Furthermore, the smooth muscle contractile properties of NmU are well documented in a variety of tissues and from a variety of species (Minamino *et al.*, 1985; Bockman *et al.*, 1989; Maggi *et al.*, 1990; Westfall *et al.*, 2001). The use of rat smooth muscle cells, therefore, provides an excellent means of studying signalling properties of endogenous NmU receptors. More specifically, due to the existence of mRNA for rNmU-R1 in rat gastro-intestinal tract (Fujii *et al.*, 2000) and the contractile properties of NmU in rodent stomach fundus and distal colon smooth muscle (Dass *et al.*, 2003b), smooth muscle cells from these organs are ideal for further investigation.

This chapter details the optimisation and characterisation of primary cultures of rat colon and fundus smooth muscle cells and assesses their feasibility for use in subsequent experiments. These cells were then used to investigate the signalling properties of endogenous NmU receptors and to explore the physiological reality of their dual G-protein coupling and the irreversible binding of NmU observed with recombinant human forms of the receptors. To alleviate any concerns over species differences, the ability of recombinant rat receptors to mediate $[Ca^{2+}]_i$ elevations in response to challenges with various NmU analogs was investigated. Further, the signalling properties of both rat and human NmU isoforms were compared in the cultured smooth muscle cells.

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All methods are exactly as described previously (see Methods, section 2.2).

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Section 5.3 Results

5.3.1 Smooth muscle cell preparation

Immediately following dissociation, the resultant individual smooth muscle cells in suspension appeared long and thin (Fig. 5.1), and resembled typical isolated smooth muscle cells (Gröschel-Steward *et al.*, 1977) when viewed at 100X magnification under the light microscope.

The initial objective of this work was to characterise aspects of NmU-mediated signalling by endogenous NmU receptors in freshly isolated smooth muscle cells from the rat colon. This would limit any cellular or expression changes associated with the culture of smooth muscle cells (discussed in more detail in Discussion, section 5.4.2). Initially, cells were left overnight to adhere to coverslips prior to analysis of NmU-mediated single-cell Ca²⁺ signalling. However, despite attempts at optimising the incubation times and temperature during the incubation, smooth muscle cells would not adhere sufficiently to untreated glass coverslips, and nearly all cells were lost in subsequent wash steps. Further, the use of either poly-D-lysine, fibronectin, 10% gelatin/90% PBS, conditioned media or rat or mouse collagen to coat the coverslips failed to provide a sub-stratum on which the smooth muscle cells would substantially adhere.

Smooth muscle cells from the rat fundus were subject to identical dissociation techniques. These also seldom adhered to glass coverslips, but on the rare occasion they did, confocal imaging of fluo-3 loaded cells during challenge with either 10nM hNmU-25 (Fig. 5.2a) resulted in robust contraction of the cell. Subsequent washing failed to return the cell to its original elongate morphology (not shown) and cells frequently detached from the coverslip following contraction. No details of $[Ca^{2+}]_i$ elevations within these cells are shown due to the cells moving on application of hNmU-25. Fluorescent images are shown in preference to phase/light images to aid in visualisation. Thus, although such studies demonstrate NmU-mediated contraction in acutely isolated smooth muscle cells, these preparations would make further studies problematic. Despite attempts, no similar data were obtained for acutely dissociated smooth muscle cells from the rat colon. As an alternative, the smooth muscle cells from both tissues were cultured (see below).



Fig. 5.1. Dissociated smooth muscle cells. Smooth muscle cells were dissociated from the distal colon of male Wistar rats by enzymatic digestion of the extracellular matrix. Photographs were taken through a light microscope at 100x magnification. Individual cells were not adhered at this point and were indeed lost on subsequent washing (not shown). Each individual image was chosen at random and is representative of three images from separate animals.



Fig. 5.2a-e. Contraction of rat fundus smooth muscle cells. Smooth muscle cells were dissociated from the fundus of male Wistar rats by enzymatic digestion of the extracellular matrix. These were placed onto untreated 25mm coverslips in 6-well plates and left to adhere overnight at 37°C. Cells were carefully washed, loaded with fluo-3-AM (30min, RT), and mounted onto the stage of a confocal microscope (a). The images are of a single isolated smooth muscle cell and were taken at 5s (b), 10s (c), 15s (d) or 30s (e) following application of 10nM hNmU-25. Images are from one experiment. Attempts to repeat the experiment with rat colon smooth muscle cells were unsuccessful.

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5.3.2 Characterisation of smooth muscle cells

Failure of acutely isolated smooth muscle cells to adhere to coverslips led to the development of culture conditions permitting the proliferation of smooth muscle cells, and their adherence to glass coverslips. Isolated colonic and fundus smooth muscle cells in suspension were cultured in Medium 231 supplemented with smooth muscle growth supplement (SMGS) (Cascade Biologics, Nottingham, U.K.) at 37° C in 5% air 95% CO₂ for up to 15 days.

Rat colonic smooth muscle cells examined at day 3 in culture had not adhered to glass coverslips. However, smooth muscle cells began to adhere to un-treated coverslips by day 5 in culture, when they also began to proliferate and develop processes (Fig. 5.3a). By between day 7 (Fig. 5.3b) and day 9 (Fig. 5.3c) cells had grown to approximately 50-70% confluency, and had an elongated morphology. After 11 days in culture (Fig. 5.3d) cells began to lose their elongated appearance and became more triangular in shape. This continued to day 13 (Fig. 5.3e) and 15 (Fig. 5.3f) where confluency reached 90-100% and cells appeared almost exclusively triangular in shape.

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Fig. 5.3. Phenotypic characterisation of cultured rat colonic smooth muscle cells. Smooth muscle cells were dissociated from the distal colon of male Wistar rats by enzymatic digestion of the extracellular matrix. These were cultured directly onto 25mm coverslips for either 5(i), 7(ii), 9(iii), 11(iv), 13(v) or 15(vi) days as indicated. Coverslips were mounted onto the stage of a PerkinElmer confocal microscope with a 40X oil emersion objective lens and phase/light images were captured. Each individual image was chosen at random and is representative of three images from separate animals.

To further characterise rat colonic smooth muscle cells as they progressed in culture, the presence of α -actin, a smooth muscle cell phenotype marker (Gröschel-Steward *et al.*, 1977; Skalli *et al.*, 1988), was determined throughout the 15-day culture period using immunocytochemistry. Through the use of an antibody specific for α -actin and a FITC-labelled secondary antibody the changes in this smooth muscle cell marker were characterised by confocal microscopy.

These studies revealed the presence of α -actin at day 5 in culture (Fig. 5.4a(i)), the earliest time at which cells adhered sufficiently to allow immunocytochemical studies. The α -actin was evident as fluorescent strands or filaments within the cytoplasmic region of the cells. This pattern of staining is characteristic of smooth muscle cells (Skalli *et al.*, 1988) and these cultures became most apparent on days 7 (Fig. 5.4a(ii)), 9 (Fig. 5.4a(iii)) and 11 (Fig. 5.4a(iv)). Although still evident towards the end of the 15-day culture period (Fig. 5.4a(v) and Fig. 5.4a(vi)), α -actin staining had lost most of its filamentous characteristics.

Wild-type HEK293 cells (Fig. 5.4b(i)) and human umbilical vascular endothelial cells (HUVECs) (Fig. 5.4b(ii)), which are endothelial in origin, were used as negative controls and did not show α -actin staining. Further, cultured rat colonic smooth muscle cells, cultured for 9-days, did not show any α -actin staining following the exclusion of the primary (α -actin) antibody during the immunocytochemical technique (Fig. 5.4b(iii)).

The morphology, confluency, and α -actin staining properties of rat colonic smooth muscle cells as they progress through the 15-day culture period have been summarised in Table 5.1.

Based on this evidence, only smooth muscle cells cultured between 7 and 9 days were used in subsequent experiments.



Fig. 5.4a. Phenotypic characterisation of cultured rat colonic smooth muscle cells. Smooth muscle cells were dissociated from the distal colon of male Wistar rats by enzymatic digestion of the extracellular matrix. These were cultured directly onto 25mm coverslips for either 5(i), 7(ii), 9(iii), 11(iv), 13(v) or 15(vi) days at $37^{\circ}C/5\%CO_2$. Cells were permeablised and fixed before α -actin antibody was applied at 1:500 overnight at 4°C. Cells were then incubated with FITC-labelled secondary antibody (1:200, 2h, RT). Coverslips were mounted onto the stage of a PerkinElmer confocal microscope with a 40X oil emersion objective lens and cells were excited at 568nm. Images were collected with a broad band RGB emission filter. Each individual image was chosen at random and is representative of 3 images each from separate animals.

a)



b)

Fig. 5.4b. Negative controls for the characterisation of α -actin staining. (i) HEK293 cells and (ii) HUVECs were cultured onto 25mm coverslips and probed with the α -actin antibody (1:500 overnight, 4°C) before being incubated with FITC-labelled secondary antibody (1:200, 2h, RT). (iii) Smooth muscle cells were dissociated from rat distal colon and cultured onto 25mm coverslips for 7 days. These were incubated (overnight, 4°C) in buffer with the exclusion of the primary α -actin antibody. Probing with the FITC-tagged secondary antibody proceeded as described (1:200, 2h, RT). Coverslips were mounted onto the stage of a PerkinElmer confocal microscope with a 40X oil emersion objective lens and phase/light images were captured. Cells were then excited at 568nm and images were collected with a broad band RGB emission filter. Images are representative of 3 experiments.

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Table. 5.1. Summary of the phenotypic characterisation of rat colonic smooth muscle cells up to 15-days culture. Data obtained from morphological appearance and α -actin staining from 3 separate cultures.

Day	Morphology	α-actin staining	Confluency
3	Did not adhere	Did not adhere	Did not adhere
5	Elongate	Positive	10-20%
7	Elongate	Positive	40-50%
9	Elongate	Positive	40-70%
11	Elongate- triangular	Positive	50-80%
13	Triangular	Positive – with loss of filamentous appearance	50-80%
15	Triangular	Positive – with loss of filamentous appearance	50-80%
HUVECs	Cobble-stone	Negative	N/A

5.3.3 G-protein coupling of endogenously expressed rat NmU receptors

Assessment of G-protein coupling using the immunoprecipitation assay (Akam *et al.*, 2002) (see also Methods, section 2.2.7 and Chapter 3, section 3.3.5) gives the opportunity to directly assess the specific G-proteins to which endogenous rat NmU-receptors couple.

In membrane preparations from cultured (7-9 days) rat colonic smooth muscle cells, binding of [35 S]-GTP γ S to immuno-precipitated G $\alpha_{q/11}$ (Fig. 5.5a) or G $\alpha_{i(1-3)}$ (Fig. 5.5b) increased by ~4 and ~3-fold over basal, for G $\alpha_{q/11}$ and G $\alpha_{i(1-3)}$ respectively, upon activation with 10nM hNmU-25. Non-specific binding using 10 μ M GTP γ S was ~40-50% of basal (un-stimulated) [35 S]-GTP γ S binding (Fig.5.5).



Fig. 5.5. G-protein coupling of endogenous rat NmU receptors. Membrane preparations (25µg) from cultured rat colonic smooth muscle cells were incubated in the presence of GDP (1µM for $G\alpha_{q/11}$ and 10µM for $G\alpha_i$), 1nM [³⁵S]-GTPγS and where applicable (stimulated) hNmU-25 (10nM). Non-specific binding (NSB) was determined using 10µM GTPγS. Immunoprecipitation was carried out using antibodies specifically against either $G\alpha_{q/11}$ (a) or $G\alpha_{i(1-3)}$ (b) subunits as indicated, and associated [³⁵S] determined. All data are mean + s.e.m., n=3.

5.3.4 Characterisation of [Ca²⁺]_i signalling by endogenously expressed rat NmU receptors

Loading of cultured rat colonic smooth muscle cells with the Ca^{2+} -sensitive dye fluo-3-AM was optimised to allow the subsequent analysis of changes in $[Ca^{2+}]_i$. Here, incubation conditions, in terms of time and temperature, were altered so as to develop a loading protocol giving even loading of fluo-3-AM. Fluo-3-AM (5µM) was diluted in KHB buffer and loaded into 7-9-day-cultured rat colonic smooth muscle cells in the presence of pluronic F-127 (see Methods, Section 2.2).

The necessity to optimise loading conditions developed from the fact that fluo-3, when viewed under the confocal microscope, often appeared as uneven, punctate fluorescent spots within the cytoplasm of the cells. This is most evident at longer incubation periods of 40min (Fig. 5.6c) and 60min (Fig. 5.6d) at all temperatures tested (4°C, RT and 37°C). Despite an increase in cytosolic fluorescence following challenge of cells with 300µM UTP with these longer loading periods (data not shown), it is clear that incubation times of 20min (Fig. 5.6a) and 30min (Fig. 5.6b) provided more suitable conditions for loading of fluo-3-AM. Here, with the exception of incubation temperatures of 4°C (Fig. 5.6a(i) and Fig. 5.6b(i)), the appearance of cytosolic punctate fluorescence was considerably less and loading appeared more uniform throughout the cells.

From these experiments, the optimal loading conditions for fluo-3-AM were determined as 30min at RT (Fig. 5.6b(ii)).



