Functional selectivity of nociceptin/orphanin FQ peptide receptor partial agonists on cardiovascular and renal function


Department of Pharmacology and Experimental Therapeutics and the Cardiovascular and Neuroscience Centers of Excellence, Louisiana State University Health Sciences Center, New Orleans, LA 70112 (D.R.K., M.A.B., H.B.G., V.A.K.)

Department of Experimental and Clinical Medicine, Section of Pharmacology and Neuroscience Center, University of Ferrara, via Fossato di Mortara, 17, 44100 Ferrara, Italy (G.C.)

Department of Pharmaceutical Sciences and Biotechnology Center, University of Ferrara, via Fossato di Mortara, 17, 44100 Ferrara, Italy (R.G.)
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Corresponding Author Address:
Daniel R. Kapusta, Ph.D.
Department of Pharmacology and Experimental Therapeutics
Louisiana State University Health Sciences Center
1901 Perdido St.
New Orleans, LA 70112
Ph (504) 568-4740; FAX (504) 568-2361
E-mail: dkapus@lsuhsc.edu

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Abbreviations: N/OFQ, nociceptin/orphanin FQ; NOP receptor, nociceptin/orphanin FQ peptide receptor; [F/G], [Phe1ψ(CH2-NH)Gly2]N/OFQ(1-13)-NH2; HEX 1, Ac-RYYRIK-NH2; HEX 2, Ac-RYYRWK-NH2; ZP120, Ac-RYYRWKKKKKKK-NH2; i.c.v., intracerebroventricular; i.v., intravenous; UNaV, urinary sodium excretion; RSNA, renal sympathetic nerve activity; V, urine flow rate; ADH, antidiuretic hormone; CNS, central nervous system

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Abstract

The opioid-like peptide, nociceptin/Orphanin FQ (N/OFQ), produces marked cardiovascular and renal responses following central or peripheral administration in rats. Due to their ability to behave as full/partial agonists or antagonists in different cellular and tissue assays, the present studies were performed to determine how compounds classified as N/OFQ peptide (NOP) receptor partial agonists ([F/G]N/OFQ(1-13)-NH$_2$, Ac-RYYRIK-NH$_2$, Ac-RYYRWK-NH$_2$) affect cardiovascular and renal function in vivo. In conscious Sprague-Dawley rats, intracerebroventricular (i.c.v.) administration of each of the three NOP receptor ligands produced profound cardiovascular (depressor), renal excretory (water diuresis), and renal sympathetic nerve activity (inhibitory) responses that were similar to those produced by i.c.v. injection of the native ligand, N/OFQ. In contrast, in other groups of rats the intravenous (i.v.) bolus injection of these same NOP receptor ligands produced responses unlike N/OFQ; N/OFQ evoked an immediate and profound bradycardia and hypotension with no change in urine output whereas all purported NOP receptor partial agonists elicited a subtle slow onset hypotension, no change in heart rate and a marked water diuresis. In other studies, i.v. bolus pretreatment of rats with NOP receptor partial agonists prevented/attenuated the cardiovascular depressor effects produced by a subsequent i.v. bolus N/OFQ challenge without affecting the cardiovascular responses to i.c.v. N/OFQ. Together, these findings demonstrate that in conscious rats NOP receptor partial agonists produce functionally selective effects on cardiovascular and renal function ranging from full agonist (i.c.v., cardiovascular depressor; i.c.v. and i.v., water diuresis), partial agonist (i.v., sub maximal hypotension) to antagonist (i.v., blockade of N/OFQ-evoked bradycardia and hypotension) behavior.
Nociceptin/Orphanin FQ (N/OFQ), an opioid-like peptide containing 17 amino acids, is the endogenous ligand for the N/OFQ peptide receptor (NOP, also referred to as ORL1; Meunier et al., 1995; Reinscheid et al., 1995; Cox et al., 2000). N/OFQ has a high degree of structural similarity to that of endogenous opioid peptides with particular resemblance to dynorphin A(1-17), the native kappa opioid peptide (KOP) receptor ligand (Civelli et al., 1997). Despite sequence similarity, N/OFQ has no appreciable affinity for classical opioid receptors and instead selectively activates its own NOP receptors (Meunier et al., 2000). Upon activating NOP receptors N/OFQ modulates second messenger pathways via $G_{i/o}$ to alter neurotransmitter and hormonal release and elicit organ responses at central and peripheral levels (Hawes et al., 2000).

