Naunyn-Schmiedeberg's Arch Pharmacol (2003) 368 : 528–537
DOI 10.1007/s00210-003-0821-5

ORIGINAL ARTICLE

**M. Kitayama · T. A. Barnes · G. Carra · J. McDonald
G. Calo · R. Guerrini · D. J. Rowbotham · G. Smith
D. G. Lambert**

Pharmacological profile of the cyclic nociceptin/orphanin FQ analogues c[Cys^{10,14}]N/OFQ(1–14)NH$_2$ and c[Nphe^1,Cys^{10,14}]N/OFQ(1–14)NH$_2$

Received: 30 May 2003 / Accepted: 8 September 2003 / Published online: 4 November 2003

Abstract In this study we describe the activity of two cyclic nociceptin/orphanin FQ (N/OFQ) peptides; c[Cys^{10,14}]N/OFQ(1–14)NH$_2$ (c[Cys^{10,14}]) and its [Nphe^1] derivative c[Nphe^1,Cys^{10,14}]N/OFQ(1–14)NH$_2$ (c[Nphe^1,Cys^{10,14}]) in native rat and mouse and recombinant human N/OFQ receptors (NOP). Cyclisation may protect the peptide from metabolic degradation.

In competition binding studies of rat, mouse and human NOP the following rank order pK$_i$ was obtained: N/OFQ(1–13)NH$_2$(reference agonist)>N/OFQ=c[Cys^{10,14}]>>c[Nphe^{1}Cys^{10,14}]. In GTPγ^{35}S studies of Chinese hamster ovary cells expressing human NOP (CHO$_{hNOP}$) c[Cys^{10,14}] (pEC$_{50}$ 8.29) and N/OFQ(1–13)NH$_2$ (pEC$_{50}$ 8.57) were full agonists whilst c[Nphe^{1}Cys^{10,14}] alone was inactive. Following 30 min pre-incubation c[Nphe^{1}Cys^{10,14}] competitively antagonised the effects of N/OFQ(1–13)NH$_2$ with a pA$_2$ and slope factor of 6.92 and 1.01 respectively. In cAMP assays c[Cys^{10,14}] (pEC$_{50}$ 9.29, E$_{max}$ 102% inhibition of the forskolin stimulated response), N/OFQ(1–13)NH$_2$ (pEC$_{50}$ 10.16, E$_{max}$ 103% inhibition) and c[Nphe^{1}Cys^{10,14}] (~80% inhibition at 10 μM) displayed agonist activity. In the mouse vas deferens c[Cys^{10,14}] (pEC$_{50}$ 6.82, E$_{max}$ 89% inhibition of electrically evoked contractions) and N/OFQ(1–13)NH$_2$ (pEC$_{50}$ 7.47, E$_{max}$ 93% inhibition) were full agonists whilst c[Nphe^{1}Cys^{10,14}] alone was inactive. c[Nphe^{1}Cys^{10,14}] (10 μM) competitively antagonised the effects of N/OFQ(1–13)NH$_2$ with a pK$_B$ of 5.66. In a crude attempt to assess metabolic stability, c[Cys^{10,14}] was incubated with rat brain membranes and then the supernatant assayed for remaining peptide. Following 60 min incubation 64% of the 1 nM added peptide was metabolised (compared with 54% for N/OFQ-NH$_2$).

In summary, we report that c[Cys^{10,14}] is a full agonist with a small reduction in potency but no improvement in stability whilst c[Nphe^{1}Cys^{10,14}] displays tissue (antagonist in the vas deferens) and assay (antagonist in the GTPγ^{35}S assay and agonist in cAMP assay) dependent activity.

Keywords Nociceptin/orphanin FQ (N/OFQ) · Cyclic N/OFQ peptides · N/OFQ receptor · Radioligand binding · GTPγ^{35}S binding · cAMP formation · Electrically stimulated vas deferens

Introduction

Since its formal identification in 1995 (Meunier et al. 1995; Reinscheid et al. 1995), nociceptin/orphanin FQ (N/OFQ) has been the subject of intense study (Mogil and Pasternak 2001; Calo et al. 2000c) with particular emphasis on the design and evaluation of high affinity selective agonists and antagonists for the N/OFQ peptide receptor (NOP; Calo et al. 2000a). Activation of NOP by N/OFQ reduces cAMP formation, enhances an outward K$^+$ conductance and closes voltage sensitive Ca^{2+} channels (Knoflach et al. 1996; Meunier et al. 1995; Reinscheid et al. 1995; Vaughan and Christie 1996). These actions decrease neurotransmission leading to a variety of organ/whole animal effects including; inhibition of neurogenic contractions in several tissues, antinociception/hyperalgesia, food intake stimulation, locomotor activity modulation, anxiolysis, diuresis and antinaturesis.

There are several novel peptide ligands currently available for the NOP receptor with varying degrees of effi-

Presented in part to The British Pharmacological Society at the Brighton, UK Meeting January 2003

M. Kitayama · T. A. Barnes · J. McDonald · D. J. Rowbotham
G. Smith · D. G. Lambert (✉)
University Department of Anaesthesia,
Critical Care and Pain Management, Leicester Royal Infirmary,
Leicester, LE1 5WW, UK
Tel.: +44-116-2585291, Fax: +44-116-2854487,
e-mail: DGL3@le.ac.uk

G. Carra · G. Calo
Department of Experimental and Clinical Medicine,
Section of Pharmacology and Neuroscience Centre,
University of Ferrara,
via Fossato di Mortara 17, 44100 Ferrara, Italy

R. Guerrini
Department of Pharmaceutical Sciences, University of Ferrara,
via Fossato di Mortara 17, 44100 Ferrara, Italy

cacy, potency and selectivity and we and others have described these in some detail elsewhere (Hashiba et al. 2001; Calo et al. 2002; Guerrini et al. 2000). The major drawback with these peptide ligands is they are less stable due to their susceptibility to enzymatic cleavage (Terenius et al. 2000). In an attempt to limit metabolism we routinely use amidated and truncated forms of the native peptide such as N/OFQ(1–13)NH_2 (Calo et al. 1996; Guerrini et al. 1997) and where practical high concentrations of peptidase inhibitors are also used (Terenius et al. 2000). One approach that has been used in the opioid peptide field involves peptide cyclisation to produce compounds such as [Dpen^{2,5}]enkephalin (Kramer et al. 1991).

Addition of cysteine residues to peptides makes them suitable for cyclisation via disulphide bridge formation. Indeed, Ambo et al. (2001) reported a structure-activity relationship series of six cyclic N/OFQ analogues and compared these to their linear counterparts. One of these cyclic peptides c[Cys^{10,14}]N/OFQ(1–14)NH_2 displayed a small reduction in potency but retained full agonist activity and represents the starting point for this study. A full characterisation was not performed.

We have synthesised c[Cys^{10,14}]N/OFQ(1–14)NH_2 (c[Cys^{10,14}]) and its [Nphe^1] derivative c[Nphe^1,Cys^{10,14}] N/OFQ(1–14)NH_2 (c[Nphe^1,Cys^{10,14}]; see structures in Fig. 1) with the former representing a cyclic agonist and the latter a putative cyclic antagonist. The latter [Nphe^1] substitution is based on our previous observations that modification of phenylalanine at position 1 of both N/OFQ(1–13)NH_2 and [Arg^{14},Lys^{15}]N/OFQ-NH_2 created two selective antagonists (Calo et al. 2000b, 2002). In this study we have performed a detailed characterisation of the pharmacological profile of these two cyclic peptides in:

1. Radioligand binding studies to rat and mouse native NOP and to recombinant human NOP, MOP, DOP and KOP receptors expressed in Chinese hamster ovary (CHO) cells
2. GTPγ^{35}S and cAMP functional assays in CHO_{hNOP} cells
3. Electrically stimulated mouse vas deferens

In addition we have performed some crude studies to assess the metabolic stability of the peptides.