Fig. 5.6a-d. Optimisation of fluo-3-AM loading in cultured rat colonic smooth muscle cells. Smooth muscle cells were dissociated from the distal colon of male Wistar rats by enzymatic digestion of the extracellular matrix and cultured on 25mm glass coverslips at $37^{\circ}C/5\%CO_2$ for 7-9 days. Cells were loaded with 5µM fluo-3-AM in the presence of 0.044% pluronic F-127 in the dark for 20 (a), 30 (b), 40 (c) or 60min (d) at either $4^{\circ}C$ (i), RT (ii) or $37^{\circ}C$ (iii). Images were captured via confocal microscopy and are representative of two further images from separate coverslips having cells cultured from the same rat.

Challenge of cells with either 10nM hNmU-25 (Fig. 5.7a) or 10nM rNmU-23 (Fig. 5.7b) resulted in robust (3-6 fold over basal) and rapid (<5s) elevations of $[Ca^{2+}]_i$ in fluo-3-loaded cells. In both instances, the rapid initial elevation in $[Ca^{2+}]_i$ was followed by a sustained but lower (1-2 fold over basal) signal in all cells, and in approximately 10-15% of cells, the $[Ca^{2+}]_i$ signal was seen to oscillate. Changes in nuclear fluorescence were often larger than changes in the cytoplasm. This most likely reflects the increased loading of fluo-3 into the nucleus, and as such, all data is representative of fluorescence changes from a combination of the cytosol and the nucleus.

Stimulation of cells with either 100nM (Fig. 5.7c) or 1 μ M (Fig. 5.7d) hNmU-25 again resulted in robust (3-6 fold over basal) and rapid (<5s) elevations of [Ca²⁺]_i. Further, similar to stimulation with lower concentrations (10nM) of hNmU-25, some cells oscillated during the sustained phase. In fact, responses to 10nM, (Fig. 5.7b) 100nM (Fig. 5.7c) and 1 μ M (Fig. 5.7d) hNmU-25 were comparable suggesting that the Ca²⁺ response was maximal at 10nM. Indeed, more complete analysis of the concentration-dependencey of this Ca²⁺ signal demonstrated maximal changes in peak [Ca²⁺]_i at ~10nM for both hNmU-25 and rNmU-23 (Fig. 5.7e). The pEC₅₀ values obtained for these responses (based on peak [Ca²⁺]_i responses) were 8.94 ± 0.42 and 8.64 ± 0.58 (mean ± s.e.m., n=3), for hNmU-25 and rNmU-23, respectively (Fig. 5.7e).

The magnitude of the initial response (3-8 fold over basal) to hNmU-25 was unaffected by the removal of extracellular Ca^{2+} (Fig. 5.7f) but the sustained signal and any oscillations were abolished. In addition, depletion of intracellular Ca^{2+} stores by pre-treating cells for 10min with 1µM thapsigargin abolished Ca^{2+} responses to hNmU-25 (Fig. 5.7g).





b)

a)



c)

d)

Fig. 5.7c-d. Single cell analysis of changes in $[Ca^{2+}]_i$ in cultured rat colonic smooth muscle cells. Cell preparation as per Fig. 5.7a-b. Cells were challenged with either 100nM hNmU-25 (d) or 1µM hNmU-25 (e) at t=30s. Traces show the change in cytosolic fluorescence of 6-10 individual cells in the field of view. Image panels A, B and C were taken at the time points indicated on the traces. Data are representative of 3 separate experiments, each from individual animals.



Fig. 5.7e. Concentration-response relationship for NmU-mediated changes in $[Ca^{2+}]_i$ in cultured rat colonic smooth muscle cells. Cell preparation as per Fig. 5.7a-b. Cells were loaded with fluo-3-AM and stimulated with varying concentrations of either hNmU-25 (*filled squares*; **n**) or rNmU-23 (*filled triangles*; \blacktriangle) ranging from 100pM-100nM. The fold change in peak $[Ca^{2+}]_i$ elevations were averaged from 6-10 cells chosen at random from the field of view. These data gave pEC₅₀ values of 8.94 ± 0.42 and 8.64 ± 0.58 for hNmU-25 and hNmU-23, respectively. Data are mean ± s.e.m., n=3, and were obtained from individual animals.



Fig. 5.7f. Changes in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} in cultured rat colonic smooth muscle cells. Cell preparation as per Fig. 5.7a-b. Cells were loaded with fluo-3-AM and imaged by confocal microscopy in Ca^{2+} -free KHB. Temperatures were maintained at 37°C with a Peltier unit and cells were challenged with 10nM hNmU-25 at t=30s. Traces show the average change in cytosolic fluorescence of 6-10 cells in the field of view chosen at random. Image panels A, B and C were taken at the time points indicated on the traces. Data are representative of at least 3 separate experiments, each from separate animals.



Fig. 5.7g. Changes in $[Ca^{2+}]_i$ following depletion of intracellular Ca^{2+} stores in cultured rat colonic smooth muscle cells. Cell preparation as per Fig. 5.7(a-b). Cells were loaded with fluo-3-AM and cytosolic fluorescence was determined by confocal microscopy and used as an index of $[Ca^{2+}]_i$. Temperatures were maintained at 37°C with a Peltier unit. Cells were treated with 1µM thapsigargin for 10min and washed by KHB perfusion (5ml min⁻¹) for 1-2min prior to assay. Cells were challenged with 10nM hNmU-25 at t=30s. Traces show the change in cytosolic fluorescence of 6-10 individual cells in the field of view. Image panels A, B and C were taken at the time points indicated on the traces. Data are representative of 3 separate experiments, each from separate animals.

5.3.5 Repetitive challenge of endogenously expressed rat NmU receptors with NmU

In experiments designed to explore Ca^{2+} signals in cultured smooth muscle cells, an increase in $[Ca^{2+}]_i$ was seen following challenge with either 10nM hNmU-25 (Fig. 5.8a) or 10nM rNmU-23 (Fig. 5.8b) challenge. In both cases, the subsequent extensive washing by perfusion with KHB (5ml min⁻¹) failed to abolish or reduce the sustained $[Ca^{2+}]_i$ elevation. Indeed, in some cells oscillations persisted. If, following washing, the cells were re-challenged with the same NmU analog, regardless of whether the cells were oscillating or not, no response was seen to the second NmU application (Fig. 5.8a-b).

In contrast, repeat Ca^2 signals were possible to repeat challenges with $100\mu M$ UTP in these cells (Fig. 5.8c). Here, any sustained signal or oscillations were abolished during the wash by perfusion of KHB. Re-challenge of cells with $100\mu M$ UTP following the wash resulted in an increase in $[Ca^{2+}]_i$, albeit ~50-60% of the magnitude of the first response.

No response to a second 10nM hNmU-25 (Fig. 5.8d) was seen even after an extended perfusion with KHB (5ml min⁻¹, 720s (12min)).

Further, the addition of rNmU-23 or hNmU-25 to naïve cells prevents the alternate ligand eliciting a $[Ca^{2+}]_i$ response on the same cells following a perfusion wash with KHB (Fig. 5.8e-f).



Fig. 5.8a-b. Ca^{2+} responses to repeated application of either hNmU-25 or rNmU-23 in cultured rat colonic smooth muscle cells. Smooth muscle cells were dissociated from the rat colon by enzymatic digestion of the extracellular matrix and cultured for 7-9 days. Cells were loaded with fluo-3-AM and changes in fluorescence were determined by confocal microscopy and used as an index of $[Ca^{2+}]_i$. Temperatures were maintained at 37°C with a Peltier unit. Cells were challenged with either 10nM hNmU-25 (a) or 10nM rNmU-23 (b) at t=30s. At t=60s cells were perfused with agonist-free buffer at a rate of 5ml min⁻¹ for 120s. At t=180s, either 10nM hNmU-25 (a) or 10nM rNmU-23 (b) were re-applied. Changes in the cytosolic fluorescence of 6-10 cells within the field of view were plotted individually. Images A, B, C and D were taken at the time points indicated. Data are representative of at least 3 experiments, each from separate animals.

a)

b)



Fig. 5.8c. Ca^{2+} responses to repeated application of UTP in cultured rat colonic smooth muscle cells. Cell preparation and loading as per Fig. 5.8a-b. Cells were challenged with 100µM UTP at t=30s. At t=60s cells were perfused with agonist-free buffer at a rate of 5ml min⁻¹ for 120s. At t=180s, 100µM UTP was re-applied. Changes in the cytosolic fluorescence of 6-10 cells within the field of view were plotted individually. Images A, B, C and D were taken at the time points indicated. Data are representative of at least 3 experiments, each from separate animals.



Fig. 5.8d. Ca^{2+} responses to repeated application of hNmU-25 in cultured rat colonic smooth muscle cells with an extended washing period between applications. Cell preparation and loading as per Fig. 5.8(a-b). Cells were challenged with 10nM hNmU-25 at t=30s. At t=60s cells were perfused with agonist-free buffer at a rate of 5ml min⁻¹ for 720s (12min). At t=780s, 10nM hNmU-25 was re-applied. Changes in the cytosolic fluorescence of 6-10 cells within the field of view were plotted individually. Images A, B, C and D were taken at the time points indicated. Data are representative of at least 3 experiments, each from separate experiments.

d)



Fig. 5.8e-f. Ca^{2+} responses to rNmU-23 and hNmU-25 following initial stimulation with the alternative NmU analog in cultured rat colonic smooth muscle cells. Cell preparation and loading as per Fig. 5.8(a-b). Cells were challenged with either 10nM hNmU-25 (e) or 10nM rNmU-23 (f) at t=30s, at t=60s cells were perfused with agonist-free buffer at a rate of 5ml min⁻¹ for 120s. At t=180s, 10nM rNmU-23 (e) or 10nM hNmU-25 (f) were applied. Changes in the cytosolic fluorescence of 6-10 cells within the field of view were plotted individually. Images A, B, C and D were taken at the time points indicated. Data are representative of at least 3 experiments, each from separate animals.

f)

e)

Exploration of NmU-mediated signalling in alternative tissues led to the isolation and culture of smooth muscle cells from rat fundus. Cells were isolated and cultured exactly as described for the rat distal colon. Single-cell analysis of changes in $[Ca^{2+}]_i$ in fluo-3-loaded cultured rat fundus smooth muscle cells revealed that 10nM hNmU-25 (Fig. 5.9a) evoked marked peak and plateau elevations of $[Ca^{2+}]_i$ similar to that seen in cultured colonic cells. Following stimulation, perfusion with agonist-free buffer reduced $[Ca^{2+}]_i$ to basal levels following stimulation with UTP (Fig. 5.9c) but not hNmU-25 (Fig. 5.9b). Furthermore, following this wash period (KHB, 5ml min⁻¹, 120s), re-application of UTP (Fig. 5.9c) but not hNmU-25 (Fig. 5.9b) resulted in a further elevation of $[Ca^{2+}]_i$.



Fig. 5.9a-b. $[Ca^{2+}]_i$ responses to repeated application of hNmU-25 in isolated rat fundus smooth muscle cells. Smooth muscle cells were dissociated from the rat stomach fundus by enzymatic digestion of the extracellular matrix and cultured for 7-9 days. Cells were loaded with fluo-3-AM and cytosolic fluorescence measured as an index of $[Ca^{2+}]_i$ using confocal microscopy. Cells were challenged with 10nM hNmU-25 (a) at t=30s. In further experiments, naïve cells were challenged with 10nM hNmU-25 (b) at t=30s. At t=60s cells were perfused with agonist-free buffer at a rate of 5ml min⁻¹ for 120s. At t=180s, 10nM hNmU-25 was re-applied. Changes in the cytosolic fluorescence of 3-6 cells within the field of view were plotted individually. Images A, B, C and where applicable D, were taken at the time points indicated. Data are representative of at least 3 experiments, each from separate animals.

b)

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Fig. 5.9c. $[Ca^{2+}]_i$ responses to repeated application and UTP in isolated rat fundus smooth muscle cells. Cell preparation and loading as per Fig. 5.9a-b. Cells were challenged with 100µM UTP at t=30s. At t=60s cells were perfused with agonist-free buffer at a rate of 5ml min⁻¹ for 120s. At t=180s, 100µM UTP was reapplied. Changes in the cytosolic fluorescence of 3-6 cells within the field of view were plotted individually. Images A, B, C and D, were taken at the time points indicated. Data are representative of at least 3 experiments, each from separate animals.

c)

5.3.6 Binding of fluorescently labelled NmU to endogenously expressed rat NmU receptors

As a second approach to determine the possible irreversible binding of NmU to its receptors, porcine NmU-8 (pNmU-8) with an *N*-terminally conjugated fluorophore, Cy3B (pNmU-8-Cy3B) (see Chapter 4) was used. In studies based on changes in $[Ca^{2+}]_i$ as measured by FLIPR, pNmU-8-Cy3B was equipotent to unlabelled pNmU-8, rNmU-23 and hNmU-25 in HEK293 cells transiently expressing either rNmU-R1 (Fig. 5.10a), or rNmU-R2 (Fig. 5.10b). A comparison of pEC₅₀ values obtained from these experiments is shown in Table 5.2.