N/OFQ produces marked changes in cardiovascular and renal function in conscious or anesthetized rats (Kapusta, 2000; Salis et al., 2000; Malinowska et al., 2002). In this regard, intracerebroventricular (i.c.v.) injection of N/OFQ produces a dose-dependent bradycardia, hypotension, diuresis and antinatriuresis (Kapusta and Kenigs, 1999; Kapusta et al., 1997; 1999; 2002; Shirasaka et al., 1999). The cardiovascular depressor responses elicited by central N/OFQ occur shortly after administration (15 sec to 1 min) and are prolonged in duration (30-40 min). Central N/OFQ also evokes a concurrent and sustained (70-80 min) increase in urine flow rate and decrease in urinary sodium excretion; however, this free water diuresis is delayed in onset, commencing after arterial blood pressure and heart rate return toward control levels (i.e., approximately 30-min after i.c.v. peptide injection) (Kapusta and Kenigs, 1999; Kapusta et al., 1997; 1999, 2002). In comparison to these centrally evoked responses, i.v. bolus N/OFQ also produces a dose-dependent hypotension that occurs with bradycardia, and not a baroreflex-evoked tachycardia (Bigoni et al., 1999; Champion et al., 1997a,b; Madeeddu et al., 1999; Kapusta, 2000; Malinowska et al., 2000a, 2002). However, these cardiovascular depressor
responses are immediate in onset and of brief duration (3-10 min). Although it remains to be fully determined, the results of pilot studies suggest that i.v. bolus N/OFQ may not affect urine output (Kapusta, unpublished). Thus, the particular profile/pattern of cardiovascular and renal responses evoked by N/OFQ depends on the route in which this peptide is administered (i.c.v. versus i.v. bolus), presumably reflecting the different tissue/cell types that N/OFQ comes in contact with following distribution.

Since the discovery of N/OFQ, considerable effort has been made to develop new ligands that bind selectively to, but do not activate NOP receptors. These antagonist compounds are required to study the importance of the endogenous N/OFQ system in different biological processes. However, certain ligands which have been reported to have NOP receptor antagonist behavior have also been shown to exert additional pharmacological properties in different assays/preparations. For instance, the compounds [Phe1ψ(CH2-NH)Gly2]N/OFQ(1-13)-NH2 ([F/G], Guerrini et al., 1998); Ac-RYYRIK-NH2 (HEX 1) and Ac-RYYRWK-NH2 (HEX 2; Dooley et al., 1997) behave as full agonists, partial agonists or antagonists of NOP receptors depending on the in vitro or in vivo system studied (Calo’ et al., 2000). Despite their mixed pharmacological profiles these compounds continue to be classified and referred to (as they will be herein) as ‘partial agonists’ of the NOP receptor (Calo’ et al., 2000). Despite their unique and cellular/tissue/system-dependent pharmacological profile, it remains to be established how NOP receptor partial agonists affect cardiovascular and renal function in conscious rats as compared to the native ligand, N/OFQ. This is of particular interest since NOP receptors which are expressed in both central (brain, spinal cord) and peripheral tissues (vasculature, kidneys, heart, sympathetic and parasympathetic nerve terminals) (Mollereau and Mouledeous, et al., 2000; Malinowska et al., 2001; 2000b) may be affected differently by various classes of NOP receptor
ligands (e.g., N/OFQ and NOP receptor partial agonists) to influence cardiovascular and renal function.

With these considerations, the present studies examined the cardiovascular and renal responses produced by the central and peripheral administration of NOP receptor partial agonists in vivo. In particular, we compared the cardiovascular, renal excretory and renal sympathetic nerve responses produced by the i.c.v. (central) and i.v. bolus (peripheral) administration of N/OFQ or NOP receptor partial agonists in conscious Sprague-Dawley rats. The three partial agonists tested were [F/G], HEX 1 and HEX 2. Considering that these NOP receptor partial agonists can prevent the biological activities of N/OFQ in certain in vitro and in vivo systems (Calo’ et al., 1998; 2000), additional studies were performed to determine how the i.v. bolus pre-treatment of conscious rats with these NOP receptor ligands modified the cardiovascular responses to a subsequent i.v. bolus N/OFQ challenge.