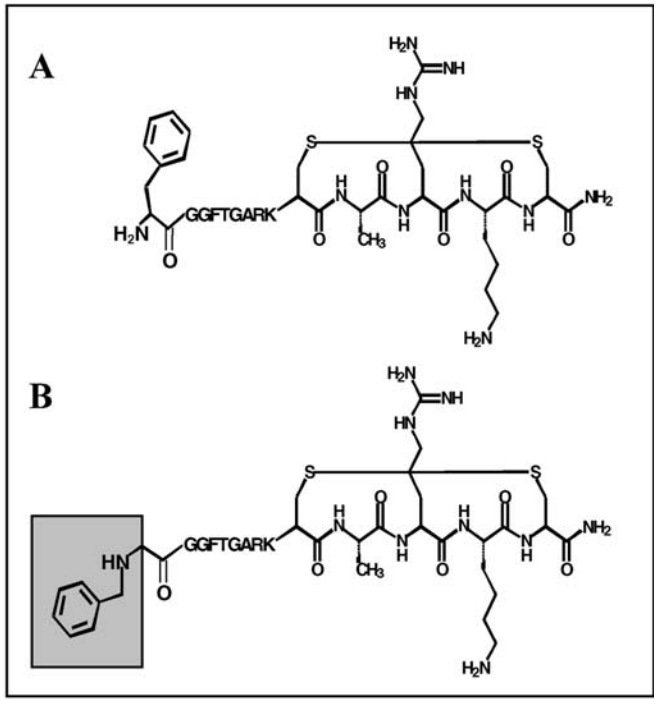

Fig. 1 Structures of **A** c[Cys^{10,14}]N/OFQ(1–14)NH_2 and **B** c[Nphe^1, Cys^{10,14}]N/OFQ(1–14)NH_2. In **B** the C to N shift to create an antagonist is highlighted

Materials and methods

Sources of reagents

All peptides (N/OFQ, N/OFQ(1–17)NH_2, N/OFQ(1–13)NH_2, UFP-101, c[Cys^{10,14}] and c[Nphe^1, Cys^{10,14}]) were synthesised at one of our institutes using standard solid-phase synthesis techniques according to previously published methods (Guerrini et al. 1997). For the synthesis of the cyclic derivatives c[Cys^{10,14}] and c[Nphe^1, Cys^{10,14}], the corresponding linear purified analogue was dissolved in a mixture of H_2O/DMSO/TFA (75:25:0.1 v/v) at a concentration of 1 mg/ml (Annis et al. 1997). The cyclisation reaction completed within one day and was monitored by analytical HPLC and Ellman test (Ellman 1959). After partial evaporation of the solvent, the product was purified by preparative HPLC to yield the desired cyclic compound after lyophilisation. Peptide stocks were dissolved and frozen at –70°C in distilled water at 2 mM. All tissue culture media and supplements were from Invitrogen (Paisley, Scotland). [*leucyl*-^{3}H]N/OFQ ([^{3}H]-N/OFQ, 150 Ci/mmol) was from Amersham Pharmacia Biotech (Buckinghamshire, UK), GTPγ^{35}S (1,250 Ci/mmol), [^{3}H]diprenorphine ([^{3}H]DPN, 50 Ci/mmol) and [^{3}H]cAMP (32 Ci/mmol) were from Perkin Elmer Life Sciences (Boston, MA, USA). All receptor ligands, bacitracin, bovine serum albumin, GDP, unlabelled GTPγS, 3-isobutyl methylxanthine, peptidase inhibitors and forskolin were from Sigma (Poole, UK). All other consumables and reagents were of the highest purity available.

Cell culture and membrane preparation

Stock cultures of CHO_{hNOP} cells (a gift from Dr F. Marshall and Mrs N. Bevan, GSK, Stevenage, UK) were maintained in DMEM: Ham F12(50:50) supplemented with 5% FCS, 100 IU/ml penicillin (P), 100 µg/ml streptomycin (S), 2.5 µg/ml fungizone, 200 µg/ml hygromycin B and 200 µg/ml G418 selection media. $CHO_{hMOP/DOP/KOP}$ cell stocks (gift from L. Toll, SRI International, Menlo Park, CA, US) were maintained in Ham F12 containing 10% FCS, 100 IU/ml P, 100 µg/ml S and 400 µg/ml G418, for CHO non-transfected cells G-418 and hygromycin B were omitted. Cell cultures were kept at 37°C in 5% CO_2/humidified air. In all cases experimental cultures were free from selection agents (hygromycin B, G418). When confluence was reached (3–4 days), cells were harvested for use by the addition of HEPES (10 mM) buffered saline (0.9%) containing EDTA (0.05%). For native NOP preparations, female Wistar rats (250–300 g) or mice (25–30g) were decapitated following cervical dislocation. The brain was removed and rapidly transferred to ice-cold buffer (Tris-HCl 50 mM, pH 7.4) and the cerebrocortex dissected. All CHO and animal tissues were homogenized (Ultra Turrax, 30 s) on ice in; Tris (50 mM), EGTA (0.2 mM), pH 7.4 (CHO_{hNOP}, GTPγ^{35}S), Tris (50 mM), $MgSO_4$ (5 mM), pH 7.4 for CHO_{hNOP}, rat and mouse cerebrocortex (saturation/competition binding) or 50 mM Tris, pH 7.4 for $CHO_{MOP/DOP/KOP}$ cells. The homogenate was centrifuged at 20,374 *g* for 10 min at 4°C. This procedure was repeated twice more with re-suspension in fresh buffer

each time. Rat and mouse tissue was frozen in small aliquots at –70°C until use. CHO membranes were prepared fresh each day.

Radioligand binding assay

[^{3}H]-N/OFQ. Approximately 10 µg of CHO_{hNOP} or 100–150 µg rat or mouse cerebrocortical membrane protein were incubated in 0.5 ml of homogenisation buffer containing 10 µM of peptidase inhibitors (captopril, amastatin, bestatin and phosphoramidon) and 0.5% BSA for 60 min at room temperature. In saturation experiments, various concentrations of [^{3}H]-N/OFQ (0.001–1 nM) were used. In competition experiments, a single fixed concentration of 0.2 nM [^{3}H]-N/OFQ was used. In all studies non-specific binding (NSB) was defined in the presence of 1 µM unlabelled N/OFQ. N/OFQ, N/OFQ(1–13)NH_2, c[$Cys^{10,14}$] and c[$Nphe^1Cys^{10,14}$] were included in various concentrations and combinations. Following incubation bound and free radioactivity was separated by vacuum filtration using a Brandel cell harvester through Whatman GF/B filters. Filters were soaked in polyethyleminine (PEI, 0.5%) to reduce NSB and loaded onto the harvester wet.

[^{3}H]-diprenorphine. This is essentially as described above. Twenty-five to fifty micrograms $CHO_{hMOP/DOP/KOP}$ membrane protein was incubated in 0.5 ml of homogenisation buffer containing a cocktail of peptidase inhibitors (10 µM as above) and BSA for 60 min at room temperature. Competition binding studies were performed using 0.3–0.5 nM [^{3}H]-DPN and N/OFQ(1–13)NH_2, c[$Cys^{10,14}$] and c[$Nphe^1Cys^{10,14}$] in various concentrations and combinations. In saturation studies up to 4 nM [^{3}H]-DPN was used. NSB was defined in the presence of 10 µM naloxone. As reference compounds, naltrindole was included for CHO_{hDOP}, norbinaltorphimine for CHO_{hKOP} and fentanyl for CHO_{hMOP} cells. Bound and free radioactivity was separated as described above.

GTPγ^{35}S binding assay

Assays were performed essentially as described by (Albrecht et al. 1998; McDonald et al. 2002). Twenty micrograms of CHO_{hNOP} membranes were incubated in 0.5 ml buffer containing Tris (50 mM), EGTA (0.2 mM), $MgCl_2$ (1 mM), NaCl (100 mM), bacitracin (0.15 µM), amastatin (10 µM), bestatin (10 µM), captopril (10 µM), phosphoramidon (10 µM), GDP (100 µM) and ~150 pM GTPγ^{35}S. Non-specific binding was determined in the presence of 10 µM unlabelled GTPγS. N/OFQ(1–13)NH_2, c[$Cys^{10,14}$] and c[$Nphe^1Cys^{10,14}$] were included in various concentrations to determine primary agonist actions. In order to probe any antagonist actions of c[$Nphe^1$,$Cys^{10,14}$] concentration response curves to N/OFQ(1–13)NH_2 were constructed in the absence and presence of this peptide (0.1, 1, 10 µM added simultaneously with the agonist). In some of these antagonist experiments CHO_{hNOP} membranes were pre-incubated for 30 min with c[$Nphe^1$,$Cys^{10,14}$] prior to N/OFQ(1–13)NH_2 addition. Reactions were allowed to proceed for 60 min at 30°C with gentle shaking. Bound and free radiolabel was separated by vacuum filtration onto Whatman GF/B filters. PEI was not used.