Addition of pNmU-8-Cy3B to 7-9-day-cultured rat colonic smooth muscle cells resulted in the appearance of fluorescence localized to the plasma membrane (Fig. 5.11a(ii)). At 1min following the addition of pNmU-8-Cy3B, the addition of 1 μ M hNmU-25 did not result in a loss of plasma membrane fluorescence (Fig. 5.11b(i-ii)). Addition of 1 μ M hNmU-25 prior to the addition of NmU-8-Cy3B abolished the appearance of plasma membrane fluorescence (Fig. 5.11c(ii)). Furthermore, no fluorescence was observed with addition of 10nM pNmU-8-Cy3B to either wild-type HEK293 cells (Fig. 5.11d(ii)) or HUVECs (Fig. 5.11e(ii)).

Following addition of 10nM NmU-8-Cy3B at 12°C (to block receptor internalisation), continuous perfusion of cells with KHB (5ml min⁻¹) for 700s did not diminish plasma membrane fluorescence (Fig. 5.11f(i-ii)).



Fig. 5.10a-b. Concentration-response relationships for the elevation of $[Ca^{2+}]_i$ by NmU analogs in HEK293 cells transiently expressing either rNmU-R1 or rNmU-R2. HEK293 cells were plated into 384-well plates and transiently transfected with either rNmU-R1 (a) or rNmU-R2 (b). $[Ca^{2+}]_i$ in cell populations was determined using fluo-4-loaded cells and a FLIPR. Peak changes in fluorescence intensity units (F.I.U) were used as an index of changes in $[Ca^{2+}]_i$. Cells were stimulated with varying concentrations of hNmU-25 (*filled squares*; **m**), rNmU-23 (*filled upwards triangles*; \blacktriangle), pNmU-8 (*open downwards triangles*; ∇) or pNmU-8-Cy3B (*open diamonds*; \Diamond). A summary of pEC₅₀ values is given in Table 5.2. Data are mean ± s.e.m., n=3.

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Ligand	rNmU-R1	rNmU-R2
Human NmU-25	9.43 ± 0.17	9.46 ± 0.09
Rat NmU-23	9.41 ± 0.11	9.69 ± 0.13
Porcine NmU-8	9.15 ± 0.13	9.27 ± 0.12
pNmU-8-Cy3B	9.72 ± 0.10	9.62 ± 0.11

Table. 5.2. The pEC₅₀ values of the peak elevation of $[Ca^{2+}]_i$ in HEK293 cells transiently expressing rat NmU-receptors. Data are mean ± s.e.m., n=3






Fig. 5.11f. Effects of KHB perfusion on binding of pNmU-8-Cy3B to cultured rat colonic smooth muscle cells. Preparation and culture as per Fig. 5.11a. Phase image (i) of cells was compared to the fluorescent image (ii and iii). pNmU-8-Cy3B (10nM) was added to cells (ii) and the cells were then perfused with buffer (5ml min⁻¹) for 700s at 12°C (iii). Data are representative of two further experiments, each obtained from separate animals.

5.3.7 Evidence for the internalisation of endogenously expressed rat NmUreceptors

Addition of 10nM pNmU-8-Cy3B to cultured rat colonic smooth muscle cells at 37°C revealed the rapid appearance of fluorescence within the cytoplasm (Fig. 5.12). This existed as punctate spots of fluorescence distributed randomly throughout the cytoplasm, suggesting internalisation of the fluorescent ligand.

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Fig. 5.12. Internalisation of pNmU-8-Cy3B. Preparation and culture as per Fig. 5.11a. Phase image (a) and fluorescent image (b) of cultured rat colonic smooth muscle cells 300s following addition of 10nM pNmU-8-Cy3B at 37°C. Images are representative of further 2 experiments, each obtained from separate animals.

5.4.1 Summary

In summary, methods were developed for the dissociation and culture of rat colonic smooth muscle cells resulting in adherent, viable cells that phenotypically resembled smooth muscle cells. This permitted an investigation into aspects of the signalling and ligand-binding characteristics of endogenously expressed rat NmUreceptors. Within these cells, direct assessment of G-protein coupling specificity demonstrated the coupling of these receptors to both $Ga_{q/11}$ and Ga_i G-proteins. The functional consequence of $Ga_{\alpha/11}$ coupling was demonstrated by robust increases in $[Ca^{2+}]_i$ to both hNmU-25 and rNmU-23, in cultured, fluo-3-loaded cells. Ca^{2+} responses were bi-phasic, with the initial peak response dependent on Ca²⁺ release from a thapsigargin-sensitive intracellular store, whilst the second, sustained signal and oscillatory Ca^{2+} signals were dependent on the presence of extracellular Ca^{2+} . Furthermore, by demonstrating the inability of repeat hNmU-25 and rNmU-23 challenges to elicit Ca^{2+} responses, and through the use of a fluorescent NmU analog (pNmU-8-Cy3B) I have confirmed that binding of NmU to endogenous NmUreceptors expressed in cultured rat colonic smooth muscle cells is effectively irreversible. Similar observations were made in cultured rat fundus smooth muscle cells. These properties are consistent with recombinant human NmU receptors (see Chapters 3 and 4) and demonstrate the physiological reality of dual coupling to multiple G-proteins and an irreversible ligand-receptor interaction.

5.4.2 Culture of rat colonic smooth muscle cells

This chapter details the dissociation of individual smooth muscle cells from intact rat colonic and fundus tissues by enzymatic digestion of the extracellular matrix. Similar methods have been described previously for other tissues (Quayle *et al.*, 1996; Lewis *et al.*, 2000; Vial *et al.*, 2000). The cells were viable, and indeed fundus smooth muscle cells contracted on agonist application. To obtain adherent cells that could be used to investigate single-cell Ca²⁺ imaging, dissociated smooth muscle cells were cultured for up to 15 days.

Culturing vielded smooth muscle cell populations, with ~100% of cells expressing characteristic phenotypic markers (see below). The cultures were essentially devoid of non-muscle cells, e.g. neural, endothelial and inflammatory Other studies have demonstrated that smooth muscle cells observed in cells. proliferative states contain few myofilament bundles and a larger number of organelles (e.g. free ribosomes and rough ER) (reviewed; Chamley-Campbell et al., 1979). It would therefore appear that in most cases a phenotypic modulation is a prerequisite for smooth muscle cell proliferation, with most de-differentiating into fibroblast-like cells. Morphological investigations into smooth muscle cells <7 days in culture have revealed a general, ribbon or spindle shape with an oval or elongate cell body containing two or more nuclei (Chamley-Campbell et al., 1979). In the current study, cells in culture showed these phenotypic characteristics. Further, antibodies against α -actin will stain the thin filaments within smooth muscle cells but not fibroblast or endothelial cells (Gröschel-Steward et al., 1977; Skalli et al., 1988). This was evident in the current study as staining was localised to long, straight, noninterrupted fibrils along the longitudinal axis. Such characteristics were lost as the cells were maintained for longer periods in culture.

A full and detailed characterisation of the cultured colonic smooth muscle cells has therefore revealed a phenotype typical of smooth muscle cells and demonstrated their suitability for use in further investigations into endogenous NmU-receptors.

Detailed analysis of the distribution of rNmU-R1 and rNmU-R2 reveal that rNmU-R1 is expressed largely in peripheral organs (Fujii *et al.*, 2000, Hosoya *et al.*, 2000; Graham *et al.*, 2003), most notably the gastro-intestinal tract (Fujii *et al.*, 2000). In comparison, rNmU-R2 is predominantly located in central tissues (Hosoya *et al.*, 2000; Howard *et al.*, 2000) (see also Introduction, section 1.2.5). Therefore, the predominating isoform is likely to be rNmU-R1, although the expression of rNmU-R2 and its possible contribution to signalling cannot be completely excluded.

5.4.3 Signalling by endogenously expressed rat NmU receptors

It is clear that recombinant human receptors couple to signalling pathways that result in an increase in $[Ca^{2+}]_i$ (Fujii *et al.*, 2000; Hedrick *et al.*, 2000; Hosoya *et al.*, 2000; Howard *et al.*, 2000; Kojima *et al.*, 2000; Raddatz *et al.*, 2000; Szekeres *et al.*, 2000; Funes *et al.*, 2002, see also Chapter 3). The only report

to date of endogenous NmU receptors generating Ca^{2+} signals has been in K562 cells (Shetzline *et al.*, 2004), although this signalling was not characterised. The data presented within this chapter demonstrate that both human and rat forms of NmU elicit Ca^{2+} signals in cells endogenously expressing rat NmU receptors and that the pEC₅₀ values for hNmU-25 and rNmU-23 were similar to those obtained from recombinant human receptors.

The role and functions of Ca^{2+} in smooth muscle has been extensively reviewed (Karaki *et al.*, 1997). Primarily, elevation of $[Ca^{2+}]_i$ plays an important role in both the development and maintenance of force within the smooth muscle cell. It is therefore likely, considering the contractile effects NmU had on isolated rat fundus smooth muscle cells and the contractile properties described elsewhere (Minamino *et al.*, 1985; Bockman *et al.*, 1989; Maggi *et al.*, 1990; Westfall *et al.*, 2001, see also Chapter 6), that the main function of the NmU-mediated Ca²⁺ signal in smooth muscle cells is one of contraction.

Similar to the actions of hNmU-25 on recombinant human receptors (see Chapter 3), both hNmU-25 and hNmU-23 generate bi-phasic Ca^{2+} signals, with the initial peak response declining into a sustained plateau phase. For both recombinant human and endogenous rat receptors, the first phase of $[Ca^{2+}]_i$ elevation is reliant on the release of Ca^{2+} from thapsigargin-sensitive intracellular stores whilst the second phase of release relies on the presence of extracellular Ca^{2+} .

In contrast to the signals mediated by recombinant receptors, the sustained Ca^{2+} signal in cultured smooth muscle cells was often observed as repetitive, transient increases in $[Ca^{2+}]_i$, or Ca^{2+} oscillations, which occurred asynchronously in adjacent cells. Approximately 30-40% of cells were seen to oscillate, and the $[Ca^{2+}]_i$ returned to its resting value between each increase. Agonist-mediated Ca^{2+} oscillations are frequently observed in vascular, airway and intestinal smooth muscle cells (Savineau and Marthan, 2000) and can occur either independently of extracellular Ca^{2+} (Guilbert *et al.*, 1996; Roux *et al.*, 1997; Hyvelin *et al.*, 1998) suggesting an internal Ca^{2+} source drives the oscillations, or as with the NmU receptors they may depend on extracellular Ca^{2+} (Wu *et al.*, 1995; Khoda *et al.*, 1996). Thus, in this and other cases, influx of Ca^{2+} across the plasma membrane is required and it has been

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suggested that the Ca^{2+} entry contributes to replenishment of the Ca^{2+} stores which are then able to maintain oscillations (Rink and Hallum, 1989, Berridge, 1993).

The mechanisms behind this Ca^{2+} influx could involve the conformational coupling model for Ca^{2+} entry (reviewed in Putney *et al.*, 1999). Here, $Ins(1,4,5)P_3$, mobilizes Ca^{2+} from the endoplasmic reticulum following activation of a $Ga_{q/11}$ G-protein. The subsequent emptying of the pool induces a conformational change in the $Ins(1,4,5)P_3$ receptor that physically interacts with and opens the plasma membrane associated Ca^{2+} channel and thus permits the influx of Ca^{2+} . However, others suggest a mechanism of Ca^{2+} influx across the plasma membrane that involves activation of diffusible second messenger that interacts with and opens plasma membrane associated Ca^{2+} channels (reviewed in Putney *et al.*, 1999). Capacitative Ca^{2+} -entry can contribute to Ca^{2+} oscillations (Berridge *et al.*, 1995, Berridge *et al.*, 1997; Parekh and Penner, 1997) by aiding in the refilling of repeatedly releasing Ca^{2+} stores.

Proposed mechanisms driving Ca^{2+} oscillations involve the Ca^{2+} -mediated regulation of the $Ins(1,4,5)P_3$ receptor or the feedback inhibition properties of PKC. In the former theory, low Ca^{2+} concentrations within the cell will cause an increase in opening of the $Ins(1,4,5)P_3$ receptor in membranes of Ca^{2+} stores and will thus cause an increase in cytosolic Ca^{2+} , but at higher concentrations Ca^{2+} inhibits opening of the $Ins(1,4,5)P_3$ receptor (Berridge *et al.*, 2000, Schuster *et al.*, 2002). These two opposing regulatory effects manifest themselves as Ca^{2+} oscillations. In the second proposal, feedback inhibition of PLC generates oscillatory patterns in $Ins(1,4,5)P_3$ production and hence generates Ca^{2+} oscillations (Taylor and Thorn, 2001). The feedback inhibition could be driven by either PKC (Nash *et al.*, 2001) or regulators of G-protein signalling (RGS) proteins (Luo *et al.*, 2001).

Oscillatory responses are often associated with lower agonist concentrations (Thomas *et al.*, 1996; Berridge, 1997; Shuttleworth, 1999) or lower GPCR expression levels (Nash *et al.*, 2002). Despite no quantitation on the expression levels of NmU receptors in the cultured rat smooth muscle cells, in the absence of any gross changes in expression throughout culture, they do represent a physiological relevant level of receptor expression.