Materials and Methods

Subjects. Male Sprague-Dawley rats (275-325 g, Harlan Sprague-Dawley Inc., Indianapolis, IN) were used in these studies. Rats were fed with normal sodium diets (Na+ content, 163 meq/kg) and were allowed tap water ad libitum. All procedures were conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Animals and were approved by the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee.
Surgery. For experiments requiring the i.c.v. administration of drugs, a stainless steel cannula was stereotaxically implanted into the right lateral cerebral ventricle of rats anesthetized with ketamine (40 mg/kg, i.m., Vedco Inc., St. Joseph, MO) in combination with xylazine (5 mg/kg, i.m.; Butler, Columbus, OH) at least 5-7 days prior to experimentation. The coordinates used to position the cannula were 0.3 mm posterior to the bregma, 1.3 mm lateral to midline, and 4.5 mm below skull surface (Paxinos and Watson, 1986). Verification of cannula position in the lateral ventricle was made by observation of cerebrospinal fluid flow from the implanted steel cannula after removal of the obturator or by observing injected dye in the lateral ventricle following completion of the study and subsequent postmortem brain section (Kapusta and Kenigs, 1999; Kapusta et al., 1997, 1999; 2002).

On the day of the study, rats were anesthetized with sodium methohexital (75 mg/kg, i.p., and supplemented with 10 mg/kg, i.v. as needed; King Pharmaceuticals, Bristol, TN) and instrumented with chronic catheters in the left femoral artery and vein (PE-10 connected to PE-50; Becton Dickinson and Company; Sparks, MD) and urinary bladder (flanged PE-240; Becton Dickinson and Company) for measurement of arterial blood pressure, administration of drugs/saline, and collection of urine, respectively. In certain studies, the rat (still anesthetized) was also implanted with a recording electrode (bipolar platinum wire; Cooner Wire Company, Chatsworth, CA) to a renal nerve branch to measure renal sympathetic nerve activity using standard techniques previously described (Kapusta and Kenigs, 1999; Kapusta et al., 1997; 1999; 2002).

Following surgical preparation, rats were placed in a rat holder (a chamber with Plexiglas ends connected by stainless steel rods) which permits forward and backward movement of the rat, allows for collection of urine, and when appropriate protects the renal nerve recording
preparation. An i.v. infusion of isotonic saline (55 µl/min) was then started and continued for the duration of the experiment. The experimental protocol commenced after rats regained full consciousness and cardiovascular and renal excretory function stabilized. Heart rate was derived from the pulse pressure with a tachograph (model 7 P4H; Grass Instruments, Quincy, MA). Heart rate and arterial pressure were continuously monitored and recorded on a Grass polygraph (model 7).

**Experimental protocols.**

**I.c.v. microinjection studies.** Studies were performed to determine the cardiovascular and renal excretory responses produced by the i.c.v. microinjection (i.e., central nervous system administration) of N/OFQ or NOP receptor partial agonists in conscious rats. In certain animals, changes in renal sympathetic nerve activity were also measured. After stabilization of cardiovascular and renal excretory function (4-6 hours) urine was collected during a 20-min control period. Groups of conscious rats then received i.c.v. microinjection of one of the NOP receptor partial agonists: HEX 1 (1 nmol = 1 µg; n=5); HEX 2 (0.73 nmol = 1 µg; n=6) or [F/G] (7.3 nmol = 10 µg; n=9). For comparative purposes, other groups of rats received either i.c.v. injection of the endogenous ligand N/OFQ (5.5 nmol = 10 µg, i.c.v.) or isotonic saline vehicle (5 µl). Immediately after i.c.v. microinjection of drug/vehicle, urine was collected for 80-min during eight consecutive 10-min experimental urine samples.

The i.c.v. doses of NOP receptor ligands used in the current studies were based on the following observations. In previous i.c.v. dose-response studies performed under similar experimental conditions we have established that N/OFQ administered to rats over a range of 0.55, 5.5 and 16.5 nmol produced relatively comparable cardiovascular depressor responses, but
a maximal diuresis at the 5.5 nmol (10 µg) dose (Kapusta et al., 1997). Similarly, in i.c.v. dose-response studies with [F/G], we have shown that a dose of 7.3 nmol (10 µg) was the minimally effective dose to produce marked changes in cardiovascular and renal function. Thus, dose-response studies with N/OFQ and [F/G] were not repeated and the 5.5 nmol and 7.3 nmol i.c.v. doses of N/OFQ and [F/G], respectively, were used in the current studies. In pilot i.c.v. dose response studies it was then established that HEX 1 and HEX 2 were considerably more potent than either N/OFQ or [F/G] in altering cardiovascular and renal function. Based on findings of these studies it was determined that the minimally effective i.c.v. dose of HEX 1 and HEX 2 to produce marked changes in cardiovascular and renal function was 1 nmol (1 µg) and 0.73 nmol (1 µg), respectively. In each of the i.c.v. dose-response studies noted above it was observed that higher i.c.v. doses of each NOP receptor ligand produced a qualitatively similar pattern of cardiovascular depressor and water diuretic properties, thus demonstrating that i.c.v. dose-responsiveness was not bimodal. However, at doses higher than those used in the current studies each NOP receptor ligand often produced sedation.