Cyclic AMP accumulation assay

For measurement of cyclic AMP, whole CHO_{hNOP} cells were incubated in 0.3 ml volumes of Krebs-HEPES buffer supplemented with BSA (0.5%), pH 7.4. cAMP formation was measured in the presence of isobutyl-methylxanthine (1 mM) and forskolin (1 µM). N/OFQ(1–13)NH_2, c[$Cys^{10,14}$] and c[$Nphe^1$,$Cys^{10,14}$] were included in various concentrations and combinations. After 15 min incubation at 37°C, reactions were terminated and cAMP extracted by addition of HCl (10 M)/NaOH (10 M)/Tris (1 M) and cyclic AMP was measured using a protein binding assay as described by Okawa et al. (1999).

Electrically stimulated mouse vas deferens

Vas deferens tissues was taken from male Swiss mice weighing 25–30 g; tissues prepared as previously described (Bigoni et al. 1999) and suspended in 5 ml organ baths containing Krebs buffer oxygenated with 95% O_2 and 5% CO_2. The temperature was set at 33°C and a resting tension of 0.3 g was applied to the tissues. Tissues were continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 ms duration and 0.05 Hz frequency. Electrically evoked contractions were measured isotonically with a strain gauge transducer (Basile 7006) and recorded with the PC based acquisition system Autotrace 2.2 (RCS, Florence, Italy). Following an equilibration period of about 60 min, the contractions induced by electrical field stimulation (twitches) were stable. Measurements of twitch size were made. At this time, cumulative concentration response curves to agonists were constructed (0.5 log unit steps) in the absence and presence of antagonists. Antagonists were added to the bath 15 min before performing cumulative concentration response curves to agonists. In our experience with mVD, 15 min equilibration is more than ample for complete equilibration.

Crude metabolism experiments

N/OFQ, N/OFQ(1–17)NH_2 and c[$Cys^{10,14}$] (1 nM added from frozen stocks) were pre-incubated in 1,000 µl of saturation/competition homogenisation buffer containing 1 mg rat cerebrocortex membranes at 37°C for 15 min, 60 min and 120 min. Incubations were centrifuged at 16,000 *g* for 3 min at room temperature, then the supernatant was removed and kept on ice. [^{3}H]-N/OFQ competition binding assays were performed with these and non-incubated stock samples as described above. At same time, a full competition curve for each peptide was constructed for subsequent use as a "standard curve". In order to inactivate all peptidase activity some reactions (with c[$Cys^{10,14}$]) were taken to pH 3.0 with 0.1 M trifluoroacetic (TFA) acid and incubated in parallel with an identical reaction at pH 7.4 for 1 h at 37°C. At the end of this period TFA was added to reaction at pH 7.4 so that both conditions contained identical quantities of TFA. Both reactions were then taken back to pH 7.4 using 2 M Tris (pH 7.4).

Data analysis

All data are expressed as mean ± SEM from ≥3 separate experiments. All curve fitting was performed using GraphPad PRISM V3.0 (San Diego, USA). In competition binding studies, the concentration of ligand producing 50% displacement of specific binding (IC_{50}) was corrected for the competing mass of [^{3}H]-N/OFQ or [^{3}H]-DPN using K_D values determined in this study or that of Hashiba et al. (2002b) to yield pK_i. Agonist potencies were measured as pEC_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist. In GTPγ^{35}S binding experiments antagonist potencies were expressed as pA_2 values from Schild regression plots, which is the negative logarithm to base 10 of the antagonist molar concentration at which the agonist concentration must be doubled to restore its original response, while in bioassays studies antagonist potencies were calculated with the Gaddum-Schild equation pKb = (CR-1)/[antagonist], assuming a slope equal to one. In metabolism experiments peptide concentrations were estimated from constructed standard curves using GraphPad PRISM. Statistical analysis has been performed using the Student's *t*-test or one-way ANOVA followed by Bonferroni correction as appropriate with p values less than 0.05 considered significant.

Table 1 Receptor binding profile of N/OFQ(1–13)NH_2, c[$Cys^{10,14}$]N/OFQ(1–14)NH_2 (c[$Cys^{10,14}$]) and c[$Nphe^1Cys^{10}Cys^{14}$]N/OFQ(1–14)NH_2(c[$Nphe^1Cys^{10,14}$]) to recombinant NOP, DOP, KOP and MOP receptors expressed in CHO cells, rat and mouse cerebrocortex ($n \geq 3$)

	CHO_{hNOP}	Rat cerebrocortex	Mouse cerebrocortex	CHO_{hMOP}	CHO_{hDOP}	CHO_{hKOP}
Radioligand	[^{3}H]-N/OFQ	[^{3}H]-N/OFQ	[^{3}H]-N/OFQ	[^{3}H]-DPN	[^{3}H]-DPN	[^{3}H]-DPN
B_{max}(fmol/mg protein)	1,352±127	157±4[a]	102±4	801±106	3,054±365	838±143
pK_d	9.65±0.19	10.34±0.02[a]	10.26±0.05	9.90±0.12	9.50±0.03	9.87±0.11
(pM)	(222)	(46)	(55)	(125)	(323)	(134)
NSB at K_D (% total)	<2%	<3%	<4%	<10%	<7%	<11%
Binding pK_i						
N/OFQ(1–13)NH_2	10.26±0.20	10.57±0.09	10.46±0.10	6.51±0.10[b]	5.37±0.13[b]	7.13±0.10[b]
selectivity				(>5,600)	(>7,700)	(>1,300)
c[Cys^{1014}]	9.68±0.27	10.04±0.12	10.08±0.17	6.52±0.11[b]	5.82±0.03[b]	6.97±0.10[b]
selectivity				(>1,400)	(>7,000)	(>500)
c[$Nphe^1Cys^{10,14}$]	7.92±0.23	7.89±0.06	8.33±0.04	<5	<5	<5
selectivity				(>800)	(>800)	(>800)
Reference ligand	N/OFQ	N/OFQ	N/OFQ	Fentanyl	Naltrindole	Norbinal-torphimine
	9.32±0.12	9.89±0.04	10.30±0.22	7.88±0.03	9.87±0.04	9.81±0.01

Data are mean ± SEM ($n \geq 4$)
[a]From Hashiba et al. (2002a)
[b]Assuming maximum of 100% displacement

Results

Radioligand binding

The binding of [^{3}H]-N/OFQ to human recombinant NOP expressed in CHO cells (CHO_{hNOP}) and native rat (Hashiba et al. 2002b) and mouse NOP expressed in cerebrocortical membranes was concentration dependent and saturable with pK_D and B_{max} values shown in Table 1. N/OFQ, N/OFQ(1–13)NH_2, c[$Cys^{10,14}$] and c[$Nphe^1Cys^{10,14}$] displaced the binding of [^{3}H]-N/OFQ to all tissues in a concentration dependent manner (Fig. 2) with pK_i values shown in Table 1. When compared to N/OFQ(1–13)NH_2, c[$Cys^{10,14}$] modification per se resulted in a modest (approximately 3-fold) loss of binding affinity whereas the effects of further [$Nphe^1$] modification produced a more substantial (about 2 orders of magnitude) loss of affinity. In all three species of NOP the following rank order pK_i was obtained: N/OFQ(1–13)NH_2 >N/OFQ = c[$Cys^{10,14}$] >>c[$Nphe^1Cys^{10,14}$].

In order to determine selectivity of c[$Cys^{10,14}$] and c[$Nphe^1Cys^{10,14}$] for NOP receptor over classical MOP/DOP/KOP receptor a series of [^{3}H]-DPN competition binding studies were performed using CHO cells expressing recombinant human MOP/DOP/KOP. The binding of [^{3}H]-DPN was concentration dependent and saturable, pK_D and B_{max} values are shown in Table 1. It can be seen that CHO_{hDOP} cells expressed the highest receptor density although the affinity of the N/OFQ analogues was weakest at this receptor More importantly, c[$Cys^{10,14}$] and c[$Nphe^1Cys^{10,14}$] showed at least 2 orders of magnitude selectivity for NOP.