Physiologically, information within Ca^{2+} oscillations is encoded in their frequency. Changes in time between successive spikes will influence Ca^{2+} -dependent enzymes and cellular processes (De Koninck *et al.*, 1998, Dolmetsch *et al.*, 1998). Through this mechanisms oscillations may control Ca^{2+} -dependent membrane ion channels, thus contributing to the membrane potential and may allow Ca^{2+} to function as a second-messenger while reducing the deleterious effect of sustained elevation of $[Ca^{2+}]_i$. Oscillations are also thought to increase the efficiency and specificity of gene expression (Dolmetsch *et al.*, 1998). Although the physiological role of oscillations in smooth muscle cells is presently poorly understood (reviewed, Savineau and Marthan, 2000), it has been proposed that they may contribute to the frequency-dependent control of smooth muscle cell responses.

5.4.4 Dual coupling to $Ga_{q/11}$ and Ga_i

Despite concerns that evidence of dual coupling to multiple G-proteins is an expression artefact (Tuček et al., 2002) it is clear that it is a physiological reality for a number of receptors (Hermans, 2003). The current study shows dual coupling to both $Ga_{a/11}$ and Ga_i by recombinant human receptors (see Chapter 3); furthermore, this chapter provides evidence for the dual coupling of endogenous rat NmU receptors. The nature of the [35S]-GTPyS immuno-precipitation assay means we cannot directly compare the coupling levels of NmU-receptors to $G\alpha_{i}$ and $G\alpha_{i+1}$. which prevents elucidation of the primary signalling pathway. Despite greater increases in [³⁵S]-GTPyS binding to recombinant receptors there were no differences in the relative coupling to $Ga_{\alpha/11}$ and Ga_i between recombinant human receptors and endogenous rat receptors suggesting similar relative coupling. Furthermore, in the absence of any data to confirm the functional relevance of Ga, coupling we can only tentatively interpolate data from recombinant systems. However, considering recombinant data, the robust increase (3-4 fold over basal) in [35]-GTPyS binding following Ga, precipitation, and especially considering the receptors are expressed endogenously it is unlikely that the coupling to Ga_i is misleading.

The functional implications of Ga_i coupling in smooth muscle cells could have numerous effects on the signalling properties of NmU-receptors. This may involve actin reorganisation (Hirshman and Emala, 1999), support of mitogenesis, (elevation of cAMP has been shown to inhibit mitogenesis) (Kanthou *et al.*, 1996) or enhancement of contractile properties (cAMP has been shown to relax smooth muscle) (e.g. Fernandez *et al.*, 1992; Eglen *et al.*, 1994). The possible signalling pathways involved in the NmU-mediated contraction of rat colonic smooth muscle is detailed and discussed further in Chapter 6.

5.4.5 Irreversible NmU binding to endogenously expressed rat NmU receptors.

The inability to generate additional Ca^{2+} responses to repeat application of either hNmU-25 or rNmU-23 by endogenous NmU receptors is consistent with the observations using recombinant human NmU receptors. Furthermore, this was not due to rapid and full desensitisation but to an inability to remove bound NmU from its receptors. This was demonstrated by sustained and oscillatory $[Ca^{2+}]_i$ signals following NmU challenge, and proved further by visualisation of the fluorescent NmU-analog, pNmU-8-Cy3B at the plasma membrane. Based on changes in $[Ca^{2+}]_i$ as measured by FLIPR, pNmU-8-Cy3B is equipotent to unlabelled pNmU, hNmU-25 and rNmU-23 at transiently expressed rat NmU receptors. This supports its use to compare with the larger NmU-analogs (hNmU-25 and rNmU-23). At <12°C, pNmU-8-Cy3B remained bound to the plasma membrane after extensive washing. Although ligand-dissociation can be adversely effected by lower temperatures, taken with evidence from single-cell $[Ca^{2+}]_i$ imaging, these studies demonstrate that the inability to see repeat [Ca²⁺]_i signals by endogenously expressed rat NmU receptors is due to an irreversible ligand-receptor interaction. This is entirely consistent with the human recombinant NmU receptors (see Chapter 4).

5.4.6 NmU internalisation

The binding and subsequent washing of pNmU-8-Cy3B was largely carried out at <12°C, as at 37°C the ligand internalised rapidly. Given the clear high-affinity binding of NmU, this almost certainly reflects receptor internalisation. Internalisation was rapid, and at 5min following application fluorescence existed predominantly within the cytoplasm. This is also entirely consistent with recombinant human NmU receptors.

This chapter explored signalling through NmU-receptors endogenously expressed in cultured rat colonic smooth muscle cells. This allowed an investigation into the activation of NmU receptors in their physiological cell background. No data exists, either within this study or others, that determines the expression level of endogenous NmU receptors. Therefore, no conclusive assumptions can be made about whether or not the HEK cell lines used throughout chapters 3 and 4 are over-expressed. However, endogenous NmU receptors showed similar characteristics to those expressed as recombinant proteins adding weight to the conclusions from chapters 3 and 4.

Chapter 6

NmU-mediated contraction of colonic smooth muscle preparations

Section 6.1 Introduction

The physiological implications of NmU-receptor activation are numerous and varied. NmU has been shown to regulate regional blood flow and blood pressure (Minamino et al., 1985a; Sumi et al., 1987; Gardiner et al., 1990) and to influence the pituitary-adrenal-cortical axis (Malendowicz et al., 1993, 1994a and 1994b). ICV administration of NmU mediates stress-related responses and also increases both arterial pressure and heart rate in conscious rats (Chu et al., 2002; Westfall et al., 2001) indicating a role in the regulation of sympathetic nervous activity and cardiovascular function. Recent studies have demonstrated important roles for NmU in the regulation of feeding behavior and energy homeostasis. Thus, in rats, ICV injection of NmU decreases food intake and body weight (Howard et al., 2000; Kojima et al., 2000; Nakazato et al., 2000; Niimi et al., 2001; Ivanov et al., 2002; Wren et al., 2002), and increases gross-locomotor activity, body temperature, heat production and oxygen consumption (Howard et al., 2000; Nakazato et al., 2000; Hananda et al., 2001). Further roles include the stimulation of gastric-acid secretion and gastric emptying (Mondal et al., 2003), pro-nociception (Cao et al., 2003; Yu et al., 2003; Nakahara et al., 2004a) and the regulation of circadian rhythm (Nakahara et al., 2004b). Further details can be found in the Introduction (Sction 1.3).

One of the first physiological roles of NmU to be investigated was that of smooth muscle contraction, and indeed the smooth muscle contractile properties of NmU are well documented in a variety of tissues and from a variety of different species. In fact, the suffix 'U' derives from NmU's ability to contract uterine smooth muscle which was originally idetified in tissues from rats (Minamino *et al.*, 1985a). Subsequent to these initial studies, NmU has been shown to mediate concentration-dependent contractions of rat (Benito-Orfila *et al.*, 1991) and mouse (Dass *et al.*, 2003b) circular smooth muscle from the fundus; colonic smooth muscle from the mouse (Dass *et al.*, 2003b) and; human smooth muscle from the ileum (Maggi *et al.*,

1990). NmU also contracts human urinary bladder (Maggi et al., 1990) whilst NmUmediated contractions of canine bladder (both *in vitro* and *in vivo*), stomach, ileum and colon have also been shown (Westfall et al., 2001).

This chapter characterises the contraction of circular and longitudinal smooth muscle preparations from the rat distal colon mediated by application of either rNmU-23 or hNmU-25. These organ-bath studies were then used as a means to investigate the irreversible binding of NmU to its receptors that was observed in isolated cells with both recombinant human receptors (see Chapter 4) and endogenous rat receptors (see Chapter 5). The use of smooth muscle preparations from rat colon will allow a direct comparison with smooth muscle cells cultured from the same tissue (see Chapter 5). This will complement the previous data by providing a comparison between recombinant receptors, endogenous receptors and intact tissue. This chapter also details the possible degradation of hNmU-25 and discusses the implications of this on NmU receptor activation, regulation and function.

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All methods are exactly as described in Methods, section 2.2 with the following inclusion.

Where appropriate, contractile studies were performed in the presence of protease inhibitor cocktail 1 and soybean trypsin-inhibitor (Calbiochem, Nottingham, U.K.). When diluted as per manufacturer's instructions the inhibitor cocktail consists of 500 μ M AEBSF HCl (4-(2-Aminoethyl)benzenesulfonylfluoride, HCl), 150nM aprotinin, 1 μ M E-64 and 0.5mM EDTA disodium salt and 1 μ M leupeptin hemisulfate. Soybean trypsin inhibitor was also used at manufacturers recommended concentration.

6.3.1 Characterisation of NmU-mediated contraction of the rat distal colon

Throughout this investigation CCh was used as a positive control for the contraction of rat circular and longitudinal colonic smooth muscle preparations. CCh has been shown to stimulate the contraction of gastrointestinal smooth muscle via phosphoinositide turnover and generation of $Ins(1,4,5)P_3$ and release of intracellular Ca²⁺ (Bielkiewicz-Vollrath *et al.*, 1987; Gardner *et al.*, 1988; Takahashi *et al.*, 1994). In the absence of electrical stimulation, application of 100μ M CCh to preparations of either circular (Fig. 6.1a) or longitudinal (Fig. 6.1b) rat colon smooth muscle (under 1g tension, see below) resulted in robust contractions of approximately 3-3.5g tension. Tension subsided on subsequent washing. The re-application of 100μ M CCh resulted in contractions of similar magnitude to the first (Fig. 6.1).

Quantitation of all subsequent NmU-mediated contractions were calculated as a percentage of that mediated by an initial challenge with 100μ M CCh. This will reduce any tissue specific variables such as the length, weight or even the part of the colon from which the tissue strip originated.

Determination of the length-tension relationship for 100μ M CCh-mediated contractions of both longitudinal and circular rat colon smooth muscle strips suggests that optimum contractions were achieved when tissue strips were stimulated under 1g tension (Fig. 6.2). Here, the magnitude of contraction (force generated) was ~3-3.5g, and was compared to contractions under 0.5g tension (generated force of 2-2.5g), 1.5g tension (2.5-3g) and 2g tension (1.5-2g). All subsequent experiments were therefore performed with tissue strips held under 1g tension.

a) Circular smooth muscle



b) Longitudinal smooth muscle



Fig. 6.1a-b. CCh-mediated contractions of longitudinal and circular rat colonic smooth muscle preparations. The distal colon of a male Wistar rat was removed, washed and cut into 1-1.5cm strips parallel to either circular (a) or longitudinal (b) smooth muscle. Strips were held between two recording electrodes under 1g tension in 5ml organ baths containing KHB at 37° C. Changes in tension were recorded by a force transducer. Tissues were challenged with 100μ M CCh via a direct bath application. Following peak contraction, tissue strips were washed twice with KHB within $30\min(1 \text{ wash at } t=0 \text{ and one at } t=15\min)$ as indicated, and stimulated again with 100μ M CCh. Data are representative of 4 experiments from 4 separate rats.



Fig. 6.2. Contraction of circular and longitudinal rat colon smooth muscle strips mediated by CCh under different starting tensions. The distal colon of a male Wistar rat was removed, washed and cut into 1-1.5cm strips parallel to either circular (*open squares*; **•**) or longitudinal (*upwards filled triangles*; **•**) smooth muscle. Strips were held between two recording electrodes under either 0.5, 1, 1.5 or 2g tension in 5ml organ baths containing KHB at 37°C and changes in tension in response to 100 μ M CCh were recorded by a force transducer. Data are mean ± s.e.m., n=3.

Application of either 1 μ M rNmU-23 (Fig. 6.3a and b) or 1 μ M hNmU-25 (Fig. 6.3c and d) initiated a contractile response in both circular (Fig. 6.3a and c) and longitudinal (Fig. 6.3b and d) colon smooth muscle preparations of approximately 60-70% of that mediated by 100 μ M CCh.

Agonist-mediated contractions of smooth muscle can occur either via direct stimulation of the muscle or through stimulation of intrinsic nervous activity. Stimulation of nerves will result in the release of neurotransmitters (predominantly acetylcholine), which stimulate contraction of the muscle. The contribution of nervous activity to any given contraction can be assessed by the addition of the muscarinic-acetylcholine receptor antagonist, atropine, or by pre-treatment of muscle preparations with tetrodotoxin (TTX). Originating from puffer fish, TTX selectively blocks Na²⁺-ion channels in nerve fibres and axons (Narahashi et al., 1960; Nakajima et al., 1962). The result of this is an inability to conduct nervous impulses, and an abolition of all nervous activity in the tissue. Neither the presence of 1µM atropine nor pre-treatment of muscle preparations with TTX (5µM, 10min) had any effects on the peak contractions mediated by application of 1µM rNmU-23 to either circular (Fig. 6.4a and b) or longitudinal (Fig. 6.4c and d) colonic smooth muscle. Quantitation revealed responses equal to those in preparations that had not been treated (Fig. 6.4e). These data demonstrate that repeat contractions of NmU are able to elicit repeat contractions of the rat distal colon. This is discussed in more detail below and in Section 6.3.2.