**Single dose i.v. bolus studies.** Studies were performed to determine the cardiovascular, renal excretory, and in certain studies the renal sympathetic nerve activity responses produced by the i.v. bolus injection (i.e., peripheral administration) of N/OFQ or NOP receptor partial agonists of conscious rats. After equilibration and collection of a control urine sample (20-min), groups of rats received an i.v. bolus injection of one of the following NOP receptor partial agonists: HEX 1 (100 nmol/kg; n=6), HEX 2 (100 nmol/kg; n=6) or [F/G] (900 nmol/kg, n=6). For comparative purposes, other groups of rats received either an i.v. bolus injection of the endogenous ligand N/OFQ (30 or 100 nmol/kg) or isotonic saline vehicle (200
μl). Immediately after i.v. bolus drug/vehicle injection, urine was collected for 80-min during eight consecutive 10-min experimental urine samples.

The doses for HEX 1 (100 nmol/kg), HEX 2 (100 nmol./kg) and [F/G] (900 nmol/kg) used in the single dose i.v. bolus studies were selected based on results of pilot studies which demonstrated that these were the minimally effective doses of each NOP receptor ligand to produce a significant increase in urine flow rate and decrease in urinary sodium excretion. N/OFQ was administered at 30 or 100 nmol/kg, i.v. since we and other investigators have previously shown that at these doses N/OFQ elicits immediate and profound cardiovascular depressor responses.

I.v. bolus dose-response studies with HEX 1. Additional studies were performed to explore the full i.v. bolus dose-response relationship of the NOP receptor partial agonist, HEX 1, on cardiovascular and renal function. For these studies, the same experimental protocol as described above for the single i.v. bolus dose HEX 1 (100 nmol/kg) study was repeated, with the exception that groups of rats received HEX 1 at i.v. bolus doses of 30 (n=4), 300 (n=5) or 900 (n=5) nmol/kg.

Cardiovascular antagonist studies. Studies were performed to determine whether i.v. bolus pretreatment of conscious rats with the NOP receptor ligands, HEX 1, HEX 2 or [F/G] modified the cardiovascular responses to a subsequent i.v. bolus N/OFQ challenge. Renal excretory function was not measured in these studies. After stabilization, baseline (control) cardiovascular (heart rate, pulsatile and mean arterial pressure) function was measured for 20-min. Rats then received an i.v. bolus pretreatment (200 μl) of the NOP receptor ligand HEX 1
(100 nmol/kg; n=8), HEX 2 (100 nmol/kg; n=6), [F/G] (900 nmol/kg; n=6) or isotonic saline vehicle (n=6). Following 5-min drug/vehicle distribution, the rats then received an i.v. bolus injection of N/OFQ (100 nmol/kg for studies with HEX 1, HEX 2 or saline pretreatment; 30 nmol/kg for studies with [F/G] or saline pretreatment). The cardiovascular responses evoked by the i.v. bolus N/OFQ challenge were then measured for 15 min. In other studies the cardiovascular responses to i.v. bolus N/OFQ (100 nmol/kg) were examined in separate rats pretreated with HEX 1 or HEX 2 (100 nmol/kg, i.v.) for longer pretreatment times (30 or 60 min). The i.v. bolus pretreatment doses for NOP receptor partial agonists used in these studies were derived from pilot studies and in the cases of HEX 2 and [F/G] were shown to be the minimally effective doses of each ligand to significantly increase urine output.