GTPγ^{35}S binding

N/OFQ(1–13)NH_2 and c[$Cys^{10,14}$] stimulated the binding of GTPγ^{35}S to CHO_{hNOP} membranes in a concentration dependent and saturable manner (Fig. 3). c[$Cys^{10,14}$] modification resulted in a modest loss of functional potency (~2 fold) without major modification of efficacy (1 μM). However, in all experiments the highest c[$Cys^{10,14}$] concentration used 10 μM (and achievable based on the stocks in use) produced a response some 22% lower than that achieved at 1 μM ($p<0.05$). c[$Nphe^1Cys^{10,14}$] alone was inactive in the GTPγ^{35}S binding assay. Full concentration response curves to N/OFQ(1–13)NH_2, c[$Cys^{10,14}$] and c[$Nphe^1Cys^{10,14}$] were constructed in non-transfected CHO cells in which all peptides failed to increase GTPγ^{35}S binding (n=3 data not shown). Moreover, no differences in GTPγ^{35}S binding at 10 μM compared to 1 μM c[$Cys^{10,14}$] were observed indicating that the difference obtained in CHO_{hNOP} membranes required the presence of the receptor.

c[$Nphe^1Cys^{10,14}$] was then tested as an antagonist where it produced a concentration dependent rightward shift in the concentration response curve to N/OFQ(1–13)NH_2 without modification of the maximum response obtained (Fig. 4A). Schild analysis of these data yielded a pA_2 and slope of 7.35 and 0.87 respectively. As this slope was less than unity it was suspected that the antagonist was not at equilibrium and hence these studies were repeated with a 30 min pre-incubation of c[$Nphe^1Cys^{10,14}$], Fig. 4B. Under these experimental conditions the pA_2 decreased to 6.92 with a slope of 1.01.

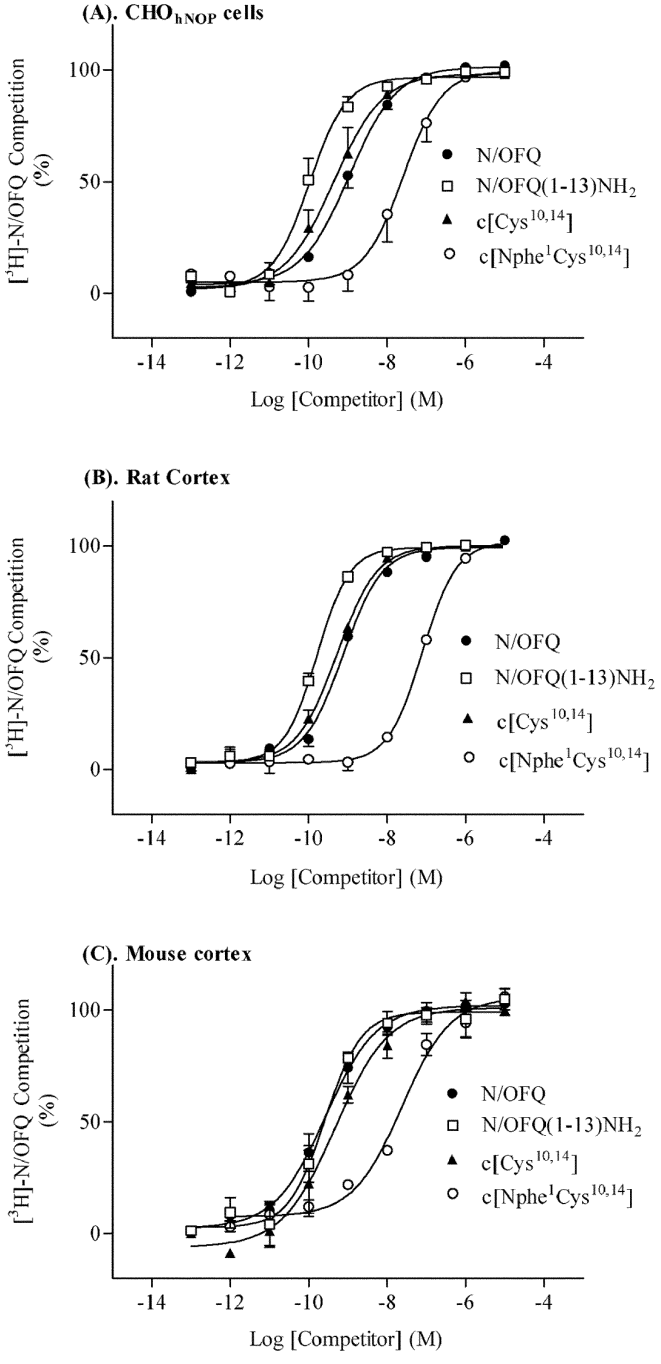

Fig. 2 Displacement of [^{3}H]-N/OFQ binding by N/OFQ, N/OFQ (1–13)NH_2, c[Cys^{10,14}] and c[Nphe^{1}Cys^{10,14}] to human NOP expressed in **A** CHO cells, **B** native NOP expressed in rat cerebrocortical membranes and **C** native NOP expressed in mouse cerebrocortical membranes. All data are mean ± SEM for n=4. pK_i values are shown in Table 1

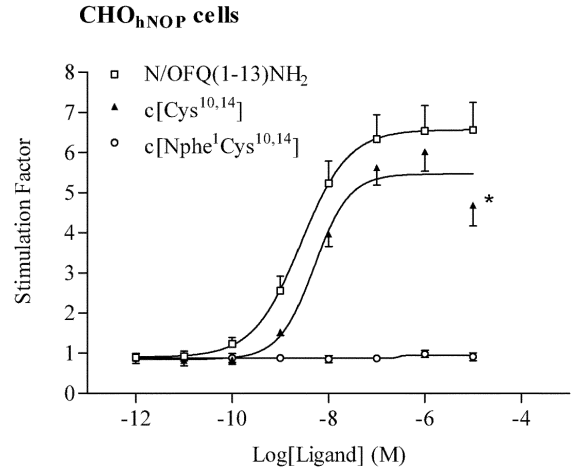

Fig. 3 N/OFQ(1–13)NH_2 and c[Cys^{10,14}] but not c[Nphe^{1}Cys^{10,14}] stimulate the binding of GTPγ^{35}S to CHO_{hNOP} membranes in a concentration dependent and saturable manner. Data are expressed as stimulation factor (ratio specific stimulated to specific basal binding) and represent mean ± SEM for n=7. pEC_{50} and E_{max} values are shown in Table 2. *Smaller than the response at 1 μM

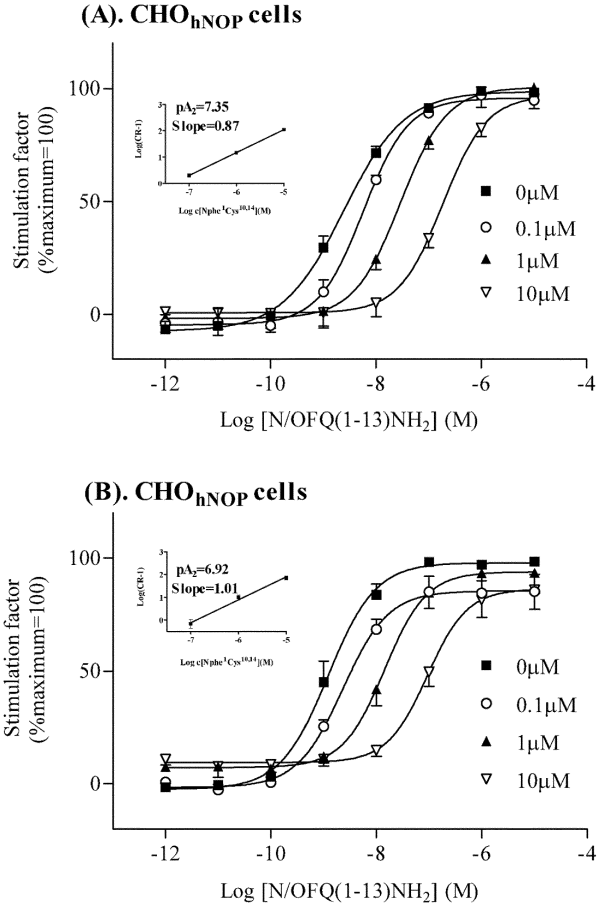

Fig. 4 Effects of c[Nphe^{1}Cys^{10,14}] on N/OFQ(1–13)NH_2 stimulated GTPγ^{35}S binding to CHO_{hNOP} membranes (stimulation factor expressed as a % of the maximum N/ OFQ(1–13)NH_2 response) either **A** without pre-incubation (i.e. added simultaneously with the agonist) or **B** with 30 min pre-incubation. In both experiments, c[Nphe^{1}Cys^{10,14}] produced a parallel rightward shift, which was used to generate the Schild plot shown as an insert in each panel. Data are mean ± SEM from n=5