Concentration-response relationships for NmU-mediated contraction (Fig. 6.5) revealed maximal responses at 1 μ M NmU (both rat and human) and generated pEC₅₀ values of 7.75 ± 0.08 and 7.53 ± 0.16 for circular and longitudinal contractions respectively, based on challenge with hNmU-25 (Fig. 6.5a), and 7.59 ± 0.17 and 7.64 ± 0.14 for circular and longitudinal contractions respectively, based on challenge with rNmU-23 (Fig. 6.5b). Data are mean ± s.e.m., n=3. A summary of pEC₅₀ values is shown in Table 6.1.

From Fig. 6.4 and experiments performed to generate concentration-response curves (Fig. 6.5) it was clear that a second contraction to repeat applications of NmU

was approximately the same magnitude as that of the first. In fact, concentrationresponse curves were generated non-cumulatively with agonist concentrations being applied randomly and separated by the standard KHB wash of 30min incorporating two complete replacements of KHB. This is in complete contrast to the increases in $[Ca^{2+}]_i$ by recombinant and endogenous NmU receptors and is investigated further below (see Section 6.3.2). a) Circular smooth muscle



b) Longitudinal smooth muscle



c) Circular smooth muscle



d) Longitudinal smooth muscle



Fig. 6.3a-d. hNmU-25- and rNmU-23-mediated contraction of circular and longitudinal rat colon smooth muscle strips. Circular (a and c) or longitudinal (b and d) colonic smooth muscle tissue strips were prepared as per Fig. 6.1. Tissue strips were challenged with 100μ M CCh and then washed twice with KHB within 30min as indicated. Tissue strips were then challenged with either 1μ M rNmU-23 (a and b) or 1μ M hNmU-25 (c and d). Data are representative of 4 experiments from 4 separate rats.



e)

Fig. 6.4e. Quantitation of rNmU-23-mediated contraction of circular and longitudinal smooth muscle strips in the presence of either TTX or atropine. Quantitation of Fig. 6.4a-d. Strips were challenged with 1 μ M rNmU-23 and then either treated with TTX (10min, 5 μ M) (*light grey filled*) or atropine (1 μ M, 10min) (*dark grey filled*) before being challenged again with 1 μ M rNmU-23. Data are expressed as the magnitude of the second rNmU-23-mediated response as a percentage of the first. Data are mean + s.e.m., n=3

a) hNmU-25



Fig. 6.5a-b. Concentration-response relationships for circular and longitudinal rat colon smooth muscle contraction by hNmU-25 and rNmU-23. Circular (upward filled triangle; \blacktriangle) or longitudinal (filled squares; \blacksquare) colonic smooth muscle tissue strips were prepared as per Fig. 6.1. Strips were challenged with 100µM CCh and subsequently washed. hNmU-25 (a) or rNmU-23 (b) at varying concentrations were applied non-cumulatively and in a random order. Each successive agonist application was separated by two complete replacements of KHB in the bath over a 30min period. The magnitude of each response was measured as a percentage of the initial CCh-mediated response. Data are mean \pm s.e.m., n=4.

Table. 6.1. pEC₅₀ values for the hNmU-25- or rNmU-23-mediated contraction of circular or longitudinal muscle strips from the rat distal colon. Data are mean ± s.e.m., n=4.

Ligand	Circular	Longitudinal	
hNmU-25	7.75 ± 0.08	7.53 ± 0.16	
rNmU-23	7.59 ± 0.17	7.64 ± 0.14	

6.3.2 Repetitive NmU-mediated contractions of the rat distal colon

Organ bath studies using intact colonic tissues demonstrated that following stimulation of either circular (Fig. 6.6a and b) or longitudinal (Fig. 6.6c and d) muscle strips with a maximal concentration $(1\mu M)$ of either hNmU-25 (Fig. 6.6a and c) or rNmU-23 (Fig. 6.6b and d) and following a KHB wash, a second $1\mu M$ rNmU-23 challenge was able to elicit a contraction that was ~100% of the initial response (Fig. 6.6e).

In these initial experiments the protocol consisted of a 30min period between NmU additions with two complete replacements of buffer at t=0s and t=15min. Using this protocol, repeat contractions of circular (Fig. 6.7a and b) and longitudinal (Fig. 6.7c and d) muscle were possible for 5 (experimental protocol included only 5 repeat challenges, although it is likely more would be possible) repetitive applications of maximal (1 μ M) concentrations of either hNmU-25 (Fig. 6.7a and c) or rNmU-23 (Fig. 6.7b and d), with no apparent loss of contractile properties and no evidence of desensitisation.

Even following a 4h stimulation with NmU, washing, and the subsequent addition of either 1 μ M hNmU-25 (Fig. 6.8a) or 1 μ M rNmU-23 (Fig. 6.8b) to circular smooth muscle strips resulted in a contraction equal in magnitude to the initial response. Further evidence from these experiments revealed that either hNmU-25 or rNmU-23 caused repetitive twitching of the muscle strips for the 4h stimulation, again with no evidence of desensitisation (Fig. 6.8).

This apparent lack of desensitisation is consistent with the ability to generate non-cumulative concentration-response curves for these contractions (see Fig. 6.5).

a) Circular smooth muscle



b) Circular smooth muscle



c) Longitudinal smooth muscle



d) Longitudinal smooth muscle



Fig. 6.6a-d. Contractions of circular and longitudinal rat colon smooth muscle strips to repeated applications of either hNmU-25 or rNmU-23. Circular (a and b) or longitudinal (c and d) colonic strips were prepared as per Fig. 6.1. Tissue strips were challenged with 100μ M CCh and then washed with KHB twice over 30min as indicated before being challenged with either 1μ M hNmU-25 (a and c) or 1μ M rNmU-23 (b and d). These were then washed again and re-stimulated with the same agonist. Data are representative of 3 experiments from 3 separate rats.



Fig. 6.6e. Quantitation of contractions of circular and longitudinal rat colon smooth muscle strips to repeated applications of either hNmU-25 or rNmU-23. Quantitation of Fig. 6.6a-d. Tissue strips were challenged with 1 μ M hNmU-25 (*light grey filled*) or 1 μ M rNmU-23 (*dark grey filled*) washed with KHB and then challenged again with the same NmU analog. Data are expressed as the peak magnitude of the second NmU-mediated response as a percentage of the first. Data are mean + s.e.m., n=3.



Fig. 6.7a-b. Contractions of circular rat colon smooth muscle strips to repetitive hNmU-25 and rNmU-23 challenge. Circular colonic smooth muscle tissue strips were prepared as per Fig. 6.1. Tissue strips were challenged with 100μ M CCh and then washed with KHB before being challenged with 1μ M hNmU-25 (a) or 1μ M rNmU-23 (b) up to 5 times. Each NmU application was separated by two complete replacements of KHB in the organ bath as indicated over a 30min period. Data are representative of 3 experiments from 3 separate rats.



Fig. 6.7c-d. Contractions of longitudinal rat colon smooth muscle strips to repetitive hNmU-25 and rNmU-23 challenge. Longitudinal colonic smooth muscle tissue strips were prepared as per Fig. 6.1. Tissue strips were challenged with 100 μ M CCh and then washed with KHB before being challenged with 1 μ M hNmU-25 (a) or 1 μ M rNmU-23 (b) up to 5 times. Each NmU application was separated by two complete replacements of KHB in the organ bath over a 30min period. Data are representative of 3 experiments from 3 separate rats.



Fig. 6.8a-b. Contractions of circular and longitudinal rat colon smooth muscle strips to sustained challenge with either hNmU-25 or rNmU-23. Circular colonic smooth muscle tissue strips were prepared as per Fig. 6.1. Tissue strips were challenged with 1 μ M hNmU-25 (a) or 1 μ M rNmU-23 (b) for 4h. Strips were then washed with KHB twice over 30min and re-stimulated with the same NmU analog. Data are representative of 3 experiments from 3 separate rats.

In intact tissues, the ability to generate repetitive functional responses to repeat NmU challenge is in contradiction to observations from single-cell experiments. Here, neither HEK293 cells recombinantly expressing either hNmU-R1 or hNmU-R2 (see Chapter 4), nor cultured rat colonic smooth muscle cells endogenously expressing rat NmU receptors, elicited Ca^{2+} responses to a second NmU-challenge when cells were washed following the initial response.

One difference between the re-challenge experiments in single-cells and those using tissue contraction is the duration of the wash period. The period between successive applications of NmU in single-cells was investigated only up to 12min. In tissue studies, applications were 30min apart. This difference could account for the discrepancy by allowing receptors sufficient time for recovery of a limiting process and to exist at the plasma membrane ligand-free and ready for another round of signalling.

Using preparations of circular smooth muscle the impact of reducing the period between consecutive applications of 1μ M rNmU-23 was explored. In these experiments, the period between repetitive 1μ M rNmU-23 applications was progressively reduced from 30min (Fig. 6.9a(vi)) to 1min (Fig. 6.9a(i)). The wash protocol was adapted to include at least one complete replacement of KHB following the initial contraction, and a second replacement of buffer at a midway time point. As the duration between successive applications was reduced there was no reduction in the magnitude of the response to re-challenge (Fig. 6.9a), even with just 3 (Fig. 6.9a(ii)) and 1 (Fig. 6.9a(i)) min wash periods. Quantitation is shown in Fig. 6.9b.



Fig. 6.9a. The effects of reducing the wash period between rNmU-23 challenges on the repetitive contractions of circular rat colon smooth muscle tissue. Circular colonic smooth muscle tissue strips were prepared as per Fig. 6.1. Muscle preparations were challenged with 100μ M CCh and washed with KHB. Cells were challenged with 1μ M rNmU-23 and washed with KHB for either 1 (i), 3 (ii), 10 (iii), 15 (iv), 20 (v) or 30 (iv) min before re-challenge with 1μ M rNmU-23. Data are representative of 3 experiments. Quantitation is detailed in Fig. 6.9b.



Fig. 6.9b. Quantitation of the effects of reducing the wash period on repetitive contractions of circular smooth muscle preparations mediated by rNmU-23. Quantitation of Fig. 6.9a. The magnitude of the second rNmU-23-mediated contraction is expressed as a percentage of the first. Data are mean + s.e.m., n=3.

6.3.3 Degradation of NmU

It is likely that many extracellular enzymes (both secreted or plasma membrane located) are present in this functional model that are absent in single-cell experiments. It is possible therefore that the ability to see repeat functional responses in intact tissues and not single-cells is due to the actions of proteases that cleave NmU or its receptors, or both. It may be possible that degradation of extracellular NmU in the immediate vicinity of the receptors or cleavage of receptor-bound NmU aids the release of ligand from its binding site, thus making the receptor available for subsequent activation.

In a simple, but somewhat inconclusive experiment, the presence of a protease inhibitor cocktail and a soybean trypsin-inhibitor had no effect of the rNmU-23mediated contractions of circular rat colonic smooth muscle (Fig. 6.10). The inhibitor cocktail contained AEBSF, (an inhibitor of serine proteases e.g. chymotrypsin, kallikrein, plasmin, thrombin, trypsin, and related thrombolytic enzymes), aprotinin (which acts as a competitive and reversible inhibitor of esterase and protease activities e.g, chymotrypsin, coagulation factors, kallikrein, plasmin, leukocyte proteinase and trypsin), E-64 (irreversible inhibitor of cysteine proteases) and leupeptin hemisulfate (inhibits trypsin-like proteases and cysteine proteases).

However, having little idea of protease activity within the colonic smooth muscle strips, I cannot simply disregard NmU degradation as an explanation for the discrepancy between single-cells and intact tissues. Ideally, what is needed is an assay to investigate the degradation of NmU (see below).



Fig. 6.10a-b. Repetitive contraction of circular rat colon smooth muscle tissue. Effects of protease inhibitors. Circular colonic smooth muscle tissue strips were prepared as per Fig. 6.1. Muscle preparations were challenged with 100μ M CCh and washed with KHB. Preparations were then challenged with 1μ M rNmU-23, washed twice with KHB within 30min and re-challenged with 1μ M rNmU-23 either in the presence (a) or absence (b) of protease and soybean trypsin inhibitors. Data are representative of 3 experiments from 3 separate rats.

NmU degradation was measured using standard protocols of trichloroacetic acid (TCA) protein precipitation. TCA is a well-known protein precipitation agent (Britton *et al.*, 1989) and works on the principle that, by interacting with peptides, TCA increases hydrophobicity of peptides that can lead to aggregation through hydrophobic interactions (Yvon *et al.*, 1989). Therefore, the larger the protein or protein fragment, the more hydrophobic it will become. If proteins are fragmented, the larger fragments will more readily precipitate in TCA and can be collected by centrifugation. By measuring the increase in soluble fragments following TCA precipitation and by comparing this to the quantity of intact substrate, an estimate of the amount of protein that has been degraded can be obtained. In summary, larger non-degraded proteins will precipitate, and can be collected in the pellet following centrifugation, whereas smaller protein fragments will remain soluble, and are retained in the supernatant.