**Analytic procedures.** Urine volume was determined gravimetrically. Urine sodium concentration was measured by flame photometry (model 943, Instrumentation Laboratories, Lexington, MA, USA). Data acquisition for renal sympathetic nerve activity measurements was performed with a commercially available software package (Acknowledge for Windows, Biopac Inc., Santa Barbara, CA). Integrated renal sympathetic nerve activity was expressed as microvolt-seconds per 1-sec intervals. For each 10-min experimental period, the values for integrated renal sympathetic nerve activity were sampled over the entire collection period and the numbers were averaged. Because of the limitations of comparing values for multifiber renal sympathetic nerve activity between animals, the data are expressed as the percent control with the control values for each animal taken as 100% (Kapusta and Kenigs, 1999; Kapusta et al., 1999; 2002).
Drugs. N/OFQ was obtained from Phoenix Pharmaceuticals (Belmont, CA, USA). Ac-RYYRIK-NH₂ (HEX 1), Ac-RYYRWK-NH₂ (HEX 2) and [Phe¹ψ(CH₂-NH)Gly²]N/OFQ(1-13)-NH₂ ([F/G]) were synthesized and provided by the laboratories of Professors R. Guerrini and S. Salvadori, Department of Pharmaceutical Sciences of the University of Ferrara, Ferrara, Italy. Stock solutions of N/OFQ and NOP receptor partial agonists used for i.v. and i.c.v. administration were prepared fresh in isotonic saline and stored frozen. Injection of drugs in isotonic saline vehicle (5 µl) into the lateral cerebroventricle of conscious rats was made via a 10 µl Hamilton syringe (Hamilton, Reno, NV, USA).

Data Analysis

Data are expressed as means ± standard error of the mean of n experiments. Statistical comparisons were made as follows. A non-repeated one-way ANOVA and posthoc Dunnett’s multiple comparison test was used to compare peak changes (delta) in cardiovascular and renal function which were produced by different NOP receptor ligands in separate groups of animals from the control group (vehicle) value. For two groups, this comparison was made by Student’s t test for paired and unpaired observations as appropriate. In the time course study, changes in physiological parameters at different time points within a group were compared to respective group control values by using a one-way ANOVA for repeated measures and posthoc Dunnett’s multiple comparison test. In each case, a P value < 0.05 was considered to be significant.
Results

We have previously reported that, in conscious rats, the i.c.v. microinjection of N/OFQ (5.5 nmol) produces bradycardia, hypotension, renal sympathoinhibition and water diuresis (Kapusta and Kenigs, 1999; Kapusta et al., 1997; 1999; 2002).

Figure 1 depicts the peak changes in cardiovascular and renal excretory function produced by the i.c.v. injection of N/OFQ (5.5 nmol = 10 µg) and each of the purported NOP receptor partial agonists, [F/G] (7.3 nmol = 10 µg), HEX 1 (1.0 nmol = 1 µg) or HEX 2 (0.73 nmol = 1 µg) in conscious Sprague-Dawley rats (see Experimental Methods for basis for drug doses). Cardiovascular and renal excretory function was measured in each rat during control (pre-drug baseline values for each animal) and immediately following i.c.v. drug injection for 80-min (consecutive 10-min experimental periods). Group data are peak (mean ±SE) changes from control in each cardiovascular and renal parameter that occurred over the 80-min period following the i.c.v. injection of isotonic saline vehicle, N/OFQ, or each NOP receptor ligand.

As illustrated (Fig. 1), the i.c.v. injection of isotonic saline vehicle failed to alter any cardiovascular or renal excretory parameter over the course of the experiment (80 min). In contrast, in conscious rats, i.c.v. injection of N/OFQ as well as [F/G], HEX 1 and HEX 2 each produced significant decreases in heart rate, mean arterial pressure and urinary sodium excretion and a profound increase in urine flow rate. Although not depicted, the pattern and magnitude of cardiovascular (onset, approx. 30 sec to 2-min; peak nadir, 10-20 min; duration, 40-50 min) and renal responses (onset, approx. 30 min; peak, 40-50 min, duration, 70-80 min) produced by the i.c.v. injection of [F/G], HEX 1 and HEX 2 were highly comparable and essentially the same as those produced by N/OFQ in this and previous studies (Kapusta et al., 1997; 1999). Similar to i.c.v. N/OFQ, it was also confirmed that the central injection of each of the 3 purported NOP
receptor partial agonists inhibited renal sympathetic nerve activity (data not shown). In the current studies HEX 1 and HEX 2 were each administered at a lower dose of 1.0 µg (1.0 nmol and 0.73 nmol, resp.) because preliminary findings revealed that the i.c.v. injection of 10-fold higher doses produced marked sedation. However, at these higher doses each NOP receptor ligand still produced cardiovascular, renal excretory and renal sympathetic nerve activity responses that were similar in pattern/direction as those elicited by i.c.v. N/OFQ (Kapusta et al., 1997; 1999, 2000, 2002; Kapusta and Kenigs, 1999).