Inhibition of cAMP formation

N/OFQ, N/OFQ(1–13)NH_2 and c[Cys^{10,14}] produced a concentration dependent inhibition of forskolin stimulated cAMP formation (Fig. 5). In this assay all peptides behaved as agonists with the following rank order potency (pEC_{50}) N/OFQ(1–13)NH_2 >N/OFQ >c[Cys^{10,14}], Table 2.

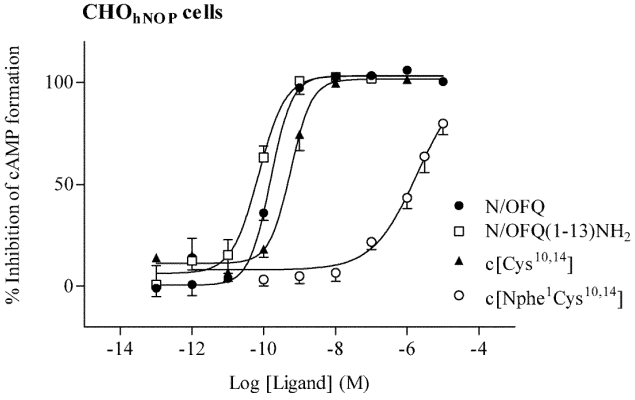

Fig. 5 N/OFQ, N/OFQ(1–13)NH_2, c[Cys^{10,14}] and c[Nphe^{1}Cys^{10,14}] produce a concentration dependent inhibition of forskolin stimulated cAMP formation in whole CHO$_{hNOP}$ cells. pEC_{50} and E_{max} values are shown in Table 2. Data are means ± SEM for n=3–7

In contrast to the GTPγ^{35}S studies described above, c[Nphe^{1}Cys^{10,14}] also produced a marked inhibition of cAMP formation (Fig. 5). However, due to the low potency of the peptide it was not possible to complete the concentration response curve to this peptide, which induced a 80% inhibition at 10 µM.

Mouse vas deferens

In the mouse vas deferens (Fig. 6A) N/OFQ(1–13)NH_2 produced a concentration dependent inhibition of electrically evoked twitches with a pEC_{50} and E_{max} of 7.47 and –93%. c[Cys^{10,14}] mimicked the inhibitory effects of the linear peptide with reduced potency but similar efficacy (Table 3). The kinetics of action of the cyclic compound were similar to those of N/OFQ(1–13)NH_2. Indeed, the action of this peptide occurred immediately after addition to the bath, was rapidly reversible after washing, and could be repeated in the same tissue (data not shown).

The effects of N/OFQ(1–13)NH_2 and c[Cys^{10,14}] were reassessed in the presence of the universal opioid receptor antagonist naloxone and of the selective NOP antagonist UFP-101 (Fig. 7). Naloxone (1 µM) did not modify the concentration response curve to N/OFQ(1–13)NH_2 or c[Cys^{10,14}], while the selective NOP receptor antagonist, UFP-101 (1 µM) clearly shifted to the right the concentration response curve to both agonists without modifying their maximal effects. pK_B values for UFP-101 vs. N/OFQ(1–13)NH_2 and c[Cys^{10,14}], respectively are shown in Table 4. c[Nphe^1, Cys^{10,14}]N/OFQ(1–14)NH_2, up to 10 µM, did not significantly modify per se the control twitches (data not shown), but was able to shift to the right the concentration response curve to N/OFQ(1–13)NH_2 without producing any change in maximal effects: a pK_B value of 5.66 (Table 4) was derived from these experiments (Fig. 6B).

Crude metabolism experiments

In an attempt to determine any increased metabolic stability inferred by cyclisation N/OFQ, N/OFQ(1–17)NH_2 and c[Cys^{10,14}] were incubated with 1 mg of a crude rat cerebrocortical membrane preparation in the absence of any peptidase inhibitors. We used a standard competition binding assay (with peptidase inhibitors) with each peptide in order to determine actual tube concentrations. For all peptides 1 nM was added which we later confirmed to vary between 0.9–1.3 nM (Table 5). There was a time dependent loss of peptide such that following 2 h incubation ~88% of N/OFQ, 71% of N/OFQ-NH_2 and 73% of c[Cys^{10,14}] was lost. Amidation appeared to provide some protection when compared with N/OFQ while cyclisation did not appear to provide any additional increase in metabolic stability (Table 5). When the pH of the reaction was reduced to 3.0 all peptidase activity ceased such that there was no statistical difference between the quantity of c[Cys^{10,14}] added and that remaining after 1 h incubation (Fig. 8).

Discussion

In this study we have shown that c[Cys^{10,14}] and c[Nphe^{1}Cys^{10,14}] bind to native (mouse and rat) and recombinant NOP expressed in CHO cells. Whilst there was a dramatic loss of affinity on c[Nphe^{1}Cys^{10,14}] modification, both peptides displayed high selectivity over classical MOP/DOP/KOP. In GTPγ^{35}S, cAMP inhibition and mouse vas

Table 2 Effects of N/OFQ, N/OFQ(1–13)NH_2, c[Cys^{10,14}]N/OFQ(1–14)NH_2 (c[Cys^{10,14}]) and c[Nphe^{1}Cys^{10,4}]N/OFQ(1–14)NH_2 (c[Nphe^{1}Cys^{10,14}]) on GTPγ^{35}S binding and inhibition of forskolin stimulated cAMP formation in CHO$_{hNOP}$ membranes and whole cells respectively

	GTPγ^{35}S binding		Cyclic AMP inhibition	
	pEC_{50}	E_{max}	pEC_{50}	E_{max} (%)
N/OFQ	8.58±0.21[a]	7.46±0.96[a]	9.79±0.08	104.6±0.79
N/OFQ(1–13)NH_2	8.57±0.09	6.37±0.61	10.16±0.07	103.0±1.25
c[Cys^{10,14}]	8.29±0.08*	5.67±0.46**	9.29±0.11	102.4±0.61
c[Nphe^{1}Cys^{10,14}]	Inactive		5.97	80% at 10 µM

Data are mean ± SEM (n=3–7)
[a]From Hashiba et al. (2002b)
*p<0.05; paired t-test compared to N/OFQ(1–13)NH_2
**p<0.01; paired t-test compared to N/OFQ(1–13)NH_2