Incubation (30min, 37°C) of 150pM [¹²⁵I]-hNmU-25 in crude homogenate (4g protein ml⁻¹) prepared from the colon of male Wistar rats resulted in an ~80% loss of ¹²⁵I in the precipitate (Fig. 6.11a(i)). This was comparable to a similar increase in soluble (fragmented/degraded) products (Fig. 6.11a(ii)). Precipitable ¹²⁵I accounted for ~18% of the total amount of [125]hNmU-25 added, whereas soluble 125] accounted for ~82%, following a 30min incubation with colonic homogenate at This was compared to ~82% precipitable and ~18% soluble prior to 37°C. Assuming that ¹²⁵I was still attached to protein fragments, this incubation. demonstrates a marked increase in soluble, and hence degraded, NmU. Importantly, heat inactivation (10min at 100°C) of the crude rat colonic homogenate abolished any changes in the soluble and precipitable fractions following incubation (30min, 37°C) product (Fig. 6.11a(iii)). Degradation of [1251]-hNmU-25 was maximal using homogenate at 4g protein ml⁻¹ and declined as the protein concentration of the homogenate was diluted (Fig, 6.11b). No degradation was seen at homogenate concentrations of 8µg ml⁻¹.

The degradation of [¹²⁵I]-hNmU-25 was not tissue specific as a similar magnitude (~80%) was degraded following incubation in both rat fundus tissue (Fig. 6.11c) and hind leg extensor skeletal muscle (devoid of NmU-receptors (Howard *et al.*,2000)) (Fig. 6.11d).

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In a rather preliminary experiment the effects of the tissue homogenate on pNmU-8-Cy3B binding to HEK293 cells expressing hNmU-R1 was examined using confocal microscopy. In HEK293 cells recombinantly expressing hNmU-R1, application of 150pM pNmU-8-Cy3B at 37°C resulted in the appearance of fluorescence at the plasma membrane (Fig. 6.12a) consistent with previous investigations using higher concentrations (see Chapter 4). Aspiration of extracellular fluid was followed by application of 500µl rat colon homogenate (400µg ml⁻¹) in the 3-4 min following pNmU-8-Cy3B addition and prior to internalisation (see Chapter 4). Application of tissue homogenate had no discernable effect on plasma membrane fluorescence (Fig. 6.12b).

This experiment was problematic and insufficient time was available to pursue this line investigation. The experiment was first carried out using tissue homogenate at 4g protein ml⁻¹ but this was detrimental to cells inducing a change in their morphology, most likely due to enzymatic activity within the homogenate. Further difficulties include internalisation. Internalisation is blocked at temperature below 12°C, but at these temperatures enzymatic activity will be greatly impaired. As an alternative, images were obtained in the few minutes prior to internalisation. To go some way to counter-act its detrimental effects the homogenate was diluted to 400µg ml⁻¹ where at this concentration degraded 60% of [¹²⁵I]-hNmU-25 over 30min (see Fig. 6.11b). The experiment was worth trying, but timing, internalisation and cell damage make it inconclusive.



Fig. 6.11a(i-iii). Degradation of $[^{125}I]$ -hNmU-25 estimated using TCA precipitation following incubation in colon homogenate. A 1.5-2cm longitudinal segment of the distal colon was removed from male Wistar rats and immediately washed in KHB. The tissue was diced into small pieces and homogenised until tissue pieces had disappeared. This was diluted to 4g protein ml⁻¹. Homogenate (250µl) was aliquoted into microfuge tubes and 250µl [^{125}I]-hNmU-25 (a(i)) added at a final concentration of 150pM. Incubation proceeded for the desired duration and was terminated by the addition of 500µl ice-cold 1M TCA. Samples were incubated on ice for 10min and then centrifuged (10000g, 10min). ^{125}I in both the supernatant (*black filled*) and pellet (*grey filled*) was determined by standard counting methods (a(ii)). Panel (iii) shows the effect of heat-treating (10min, 100°C) the homogenate prior to incubation with [^{125}I]-hNmU-25. Data are mean + s.e.m., n=3.

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Protein concentration of rat colon homogenate

4g mt ¹	400g m ¹	40µg m ¹
2g mt ¹	200g m ¹	8µg m ¹
800µg m ¹	80µg mt ¹	4µg mt ¹

Fig. 6.11b. Assessment of the degradation of [125 I]-hNmU-25 using TCA precipitation following incubation in colon homogenate at varying protein concentrations. Homogenate was prepared as per Fig. 6.11a. Tissue homogenate was diluted in KHB to give final concentrations of 4µg ml⁻¹ - 4g ml⁻¹ as indicated. Homogenate (250µl) was aliquoted into microfuge tubes and 250µl [125 I]-hNmU-25 added at a final concentration of 150pM. Incubation proceeded for 0min (stopped immediately) or 30min as indicated and was terminated by the addition of 500µl ice-cold 1M TCA. Samples were incubated on ice for 10min and then centrifuged (10000g, 10min). ¹²⁵I in both the supernatant and pellet was determined by standard counting methods. Data are mean + s.e.m., n=3.

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Fig. 6.11c-d. Determination of the degradation of $[^{125}I]$ -hNmU-25 by homogenate prepared from either the rat fundus or rat skeletal muscle. Tissue was removed from the fundus (c) or hind leg extensor muscle (d) and prepared as per Fig. 6.11a. Tissues were diluted to protein concentrations of 4g protein ml⁻¹ and TCA precipitation of $[^{125}I]$ -hNmU-25 proceeded as per Fig. 6.11a. Data are mean + s.e.m., n=3.





Fig. 6.12a-b. The effects of rat colon homogenate on the binding of pNmU-8-Cy3B to recombinantly expressed hNmU-R1. HEK293 cells expressing hNmU-R1 were cultured on glass coverslips, excited at 568nm and imaged using confocal microscopy. pNmU-8-Cy3B (150pM) was added to cells at 37° C (e). The extracellular fluid was aspirated and was immediately replaced by 500µl of rat colon homogenate diluted to 400µg protein ml⁻¹ in KHB and pre-warmed to 37° C. Image (f) was captured after 1 min prior to internalisation. The images are representative of 3 separate experiments.

e)

f)

imaging of pNmU-8-Cy3B binding revealed fluorescence localised to the plasma membrane. Application of tissue homogenate had no effect on plasma membrane-associated fluorescence.

6.4.2 NmU-mediated contraction of the rat distal colon

These studies revealed a concentration-dependent contraction of rat circular and longitudinal smooth muscle preparations by either hNmU-25 or rNmU-23. Contractions were direct, involving neither nervous stimulation nor cholinergic activation. This is consistent with contractions of rat circular smooth muscle from the fundus (Benito-Orfila *et al.*, 1991), human ileum and urinary bladder (Maggi *et al.*, 1990), canine bladder, stomach, ileum and colon (Westfall *et al.*, 2001), all of which are resistant to both atropine and TTX.

The present study demonstrates that in the continued presence of NmU, circular muscle strips twitched repetitively. This has been reported previously for other tissues in repsonse to NmU (Minamino *et al.*, 1985a) and has been suggested to be the result of the NmU NH₂-terminus as smaller NmU analogs (i.e. pNmU-8) generate only transient contractions. The NH₂-termini of the longer NmU forms may be involved in strengthening and prolonging stimulant activity (Minamino *et al.*, 1985a) (see Introduction, Section 1.1.2).

The exact signalling pathway(s) involved in the NmU-mediated contraction of the rat distal colon was not investigated in this study. However, possibilities can be extrapolated from signalling pathways explored in Chapters 3 and 5 (especially Chapter 5 as this characterised signalling in smooth muscle cells cultured from the rat colon). Thus, NmU receptors in rat smooth muscle cells couple to both $G\alpha_{q/11}$ and $G\alpha_i$ causing the release of Ca^{2+} and inhibition of cAMP respectively. These signalling pathways could act independently or co-operatively to contribute to the contractile response. The role and functions of Ca^{2+} in smooth muscle contractions have been extensively reviewed (Karaki *et al.*, 1997). Primarily, elevation of $[Ca^{2+}]_i$ plays an important role in both the development and maintenance of force within smooth muscle cells. It is likely therefore that contraction is mediated by elevations of $[Ca^{2+}]_i$ as a result of $G\alpha_{q/11}$ activation by NmU-receptors. This is a common pathway involved in smooth muscle contraction. As an example, CCh has been shown to stimulate the contraction of gastrointestinal smooth muscle via membrane phosphoinositide turnover, generation of $Ins(1,4,5)P_3$ and subsequent Ca^{2+} release (Bielkiewicz-Vollrath *et al.*, 1987; Takahashi *et al.*, 1994).

Activation of Ga_i and the subsequent inhibition of cAMP could also have a role in either the strengthening or production of contractile forces. Smooth muscle relaxation is mediated by adenylyl cyclase activation and production of cAMP (e.g. Bitar and Makhlouf, 1982; Fernandez *et al.*, 1992; Eglen *et al.*, 1994). In rat gastric smooth muscle cells, cAMP inhibits CCh-induced muscle contraction via inhibition of Ins(1,4,5)P₃-induced [Ca²⁺]_i release from intracellular stores (Ohta *et al.*, 1992). It is possible therefore that any inhibition of relaxation will enhance contractile properties by offsetting relaxation. This could be achieved by inhibition of cAMP production (through Ga_i activation), and thus inhibition of relaxation. As with NmU receptors, muscarinic M₃ receptors have been shown to increase intracellular [Ca²⁺]_i and reduce cAMP in smooth muscle (Takahashi *et al.*, 1994) and thus these receptors may enhance contraction by inhibiting the relaxation properties of cAMP (Ashkenazi *et al.*, 1987; Takahashi *et al.*, 1994). The PTX-sensitivity (and thus the involvement of Ga_i coupling), of NmU-mediated contraction of rat distal colon was not investigated in this study.

A comparison of the pEC₅₀ values for NmU-mediated $[Ca^{2+}]_i$ increases in colonic smooth muscle cells and tissue contraction reveal a ~1000 fold decrease in potency of hNmU-25 and rNmU-23 to contract the isolated colon. For example, based on experiments with hNmU-25, NmU evoked Ca^{2+} responses in cultured rat colonic smooth muscle cells with a pEC₅₀ value of 8.94 ± 0.42, whereas contraction of circular smooth muscle isolated from the rat distal colon was mediated with a pEC₅₀ value of 7.75 ± 0.08 (all data are mean ± s.e.m., n=3). This is paradoxical because signalling processes are generally amplified so that potency is increased as signalling pathways move downstream. Possible reasons for this discrepancy could involve the accessibility of receptors to NmU in these organ bath studies. The colonic smooth muscle preparations are crude and most likely lined with endothelial cells and other fatty tissues. Thus, the accessibility of ligand to receptors is not truly representative of physiological conditions, where agonists such as NmU would be released from nerves in very close proximity to target muscles. However, although accessibility will contribute to decreased potency, the proteolytic degradation of NmU is likely to be a key player. Degradation could decrease the quantity of biologically active NmU peptide able to bind to and activate receptors, and this would certainly lead to a decrease in potency. Degradation is discussed further in Section 6.4.4.

6.4.3 Repetitive contractions of the rat colon

Evidence within this chapter demonstrates that repeat applications of both hNmU-25 and rNmU-23 could elicit repeat contractions of the rat colon in organbath studies. Successive applications of NmU were separated by washing and there was no desensitisation of the contractile response between subsequent agonist challenges. This ability to generate repetitive contractions is in contrast to Ca^{2+} signalling in single-cell studies using either recombinant human or endogenous rat NmU receptors. In those studies the lack of a Ca^{2+} response to a second NmU challenge was attributed to NmU binding irreversibly to its receptors (see Chapters 5 and 6). Evidence from this chapter would suggest that NmU-binding in intact tissues is rapidly and easily reversible, and thus permits ligand-free receptors to respond to subsequent applications of NmU.

There is no evidence from the literature that directly examines the reversibility of NmU-binding either in single-cells or tissues. In fact, much of the literature investigating the numerous physiological roles of NmU fails to address the phenomenon of repetitive responses to repetitive NmU challenge. For example, in studies demonstrating a concentration-response relationship for the regional haemodynamic effects of NmU in conscious rats (Gardiner *et al.*, 1990) or unconscious dogs (Sumi *et al.*, 1987), the effects were studied cumulatively by the addition of increasing concentrations of NmU. Several investigations into the NmU-mediated contraction of various smooth muscle tissues also fall short in addressing this issue. The authors investigating the NmU-mediated contraction of the rat uterus (Minamino *et al.*, 1985) did not examine the concentration-dependence of this response. In further studies, the concentration-response relationship for the NmU-mediated contraction of rat fundus (Benito-Orfila *et al.*, 1991) was explored using cumulative additions of increasing concentrations of NmU.

However, despite not directly exploring the issue of repetitive responses, previous investigations into the NmU-mediated contraction of smooth muscle have

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reported results consistent with those in this chapter. Thus, the concentrationresponse relationship for both the NmU-mediated contraction of isolated human urinary bladder and ileum (Maggi *et al.*, 1990) and canine urinary bladder (Westfall *et al.*, 2002) were explored by the random addition of various NmU concentrations with each subsequent challenge being separated by agonist removal by washing.

These data suggest that NmU-binding to receptors in tissues is fully and rapidly reversible. The reasons for the difference in ligand-binding between cells and tissues is currently unknown, however, one possible explanation is the degradation of NmU (see Section 6.4.4, below). It is also possible that NmU-binding to its receptors in tissues exhibits irreversible characteristics as it does in single-cells but alternative mechanisms contribute to the repetitive response and apparent availability of ligandfree receptors. Explanations here could include rapid receptor recycling or a readily accessible pool or reserve of NmU receptors. To elaborate on this latter explanation, several family-1 GPCRs have been reported as being 'cryptic, hidden, masked or compartmentalised.' This describes a proportion of receptors that are not membraneassociated and are not accessible to ligand, yet retain the potential to become so. Receptors such as the pituitary luteinizing hormone releasing-hormone (LHRH) (Leblanc et al., 1994), PARs (Kawabata and Kuroda, 2000), α₂-adrenergic receptors (Adler et al., 1987) and the 5-HT_{1B} receptor (Adham et al., 1993) have all been reported as having a proportion of so-called 'hidden' receptors. In some cases, such as the 5-HT_{1B} receptor hidden receptors can constitute up to 90% of binding sites (Adler et al., 1993) and are often detected by comparing radioligand binding to cell populations and homogenised cell membrane preparations. Our understanding of what signals (either extracellular or intracellular) are required to transform masked receptors into ligand-accessible cell-surface receptors, and the speed at which this occurs is currently poorly understood. Furthermore, the existence of masked NmUreceptors and their involvement in the repetitive NmU-mediated contractions of the rat colon is at present only a possibility, but may offer a route for further research.

6.4.4 NmU degradation

This study provides evidence for the degradation of NmU. TCA precipitation of $[^{125}I]$ -hNmU-25 following incubation with rat colon homogenate revealed that a considerable proportion (~80%) of $[^{125}I]$ -hNmU-25 was degraded within 30min. It is

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currently unclear where in its amino acid sequence NmU is cleaved and as such the degradation products are unclear. In addition, the extent of degradation required (i.e. the size of the peptide) to prevent TCA precipitation is also known. As such, this TCA precipitation method is crude. However, it is useful as a starting point to explore NmU degradation.

The proteolytic activity was not specific to rat distal colon as similar results were obtained with rat fundus and skeletal muscle. There is no NmU-R expression within skeletal muscle (Howard *et al.*, 2000) however this crude homogenate is likely to contain vascular and other tissues that may contain a source of proteolytic activity. It is unclear if the proteolytic enzymes involved are specific or non-specific in degrading NmU, or indeed if they intracellular or extracellular or involved in the physiological processes of NmU.

The TCA method may overestimate degradation (Skough, 1982). However, considering no degradation was seen following incubation in heat-inactivated tissue, these data would suggest NmU is subject to some form of proteolytic activity. The only previous study or NmU-degradation showed that $[^{125}I]$ -rNmU-23 could be degraded by ~80% following incubation in rat uterine membrane-preparations for 30min (Nandha *et al.*, 1993).

Interestingly, dog NmU-8 is more potent than pNmU-8 in contraction of chicken crop smooth muscle (Sakura *et al.*, 1995). Structurally, dog NmU, but not pNmU, contains a pyroglutamic acid residue (pGlu) at its *N*-terminus. pGlu has been shown to offer protection of other peptides against proteolytic activities (Van Coilli *et al.*, 1998), therefore dog NmU-8 is likely to be more potent due to resistance to degradation by aminopeptidases. Indeed, modification of the *N*-terminal of pNmU-8 to give aminopeptidase resistance increased the contractile activity on chicken crop smooth muscle (Sakura *et al.*, 1995).

Neuropeptides are often subject to proteolytic degradation (Tam and Caughey, 1990) and proteolytic events at the plasma membrane can act to terminate signalling by GPCRs and their ligands (reviewed in Böhm *et al.*, 1997; Defea *et al.*, 2000). One such family of peptidases, namely neutral endopeptidases (NEPs) are cell-surface enzymes that degrade many neuropeptides and have been extensively studied (reviewed, Roques *et al.*, 1993). NEPs project into the extracellular fluid and are ideally placed to degrade neuropeptides at the cell surface in the vicinity of their

receptors (reviewed; Roques *et al.*, 1993). Substance P is a major substrate of NEP, and NEP will attenuate substance P binding and signalling if co-expressed in the same cell as substance P receptors (Okamoto *et al.*, 1994). NEPs prefer substrates with a NH₂ terminal residue that is hydrophobic (e.g. Phe, Leu, Val, Tyr and Trp) (Matsas *et al.*, 1984; Bunnett *et al.*, 1988; Gu *et al.*, 1992). The majority of NmU analogs terminate at the NH₂ terminus with either Phe, Leu or Tyr residues, thus making them ideal substrates for NEP-mediated degradation.

Peptidase inhibitors have previously been shown to inhibit the degradation of numerous peptides, including substance P (Russell *et al.*, 1996). In this chapter, the inclusion of protease inhibitors (described in Section 6.3.3) had no effect on the contractions mediated by repetitive applications of NmU. Similarly, the inclusion of the peptidase inhibitors (bacitracin and phosphormidon) had no effect on the hNmU-25-mediated contraction of isolated canine urinary bladder preparations (Westfall *et al.*, 2002). It is, however, unknown whether NEPs would be inhibited by the protease inhibitors discussed here and despite their lack of effect, taken with the conclusions from this chapter, evidence would suggest that NmU is unlikely to be exempt from the actions of proteolytic enzymes. An important question is whether this proteolytic activity is responsible for removing NmU from its receptors at the extracellular membrane. The role of NEPs and indeed other peptidases is often one of limiting the amount of extracellular ligand in the vicinity of receptors, and it is this that regulates signalling. There is no evidence either proving or disproving that proteolytic degradation can act in removing bound-ligand from its receptor.

Identification of the protease(s) responsible for degrading NmU would enable the effects of NmU degradation in both single-cells (by inclusion of the protease in the assay) or tissues (by inclusion of inhibitors of its activity) to be explored. This is discussed further in Chapter 7.

Many enzymes and peptidases have specific amino acids or sequences of amino acids on which they exert their biological effects. Using the on-line proteomics tool; ExPASy Peptidecutter, the 25 amino acid sequence of hNmU-25 is shown to contains potential cleavage sites for numerous peptidases (Fig. 6.13). The preferred substrates for enzymes such as Arg-C proteinase, glutamyl endopeptidase, pepsin, proteinase K, trypsin as well as many others (see Fig. 6.13) contain amino acids and

amino acid sequences that exist within the sequence of hNmU-25. Further, the degradation products may have physiological roles. The products may act as agonists or even antagonists at the NmU receptors, or maybe other, receptors. Much of the biological activity of hNmU-25 (and indeed other NmU analogs) exists within the C-terminus of the peptide (see Introduction, section 1.1.2) and cleavage outside this region may result in cleavage products that are still biologically active. Information about the activity and physiological roles of all the predicted enzymes is beyond the remit of this discussion but this exercise is useful to highlight the degradation potential of NmU.



b)

Enzyme	Name of Enzyme	No. of	Position of cleavage		
no.	••••••••••••••••••••••••••••••••••••••	cleavages	sites		
1	Arg-C-Proteinase	4	2 16 22 24		
2	Asp-N endopeptidase	1	6		
3	Chymotrypsin-high specificity (C- term to[FYWML], not before P)	6	1 7 11 18 19 21		
4	Chymotrypsin-low specificity (C- term to[FYWML], not before P	7	17 11 18 19 20 21		
5	Clostripain	4	2 16 22 24		
6	Glutamyl endopeptidase	2	56		
7	Pepsin (pH 1.3)	9	1 6 7 10 17 19 20		
8	Pepsin (pH >2.0)	8	1 6 7 10 19 20		
9	Proline-endopeptidase	1	23		
10	Proteinase K	9	1 3 7 11 12 18 19 20 21		
11	Staphylococcal peptidase I	1	5		
12	Thermolysin	6	2 10 11 18 19 20		
13	Trypsin	3	2 16 24		

Fig.	6.13.	Prediction	of cleav	vage sites	within	hNmU-25	using	ExPASy
Pepti	decutter	. The amin	o acid sec	quence for i	hNmU-25	5 was obtair	ned (Aus	tin et al.,
1995) and an	alysed using	the on-l	ine proteor	nics tool;	; ExPASy l	Peptidec	utter. a)
detail	s the seq	uence of hN	lmU-25 a	ind the site	s of actio	n of numer	ous enzy	ymes. b)
The e	nzyme n	umbers and t	he positio	ons of cleav	age sites.			

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The chapter identifies a major discrepancy between NmU-receptors expressed in single-cells (both recombinantly and endogenously) and in tissues. Thus, NmU binds irreversibly to its receptors in single-cells but at least the actions of NmU are fully and rapidly reversible in whole tissues. The discrepancy between single-cell studies and intact tissue therefore remains unexplained. One possible explanation for the discrepancy could be the extracellular degradation of NmU. Evidence here demonstrates that NmU is degraded by protease activity within a crude homogenate prepared from the rat colon. The protease(s) responsible are unknown and this is clearly a starting point for further investigations.

Chapter 7

Final Discussion

7.1 Summary of research

These studies have focused on the activation and regulation of mammalian neuromedin U receptor isoforms. Initial work characterised the signalling of recombinant human forms of the two NmU receptors (hNmU-R1 and hNmU-R2) when expressed in HEK293 cells (Chapter 3). This was extended further by an exploration into signalling by endogenous rat receptors in cultured rat colonic smooth muscle cells (Chapter 5). From these single-cell studies it became apparent that a second addition of NmU did not evoke a Ca^{2+} response when the first was followed by extensive washing with agonist-free buffer. This was not thought to be due to full and rapid receptor desensitisation but due to an irreversible interaction between NmU and its receptors. This irreversible binding was explored not only in recombinant cell models (Chapter 2) but also in cells endogenously expressing rat NmU receptors (Chapter 5). The phenomenon of ligand-binding and repetitive functional responses was investigated further by studying the NmU-mediated contraction of the rat colon in organ-bath studies (Chapter 6). The experiments complemented each other by providing a comparative characterisation of NmUreceptors recombinantly expressed in HEK293, endogenously expressed in cultured rat colonic smooth muscle cells and in intact rat colon.

7.2. Summary of results

The data demonstrate that activation of either human NmU receptor type expressed recombinantly in HEK293 cells results in the activation of both PLC and inhibition of adenylyl cyclase. This was demonstrated by PTX-insensitive increases in $[Ca^{2+}]_i$, $Ins(1,4,5)P_3$ and $[^{3}H]$ -InsP_x, showing activation of PLC, and by a PTX-sensitive reduction in FSK-elevated cAMP showing inhibition of adenylyl cyclase. By direct assessment of G-protein coupling in membranes prepared from HEK293 cells expressing either hNmU-R1 or hNmU-R2 using a $[^{35}S]$ -GTP γ S binding assay and antisera raised specifically against G α subunits, both hNmU-receptors were

shown to couple to $Ga_{q/11}$ and Ga_i G-proteins. However, and consistent with a lack of elevation of basal cAMP levels, neither receptor coupled to Ga_s . Both hNmU-R1 and hNmU-R2 also activated MAP kinase. This response was PTX-insensitive suggesting activation was mediated through $Ga_{q/11}$. Within cultured rat colonic smooth muscle cells endogenously expressing rat NmU-receptors, most likely NmU-R1, direct assessment of G-protein coupling also demonstrated coupling to both $Ga_{q/11}$ and Ga_i , G-proteins consistent with recombinant receptors. The functional consequence of this $Ga_{q/11}$ coupling was confirmed by robust increases in $[Ca^{2+}]_i$ to both hNmU-25 and rNmU-23, in fluo-3-loaded cells.

Following an exploration of single-cell Ca²⁺ signalling by both recombinant and endogenous NmU receptors it became apparent that NmU did not elicit Ca2+ signals to a second challenge when the successive applications were separated by extensive washing with buffer. This was shown to be due to the failure of the wash to remove bound-NmU from its receptors. In both hNmU-R1 and hNmU-R2 expressing HEK293 cells, this was confirmed by deomstrating that washing had no effect on either the accumulation of $[{}^{3}H]$ -InsP_x, the sustained Ca²⁺ signal or the binding of fluorescently labelled NmU. In further experiments, the phenomenon of receptor cross-talk (Werry et al., 2003) was employed to assess whether or not NmUreceptors were still active, and indeed continued to signal despite washing of the cells with agonist-free buffer. Finally, in membrane preparations, [¹²⁵I]-hNmU-25 could not be displaced by maximal concentrations of unlabelled hNmU-25. In all cases, the evidence suggests an irreversible ligand-receptor interaction. Indeed, only using washing buffers with an acidity of pH 2.0 could fluorescently-labelled NmU be removed from either receptor type. In directly comparable experiments, there was no difference between recombinant human receptors expressed in HEK293 cells and endogenous rat receptors expressed in cultured rat colonic smooth muscle cells.

In organ-bath studies, NmU-mediated concentration-dependent contractions of circular and longitudinal smooth muscle strips prepared from the distal colon of male Wistar rats. This contraction was independent of nervous activity as neither the muscarinic acytlcholine receptor antagonist, atropine, or inhibition of nervous activity with TTX had any effect. In contrast to single-cells, repetitive applications of NmU to the rat colon did elicit repeat contractions when successive challenges were separated by washing with agonist-free buffer. This suggests that in the rat

colon NmU binding is fully and rapidly reversible. Repetitive contractions were consistent and showed no sign of desensitisation. Using TCA to precipitate undegraded NmU, a crude homogenate of rat distal colon degraded ~80% of [125 I]-hNmU-25 within 30mins at 37°C.