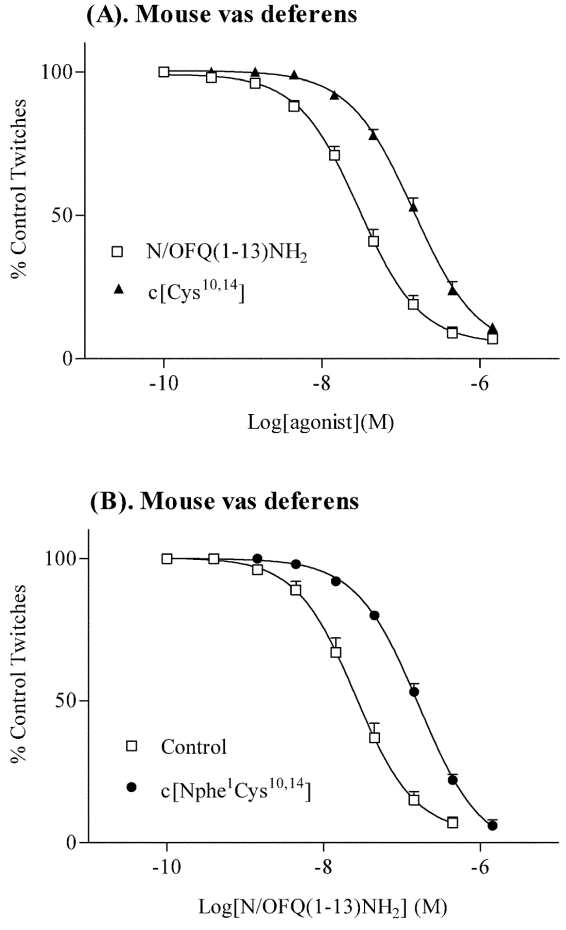

Fig. 6 **A** Comparison of N/OFQ(1–13)NH_2, c[$Cys^{10,14}$] actions in isolated mVD. **B** Antagonism of N/OFQ(1–13)NH_2 inhibition of the electrically evoked contractile response by c[$Nphe^1$, $Cys^{10,14}$]N/OFQ(1–14)NH_2 (10 µM). pEC_{50}, E_{max} and pK_B values are shown in Tables 3 and 4. Data are inhibition of twitch size (% twitches) and mean ± SEM for $n \geq 4$

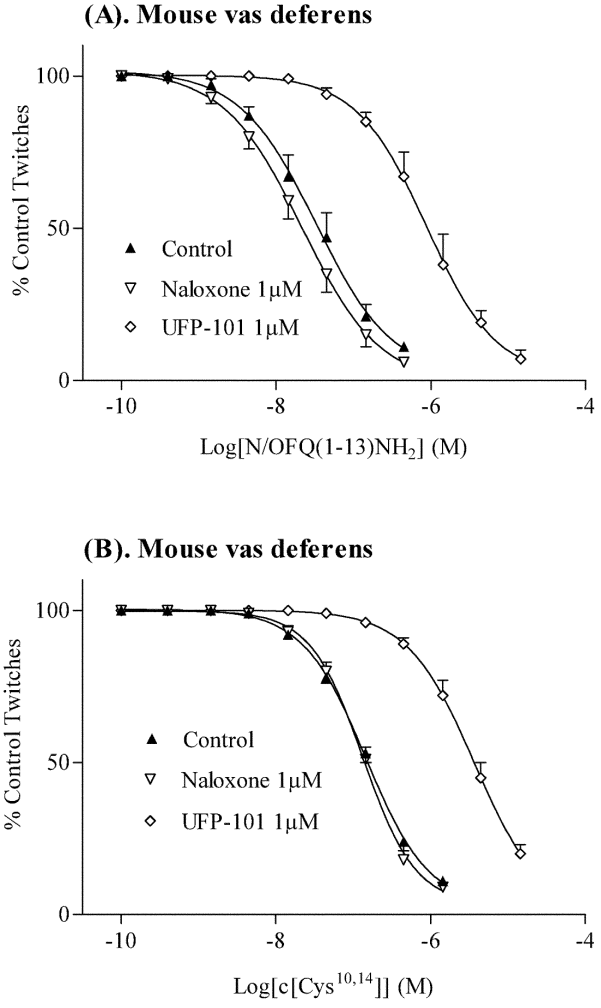

Fig. 7 Effects of naloxone (1 µM) and UFP-101 (1 µM) on **A** N/OFQ(1–13)NH_2 and **B** c[$Cys^{10,14}$] inhibition of electrically evoked contractions of the mouse vas deferens. pK_B values are summarised in Table 4. Data are mean ± SEM for $n \geq 4$

Table 3 Effects of N/OFQ(1–13)NH_2, c[$Cys^{10,14}$] and c[$Nphe^1$ $Cys^{10,14}$] in the electrically stimulated mouse vas deferens

Agonist	pEC_{50}	E_{max}(%)
N/OFQ(1–13)NH_2	7.47±0.05	–93±2
c[$Cys^{10,14}$]N/OFQ(1–14)NH_2	6.82±0.05	–89±1
c[$Nphe^1$, $Cys^{10,14}$]N/OFQ(1–14)NH_2	Inactive	

Data are mean ±SEM ($n \geq 4$)

Table 4 Effects of naloxone (1 µM), UFP-101 (1 µM) and c[$Nphe^1$ $Cys^{10,4}$]N/OFQ(1–14)NH_2(c[$Nphe^1$,$Cys^{10,14}$], 10 µM) on N/OFQ (1–13)NH_2, c[$Cys^{10,14}$]N/OFQ(1–14)NH_2 inhibitory actions in the electrically stimulated mouse vas deferens

Agonist	Naloxone pK_B	UFP-101 pK_B	c[$Nphe^1$,$Cys^{10,14}$] pK_B
N/OFQ(1–13)NH_2	<6	7.46±0.13	5.66±0.06
c[$Cys^{10,14}$]N/OFQ(1–14)NH_2	<6	7.37±0.07	Not determined

Data are mean ± SEM ($n \geq 4$)

deferens (mVD) assays c[$Cys^{10,14}$] displayed agonist activity. In the GTPγ^{35}S and mVD assays c[$Nphe^1Cys^{10,14}$] behaved as a competitive antagonist. However, in cAMP assays where signal amplification is apparent, this peptide behaved as an agonist (with reduced pEC_{50}). Aware of these tissue and assay differences we conclude that c[$Nphe^1Cys^{10,14}$] is most likely to be a partial agonist. The initial premise of this study was that cyclisation would produce a peptide with improved metabolic stability and therefore be of more use in vivo. However, in a series of crude metabolic stability studies cyclisation provided no increased enzymatic protection when compared with its amidated equivalent, N/OFQ-NH_2.

In radioligand binding studies c[$Cys^{10,14}$] shows a high degree of selectivity over classical MOP/DOP/KOP. Indeed this is confirmed with isolated tissue studies. In the mVD the effects of c[$Cys^{10,14}$] and N/OFQ(1–13)NH_2 are unaffected by the classical opioid receptor antagonist naloxone but competitively antagonised by the selective NOP

Table 5 Assessment of N/OFQ, N/OFQ-NH_2 and c[$Cys^{10,14}$]N/OFQ(1–14)NH_2 (c[$Cys^{10,14}$]) metabolism by rat cerebrocortical membranes. *ND* not determined

Agonist	Added (nM)	Measured (nM)	Concentration (nM) following incubation (% peptide loss)		
			15 min	60 min	120 min
N/OFQ	1.0	1.35±0.16	ND	ND	0.18±0.05* (87.5±1.7)
N/OFQ-NH_2	1.0	0.88±0.08	0.68±0.23 (19.0±8.7)	0.38±0.11 (53.7±2.6)	0.26±0.03*, ** (71.0±1.8)
c[$Cys^{10,14}$]	1.0	0.89±0.04	0.57±0.10* (42.2±9.2)	0.36±0.06* (64.1±4.2)	0.24±0.03*, ** (73.4±1.9)

Data are expressed as mean ± SEM ($n \geq 3$)
*$p<0.05$ vs. corresponding measured concentration
**$p<0.01$ vs. N/OFQ by ANOVA with Bonferroni correction. N/OFQ-NH_2 and c[$Cys^{10,14}$] whole time course $p<0.05$ ANOVA

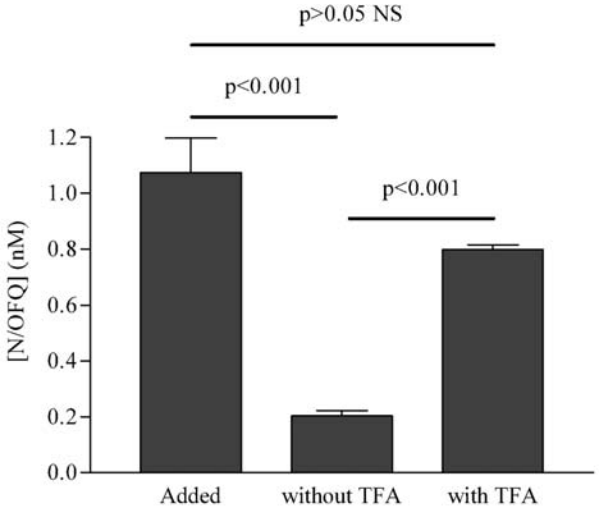

Fig. 8 Reducing buffer pH to 3.0 with trifluoroacetic acid (*TFA*) prevents metabolism of 1 nM (added) c[$Cys^{10,14}$]. Data are mean ± SEM for n=4

antagonist, UFP-101 with a pK_B (7.3) value consistent to that reported previously by us (Calo et al. 2002) thus demonstrating that the actions of both peptides are exclusively due to NOP receptor activation.

c[$Cys^{10,14}$] displays a small reduction in binding affinity that is likely to result from modification of the C-terminal address domain (responsible for receptor binding) of the peptide (Guerrini et al. 1997) where the cyclisation straddles Arg^{11} and Lys^{12}. The importance of these C-terminally located basic residues is confirmed though studies demonstrating N/OFQ(1–13)NH_2 as the shortest fragment that retains biological activity of the full length peptide and from creation of the first peptide with greater potency/affinity than N/OFQ, containing a triple Arg-Lys repeat motif (Okada et al. 2000). Indeed we have used this Arg-Lys triple repeat strategy to design UFP-101 (Calo et al. 2002). There was also a very small overall reduction in efficacy in the GTPγ^{35}S but not the amplified cAMP assay. This discrepancy is a common feature of this system (Bigoni et al. 2002). These data are largely consistent with the paper of Ambo et al. where c[$Cys^{10,14}$] was evaluated using NOP in HEK-293 cells (Ambo et al. 2001). In this brief article there was a small increase in binding affinity relative to N/OFQ(1–13)NH_2 and [$Cys^{10,14}$]N/OFQ(1–14)NH_2, the linear version of this cyclic peptide. In GTPγ^{35}S assays there was a small reduction in functional potency but all analogues were full agonists.

We have previously reported that [$Nphe^1$] substitution in the N/OFQ(1–13)NH_2 and [Arg^{14},Lys^{15}]N/OFQ-NH_2 sequences results in highly selective NOP antagonists with the latter molecule displaying improved affinity (Calo et al. 2000b, 2002; McDonald et al. 2003a). In this study we hoped to make a more metabolically stable cyclic antagonist, c[$Nphe^1Cys^{10,14}$]. In GTPγ^{35}S and mVD studies clear competitive but relatively low affinity antagonism was observed. However, there appeared to be some equilibration problems encountered with c[$Nphe^1Cys^{10,14}$] since in initial studies a slope <1 was obtained which was increased to unity by pre-incubation. Whilst we have no evidence to support this notion these data may suggest that cyclisation and [$Nphe^1$] substitution may modify the kinetics (on and/or off rates) of binding of c[$Nphe^1Cys^{10,14}$] to the receptor.

In GTPγ^{35}S and mouse vas deferens assays there was no indication of residual agonist activity with c[$Nphe^1Cys^{10,14}$] but in cAMP studies this peptide was an agonist, such that at the highest concentration tested there was an 80% inhibition of the forskolin stimulated response with a crudely estimated agonist potency (based on a theoretical 100% maximum inhibition) of 5.76.

It is worthy of note that the [$Nphe^1$] modification produces pure antagonists (Calo et al. 2000b) when applied to the linear templates N/OFQ(1–13)NH_2 and [Arg^{14},Lys^{15}] N/OFQ-NH_2 (Calo et al. 2002) but when applied to the cyclic template c[$Cys^{10,14}$] antagonism is observed in GTPγ^{35}S and mVD assays but agonism is observed in (amplified) cAMP assays (present data). This difference is not easy to interpret. However, the C-terminal region of N/OFQ (i.e. the address domain) can assume an alpha helix structure (Zhang et al. 2002). This is likely to be important for NOP binding particularly with the second extra-cellular loop which contains several acidic residues (Topham et al. 1998), reminiscent to that of dynorphin binding to KOP receptor (Paterlini et al. 1997). Cyclisation of the 10–14 region of the address domain may interfere with the ability to assume the alpha helix conformation and this may affect the relative spatial orientation between the message (where Phe^1 or $Nphe^1$ modification is located) and the address domains. These changes may be negligible for agonist activity while they may limit the

ability of the Nphe[1] modification to reduce efficacy possibly generating a partial agonist.

For a partial agonist pEC_{50} should predict pA_2/pK_B and in general pK_i should also predict pA_2/pK_B. In binding experiments c[Nphe[1]Cys[10,14]] displayed a pK_i of ~8 in GTPγ[35]S a pA_2 of ~7 and in cAMP studies a very rough estimate of pEC_{50} of ~5.8 can be obtained. This latter, pEC50 measured downstream in whole cells agrees well the pK_B value obtained in mVD of 5.7. These discrepancies and differences with accepted theory are common to this system and are likely to result from buffer and assay sensitivity differences (Calo et al. 2000c). For example, Na^+ would reduce binding affinity (Dooley and Houghten 2000) but this is necessary for GTPγ[35]S binding and cAMP/mVD assays use whole cells/tissues with relatively high concentrations of guanine nucleotides. Thus in comparison with the Na^+ free receptor binding system a reduced pA_2 in GTPγ[35]S might be predicted and further reductions in binding affinity for whole cells could also be anticipated. Indeed, we have discussed this previously (Calo et al. 2000c).

The high agonist activity of c[Nphe[1]Cys[10,14]] for inhibition of forskolin stimulated cAMP formation is worthy of a further contextual note. As cAMP formation is downstream in the signal transduction cascade this functional assay is liable to significant signal amplification. The net result of this being the creation of a receptor reserve, with partial agonists behaving as full agonists (Kenakin 2002; McDonald et al. 2003b). Indeed, we have previously reported that the partial agonists Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2 (Okawa et al. 1998) and [F/G]N/OFQ(1–13)NH_2 (Okawa et al. 1999) behave as full agonists in this assay system. Moreover, using an ecdysone inducible expression system for hNOP we were able to vary intrinsic activity of [F/G]N/OFQ(1–13)NH_2 relative to N/OFQ(1–13)NH_2 by increasing receptor density in cAMP but not GTPγ[35]S assays (McDonald et al. 2003b). Again these studies underscore the need for caution in defining pharmacology based on a single functional end point.

The initial premise of this cyclisation study was to generate a peptide with improved metabolic stability. In a crude attempt to assess metabolic stability we exposed the agonist template c[Cys[10,14]] to rat cerebrocortical membranes as a potential source of peptidases for increasing times. Peptide remaining in the supernatant was assessed using a simple competition binding "bioassay". There was a time dependent metabolism of N/OFQ, which could be reduced by amidation, but to our surprise cyclisation offered no additional protection. The drastic step of reducing buffer pH to 3.0 was able to prevent c[Cys[10,14]] metabolism. In essence, in this system the peptide did not have improved metabolic stability. There are several potential problems with this series of experiments in that we have not used N/OFQ(1–14)NH_2 or a linear version of c[Cys[10,14]] as reference standards although we feel it is unlikely that this will make a significant difference. However, and potentially of more concern is the source of metabolic "activity" (i.e., rat brain homogenate); is this a representative example of in vivo peptide metabolism? Certainly most of our in vivo studies use direct i.c.v. injection so in this respect this is a representative model. However, it might be useful to perform future studies with plasma as a representation of i.v. administration. Moreover, the real test is simple in vivo administration at both i.c.v. and i.v. sites.

In summary, we have reported that cyclisation at positions 10 and 14 produced an agonist with slightly reduced potency (pEC_{50}) in GTPγ[35]S and vas deferens assays and [Nphe[1]] substitution of that agonist, to yield c[Nphe[1]Cys[10,14]], produced an antagonist in GTPγ[35]S and vas deferens assays and an agonist in cAMP assays. With uncertainty regarding metabolic activity, in vivo studies are clearly warranted.

Acknowledgement The authors would like to thank the International Association for the Study of Pain for provision of a collaborative travel grant between Leicester (UK) and Ferrara (Italy).