7.3 Discussion and implications for further research

It is clear from these investigations that both hNmU-R1 and hNmU-R2 as recombinant proteins expressed in HEK293 cells couple to $Ga_{\alpha/11}$ and Ga_i . This dual coupling was demonstrated by increases in Ca^{2+} , $Ins(1,4,5)P_3$ and total $InsP_x$ ($Ga_{q/11}$) and by inhibition of FSK-elevated cAMP (Gai), and by direct assessment of Gprotein coupling using a $[^{35}S]$ -GTP γS binding assay using antisera raised specifically against Ga subunits. These studies demonstrate $Ga_{\alpha/11}$ coupling that is consistent with previous reports from recombinant NmU-receptors of human and rodent origin (Fujii et al., 2000; Hedrick et al., 2000; Hosova et al., 2000; Howard et al., 2000; Kojima et al., 2000; Raddatz et al., 2000; Shan et al., 2000; Szekeres et al., 2000; Funes et al., 2002). However, transient expression of hNmU-R1 in HEK293 cells failed to inhibit FSK-elevated cAMP (Szekeres et al., 2000), but has been previously reported with stably expressed hNmU-R2 in CHO cells (Hosoya et al., 2000). Multiplicity of G-protein coupling has been reported previously for many other receptors (Hermans et al., 2003) and is often affected by many experimental parameters (Tuček et al., 2002). One key factor here is that over-expression of receptors often promotes coupling to multiple G-proteins and is a major concern in extrapolating results into physiological systems. With this in mind, this research not only demonstrated $Ga_{q/11}$ and Ga_i coupling by hNmU-R1 and hNmU-R2 expressed at lower levels in HEK293 cells, but also by rat NmU receptors endogenously expressed in cultured rat colonic smooth muscle cells. At present however, the physiological and therapeutic consequence of any dual coupling by NmU receptors remains to be established.

Experiments with endogenous receptors were important because investigations using recombinant receptors may not truly reflect physiological systems (Kenakin, 1997). As such, a major part of chapter 5 involved optimising and validating primary cultures of rat colonic and fundus smooth muscle cells that would provide an

opportunity to compare certain aspects of hNmU receptor mediated signalling between recombinant and endogenous receptors. This was key in confirming the physiological relevance of signalling and dual coupling demonstrated by recombinant receptors.

These studies also demonstrate that activation of either hNmU-R1 or hNmU-R2 in HEK293 cells activate ERK1 and ERK2, suggesting that NmU-mediated signalling may be involved in mitogenesis and protein expression. Activation of the MAP kinase cascade by G-protein coupled receptors is a well-known but complex phenomenon (Sugden and Clerk, 1997; Belcheva and Coscia, 2002). The effects mediated by hNmU-R1 and hNmU-R2 in HEK293 cells were insensitive to PTX treatment suggesting activation was mediated through $Ga_{q/11}$ G-proteins but the actual mechanism involved was not explored further. This provides a source of potential further investigation, not only in terms of the signalling pathways involved but also in the physiological relevance and consequence of MAP kinase activation.

The internalisation of NmU-receptors was also demonstrated but not fully explored in these investigations. ConA inhibited internalisation suggesting the involvement of clathrin, however, nothing is known of the eventual fate of either the receptors or the ligand once inside the cell. This could have major implications in the signalling, recycling, resensitisation and responsiveness of NmU receptors and is certainly of interest in further study.

An examination of Ca^{2+} signalling in both HEK293 cells recombinantly expressing hNmU-R1 or hNmU-R2 or in cultured rat colonic smooth muscle cells endogenously expressing NmU-receptors revealed a lack of response to repeat challenges with NmU when each was separated by a wash step. This was due to NmU binding irreversibly to its receptors. However, the physiological consequences of this interaction are unclear but are certain to limit the responsiveness of cells to repeated NmU challenge.

In contrast, repetitive applications of NmU cause repeat contractions of the rat distal colon in organ-bath studies. The reasons why the characteristics of NmU

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receptors are different in cells and tissues was not confirmed and this major discrepancy provides probably the most intriguing question and source for further investigation. One possibility proposed was the action of proteases acting to degrade NmU and thus aid in cleaving and removing NmU from its receptors or in someway lowering the effective concentration around the receptor to aid ligand dissociation. Evidence was provided for the degradation of NmU in homogenate prepared from the rat distal colon suggesting that it is subject to some form of proteolytic activity. However, the specific protease(s) involved, their location (intacellular or extracellular), the degradation products and their role in NmU signalling and binding are currently unknown. Identification of the protease(s) involved in degrading NmU would be a major step in elucidating the role of degradation in NmU-receptor signalling. HPLC is a common method employed to fractionate a mix of reagents, but its application in separating the constituents of a crude homogenate of the rat distal colon is somewhat limited due to the vast number of proteins and chemicals that constitute this preparation. The protease could be identified by screening a plethora of inhibitors against NmU degradation as measured by TCA precipitation methods. Successful identification would enable the effects of NmU degradation in both single-cells (by inclusion of the protease in the assay) or tissues (by inclusion of inhibitors of its activity) to be explored.

hNmU-R1 and hNmU-R2 show approximately 50% amino acid homology with each other and exhibit differing potential sites for phosphorylation and regulation (Raddatz *et al.*, 2000, see also Introduction, section 1.2), yet despite these structural differences, the signalling properties of hNmU-R1 and hNmU-R2 when recombinantly expressed in HEK293 cells are essentially identical. Both couple to the same signalling pathways with similar potency with the same degree of desensitisation and internalisation. Therefore, the receptors either have functionally similar roles of rely on other differences to exert their functional effects. Most likely, is the different pattern of receptor distribution; hNmU-R1 exists predominantly in peripheral organs (Hedrick *et al.*, 2000; Howard *et al.*, 2000; Raddatz *et al.*, 2000; Szekeres *et al.*, 2000, Raddatz *et al.*, 2000; Shan *et al.*, 2000). There is a certain degree of overlap and neither receptor sub-type exists exclusively in certain tissues. However, the predominating receptor is likely to be the main instigator of certain physiological roles. For example, smooth muscle contraction (Minamino et al., 1985a; Brown and Quito, 1988; Bockman et al., 1989; Maggi et al., 1990; Benito-Orfila et al., 1991; Westfall et al., 2000) is likely to be mediated predominantly by NmU-R1 due to its higher levels of expression within these tissues. Conversely, hNmU-R2 is likely to be the main instigator of central effects such as energy homeostasis (Kojima et al., 2000; Nakazato et al., 2000; Niimi et al., 2001; Ivanov et al., 2002; Hanada et al., 2003), stress (Hananda et al., 2001;) and vascular changes (Chu et al., 2002). The differing expression patterns also give rise to the possibilities of cell-specific responses, and it may be this that governs the different functional responses. Each cell has its own unique proteins that are expressed at certain relative levels to each other and so protein stoichiometry in certain cells may play some part in NmU-receptor mediated signalling and physiological roles. The lack of apparent differences between hNmU-R1 and hNmU-R2 combined with the fact that the physiological roles of NmU are numerous and varied may makes the use of these receptors as selective therapeutic targets problematic.

In summary, this research has demonstrated novel features that add to the growing interest in these receptors. It has opened up numerous possibilities for further research that will complement and extend the data presented here and will add to the body of information necessary to fully understand the signalling and physiological roles of NmU and the NmU-receptors. Clearly, what is required is tools that will selectively activate or inhibit either hNmU-R1 or hNmU-R2 that could further help to describe the roles of NmU and may highlight the receptors as important therapeutic targets.

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Appendix 1

Throughout this thesis several figures are shown as representative data. Concerning mainly single-cell Ca^{2+} imaging in HEK293 cells, this data does not provide information on how representative the representative data actually is. Tables 1 (concerning representative data from chapter 3) and 2 (concerning representative data from chapter 4) below detail the variance of individual Ca^{2+} data. Data are normalised as the fold increase in cytosolic fluorescence relative to basal levels prior to agonist addition, and taken at specific time points as indicated. Data are mean \pm s.e.m, n=3.
Variation of representative data from Chapter 3

Table 1

Fig	Page	Experiment	Time	Mean	+ s.e.m					
3.11a	117	hNmU-R1-mediated activation of PLC	30s	3	0.7					
			250s	1.5	0.1					
3.11b	117	hNmU-R2-mediated activation of PLC	30s	2.5	0.6					
			250s	1.4	0.2					
3.12a	119	hNmU-R1 mediated Ca ²⁺ response	30s	3.1	0.8					
			180s	1.2	0.1					
3.12Ь	119	hNmU-R2 mediated Ca ²⁺ response	30s	2.5	0.7					
			180s	1.3	0.1					
3 120	120	hNmU-R1 mediated Ca ²⁺ response in the absence of extracellular Ca ²⁺	30s	2.3	0.8					
5.1120			180s	1	0.1					
3 124	120	hNmU-R2 mediated Ca ²⁺ response in the absence of extracellular Ca ²⁺	30s	2.8	0.9					
5.124			180s	1	0.1					
	121	Removal of extracellular Ca ²⁺ during sustained Ca ²⁺ signal of hNmU-R1	30s	3.5	0.8					
3.12e			120s	1.3	0.1					
			240s	1	0.1					
	121	Removal of extracellular Ca ²⁺ during sustained Ca ²⁺ signal of hNmU-R2	30s	2.5	0.1					
3.12f			120s	1.2	0.1					
			240s	1	0.1					
3.12g	122	hNmU-R1-mediated Ca ²⁺ signal following thapsigargin treatment	30s	1	0.1					
			180s	1	0.1					
3. 12h	122	hNmU-R2-mediated Ca ²⁺ signal following thapsigargin treatment	30s	1	0.1					
			180s	1	0.1					

Variation of representative data from Chapter 4

Table 2

Fig	Page	Experiment	Time	Mean	+ s.e.m
4.1a		2+	30s	2.8	0.8
	142	[Ca ^{**}]; responses to repeated application of NmU in hNmU-R1	120s	1.2	0.1
			210s	1.3	0.1
4.1b	142	[Ca ²⁺]; responses to repeated application of NmU in hNmU-R2	30s	3.1	0.9
			120s	1.3	0.1
			210s	1.3	0.1
4.1c	143	[Ca ²⁺] _i responses to repeated application of CCh	30s	3.2	0.8
			120s	1	0.1
			180s	2	0.4
4.1d	144	[Ca ²⁺]; responses to repeated NmU following 12min wash (hNmU-R1)	30s	3.2	0.8
			300s	1.4	0.2
			720s	1.3	0.1
	144	[Ca ²⁺]; responses to repeated NmU following 12min wash (hNmU-R2)	30s	2.9	0.7
4.1e			300s	1.4	0.1
			720s	1.3	0.1
	146	[Ca ²⁺] _i responses to application of hNmU-25 following CCh challenge	30s	3.2	0.8
4.3a			120s	1.6	0.4
			210s	2.5	0.6
		$[Ca^{2^+}]_i$ responses to application of hNmU-25 following CCh challenge and wash	30%	35	0.7
4.3b	146		120s	1	0.1
			210s	3	0.3
	147	[Ca ²⁺]; responses to application of CCh following hNmU-25 challenge	300	3.3	0.7
4.3c			120%	15	0.7
			2108	3	0.9
	147	$[Ca^{2^+}]_i$ responses to application of CCh following hNmU-25 challenge and wash	200	22	0.0
4.3d			120	3.3	0.9
			2108	1.5	0.2
			300	35	0.6
4.4a	149	Effects of washing with KHB on sustained $Ca^{4^{\tau}}$ signalling by hNmU-R1	120	13	0.0
	149	Effects of washing with KHB on sustained Ca ²⁺ signalling by hNmU-R1	20-	24	0.4
4.4b			1200	12	0.0
	····		1203	1.2	0.1
		<u> </u>	20.		0.1
4.6a	152	Ca ²⁺ signalling by β_2 -adrenoceptors in HEK293 cells	120-		0.1
			- 1208	26	0.1
4.6b	153	Cross-talk between mucarinic M3-receptors and B2-adrenoceptors	190-	3.0 2	0.8
	153	Cross-talk between mucarinic M_3 -receptors and β_2 -adrenoceptors including wash	1005		0.5
4.6c			180	3.2 1	0.0
	154	Cross-talk between hNmU-R1 and β_2 -adrenoceptors	20-	24	0.1
4.6d			180	4.4 19	0.0
4.6e	154	Cross-talk between hNmU-R1 and β_2 -adrenoceptors including wash	200	1.0 2.9	0.5
			1205	40 22	0.0
4.7f	155	Cross-talk between hNmU-R2 and β_2 -adrenoceptors	200	22	0.4
			180-	5.5 2	0.7
├ ──┤			200	22	0.5
4.7g	155	Cross-talk between hNmU-R2 and β_2 -adrenoceptors including wash	1205	5.2 21	0.8
			1008	2.1	0.5

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