References

Albrecht E, Samovilova NN, Oswald S, Baeger I, Berger H (1998) Nociceptin (orphanin FQ): high-affinity and high-capacity binding site coupled to low-potency stimulation of guanylyl-5'-O-(gamma-thio)-triphosphate binding in rat brain membranes. J Pharmacol Exp Ther 286:896–902

Ambo A, Hamazaki N, Yamada Y, Nakata E, Sasaki Y (2001) Structure-activity studies on nociceptin analogues: ORL1 receptor binding and biological activity of cyclic disulfide-containing analogues of nociceptin peptides. J Med Chem 44: 4015–4018

Annis I, Hargittai B, Barany G (1997) Disulfide bond formation in peptides. Methods Enzymol 289:198–221

Bigoni R, Giuliani S, Calo G, Rizzi A, Guerrini R, Salvadori S, Regoli D, Maggi CA (1999) Characterization of nociceptin receptors in the periphery: in vitro and in vivo studies. Naunyn-Schmiedebergs Arch Pharmacol 359:160–167

Bigoni R, Cao G, Rizzi A, Okawa H, Regoli D, Smart D, Lambert DG (2002) Effects of naloxone benzoylhydrazone on native and recombinant nociceptin/orphanin FQ receptors. Can J Physiol Pharmacol 80:407–412

Calo G, Rizzi A, Bogoni G, Neugebauer V, Salvadori S, Guerrini R, Bianchi C, Regoli D (1996) The mouse vas deferens: a pharmacological preparation sensitive to nociceptin. Eur J Pharmacol 311:R3–R5

Calo G, Bigoni R, Rizzi A, Guerrini R, Salvadori S, Regoli D (2000a) Nociceptin/orphanin FQ receptor ligands. Peptides 21: 935–947

Calo G, Guerrini R, Bigoni R, Rizzi A, Marzola G, Okawa H, Bianchi C, Lambert DG, Salvadori S, Regoli D (2000b) Characterization of [Nphe[1]]nociceptin(1–13)NH_2, a new selective nociceptin receptor antagonist. Br J Pharmacol 129:1183–1193

Calo G, Guerrini R, Rizzi A, Salvadori S, Regoli D (2000c) Pharmacology of nociceptin and its receptor: a novel therapeutic target. Br J Pharmacol 129:1261–1283

Calo G, Rizzi A, Rizzi D, Bigoni R, Guerrini R, Marzola G, Marti M, McDonald J, Morari M, Lambert DG, Salvadori S, Regoli D (2002) [Nphe[1],Arg[14],Lys[15]]Nociceptin-NH_2, a novel potent and selective antagonist of the nociceptin/orphanin FQ receptor. Br J Pharmacol 136:303–311

Dooley CT, Houghten RA (2000) Orphanin FQ/nociceptin receptor binding studies. Peptides 21:949–960

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77

Guerrini R, Calo G, Rizzi A, Bianchi C, Lazarus LH, Salvadori S, Temussi PA, Regoli D (1997) Address and message sequences for the nociceptin receptor: a structure-activity study of nociceptin-(1–13)-peptide amide. J Med Chem 40:1789–1793

Guerrini R, Calo G, Rizzi A, Bigoni R, Rizzi D, Regoli D, Salvadori S (2000) Structure-activity relationships of nociceptin and related peptides: comparison with dynorphin A. Peptides 21:923–933

Hashiba E, Harrison C, Calo G, Guerrini R, Rowbotham DJ, Smith G, Lambert DG (2001) Characterisation and comparison of novel ligands for the nociceptin/orphanin FQ receptor. Naunyn-Schmiedebergs Arch Pharmacol 363:28–33

Hashiba E, Lambert DG, Farkas J, Toth G, Smith G (2002a) Comparison of the binding of [(3)H]nociceptin/orphaninFQ(1–13)NH(2), [(3)H]nociceptin/orphaninFQ(1–17)OH and [(125)I]Tyr(14)nociceptin/orphaninFQ(1–17)OH to recombinant human and native rat cerebrocortical nociceptin/orphanin FQ receptors. Neurosci Lett 328:5–8

Hashiba E, Lambert DG, Jenck F, Wichmann J, Smith G (2002b) Characterisation of the non-peptide nociceptin receptor agonist, Ro64–6198 in Chinese hamster ovary cells expressing recombinant human nociceptin receptors. Life Sci 70:1719–1725

Kenakin T (2002) Drug efficacy at G protein-coupled receptors. Annu Rev Pharmacol Toxicol 42:349–379

Knoflach F, Reinscheid RK, Civelli O, Kemp JA (1996) Modulation of voltage-gated calcium channels by orphanin FQ in freshly dissociated hippocampal neurons. J Neurosci 16:6657–6664

Kramer TH, Toth G, Haaseth RC, Matsunaga TO, Davis P, Hruby VJ, Burks TF (1991) Influence of peptidase inhibitors on the apparent agonist potency of delta selective opioid peptides in vitro. Life Sci 48:881–886

McDonald J, Barnes TA, Calo G, Guerrini R, Rowbotham DJ, Lambert DG (2002) Effects of [(pF)Phe(4)]nociceptin/orphanin FQ-(1–13)NH(2) on GTPgamma35S binding and cAMP formation in Chinese hamster ovary cells expressing the human nociceptin/orphanin FQ receptor. Eur J Pharmacol 443:7–12

McDonald J, Calo G, Guerrini R, Lambert DG (2003a) UFP-101, a high affinity antagonist for the nociceptin/orphanin FQ receptor: radioligand and GTPgamma(35)S binding studies. Naunyn-Schmiedebergs Arch Pharmacol 367:183–187

McDonald J, Barnes TA, Okawa H, Williams J, Calo G, Rowbotham DJ, Lambert DG (2003b) Partial agonist behaviour depends upon the level of nociceptin/orphanin FQ receptor expression. Studies using the ecdysone inducible mammalian expression system. Br J Pharmacol 140:61–70

Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, Butour JL, Guillemot JC, Ferrara P, Monsarrat B et al (1995) Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature 377:532–535

Mogil JS, Pasternak GW (2001) The molecular and behavioral pharmacology of the orphanin FQ/nociceptin peptide and receptor family. Pharmacol Rev 53:381–415

Okada K, Sujaku T, Chuman Y, Nakashima R, Nose T, Costa T, Yamada Y, Yokoyama M, Nagahisa A, Shimohigashi Y (2000) Highly potent nociceptin analog containing the Arg-Lys triple repeat. Biochem Biophys Res Commun 278:493–498

Okawa H, Hirst RA, Smart D, McKnight AT, Lambert DG (1998) Rat central ORL-1 receptor uncouples from adenylyl cyclase during membrane preparation. Neurosci Lett 246:49–52

Okawa H, Nicol B, Bigoni R, Hirst RA, Calo G, Guerrini R, Rowbotham DJ, Smart D, McKnight AT, Lambert DG (1999) Comparison of the effects of [Phe1psi(CH2-NH)Gly2]nociceptin(1–13)NH2 in rat brain, rat vas deferens and CHO cells expressing recombinant human nociceptin receptors. Br J Pharmacol 127:123–130

Paterlini G, Portoghese PS, Ferguson DM (1997) Molecular simulation of dynorphin A-(1–10) binding to extracellular loop 2 of the kappa-opioid receptor. A model for receptor activation. J Med Chem 40:3254–3262

Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, Grandy DK, Langen H, Monsma Jr FJ, Civelli O (1995) Orphanin FQ: a neuropeptide that activates an opioid-like G protein-coupled receptor. Science 270:792–794

Terenius L, Sandin J, Sakurada T (2000) Nociceptin/orphanin FQ metabolism and bioactive metabolites. Peptides 21:919–922

Topham CM, Mouledous L, Poda G, Maigret B, Meunier JC (1998) Molecular modelling of the ORL1 receptor and its complex with nociceptin. Protein Eng 11:1163–1179

Vaughan CW, Christie MJ (1996) Increase by the ORL1 receptor (opioid receptor-like1) ligand, nociceptin, of inwardly rectifying K conductance in dorsal raphe nucleus neurones. Br J Pharmacol 117:1609–1611

Zhang C, Miller W, Valenzano KJ, Kyle DJ (2002) Novel, potent ORL-1 receptor agonist peptides containing alpha-Helix-promoting conformational constraints. J Med Chem 45:5280–5286