Figure 2 depicts the peak changes in cardiovascular (Figs. 2A and 2B) and renal excretory function (Fig. 2C) produced by the i.v. bolus injection of N/OFQ and the purported NOP receptor partial agonists in conscious Sprague-Dawley rats. Cardiovascular and renal function was measured in each rat during control and immediately following i.v. bolus drug injection for 80-min (consecutive 10-min experimental periods). In these studies it was demonstrated that there were differences in time to onset and peak effects for N/OFQ (rapid onset and short duration) versus the other NOP receptor ligands (slow onset and long duration). As such, cardiovascular data are presented as peak changes (mean ± SE) from control that occurred within the first 10 minutes immediately following i.v. bolus drug/saline injection (Fig. 2A) and the peak changes that were observed over the remaining 70-min protocol (Fig. 2B; time points 20-80 min). As shown in Fig. 2C, data for urine flow rate and urinary sodium excretion are the peak changes from pre-drug baseline control that occurred over the entire 80-min study (consecutive 10-min periods).

Baseline cardiovascular and renal function in groups of rats treated with N/OFQ or each NOP receptor partial agonists (Figs. 2A – 2C) were not significantly different than those observed in isotonic saline-treated animals (HR, 417±16 bpm; MAP, 117±5 mmHg; V, 54±8
µl/min; UNaV, 8.3±0.6 µeq/min). Following i.v. bolus injection, N/OFQ (100 nmol/kg) produced an immediate and marked reduction in heart rate and mean arterial pressure (Fig. 2A) with these responses returning to, and remaining at, pre-drug control levels approximately 10-min post injection (Fig. 2B). In comparison to N/OFQ, the i.v. bolus injection of [F/G] (900 nmol/kg), HEX 1 (100 nmol/kg) or HEX 2 (100 nmol/kg) produced a substantially different cardiovascular response profile. For instance, each of these 3 NOP receptor ligands failed to produce an immediate and marked bradycardia or hypotension following i.v. bolus injection (Fig. 2A). In fact, at this dose (100 nmol/kg, i.v.) these ligands did not significantly alter baseline cardiovascular function over the first 10-min following administration. However, by approximately 10-15 min following injection each of these ligands produced a subtle (but statistically significant) hypotensive response (ranging from 10 to 20 mmHg) that persisted for 60-70 min (Fig. 2B; periods 10-80 min; see Fig. 3 for time course data for HEX 1). Fig. 2C demonstrates that N/OFQ and the purported NOP receptor partial agonists also differed in their ability to affect urine output, but not urinary sodium excretion. Thus, i.v. N/OFQ failed to increase urine flow rate over the entire experimental period (80 min) at this (100 nmol/kg) or other doses (10, 30 or 300 nmol/kg, data not shown) tested. In contrast, at an equivalent dose (100 nmol/kg) i.v. bolus injection of [F/G], HEX 1 and HEX 2 each produced a significant diuresis over the 80-min study (Fig. 2C, and see Fig. 3 for time course data for HEX 1). The peak increases in urine flow rate and urinary sodium produced by [F/G] and HEX 2 occurred 30-min after injection, whereas peak renal excretory responses to HEX 1 were evident by 40-min. The renal excretory responses produced by [F/G] and HEX 2 were fully recovered by 50-min (data not shown) after drug injection, whereas changes elicited by HEX 1 returned to control levels by 60-min (Fig 3). Note that the changes in renal excretory function depicted in Fig. 2C do
not reflect the potential maximum diuretic or antinatriuretic responses evoked by each NOP receptor partial agonist (i.e., as may be obtained from dose-response studies; see Fig. 3), but simply illustrate that at an i.v. bolus dose of 100 nmol/kg or above (e.g., doses in which N/OFQ elicits marked cardiovascular depressor effects), these NOP receptor ligands elicited an entirely different profile of cardiovascular and renal responses than N/OFQ. Finally, in certain animals it was observed that immediately following i.v. injection each NOP receptor ligand produced a marked increase (spike and plateau) in renal sympathetic nerve activity, a response similar to that elicited by i.v. bolus N/OFQ, but in opposition to the renal sympathoinhibitory response that occurred in other rats when the same drug was injected into the brain (data not shown).

Additional studies were performed to thoroughly investigate whether a high i.v. bolus dose of a purported NOP receptor partial agonist would elicit changes in cardiovascular or renal function similar to i.v. bolus N/OFQ. The results of these dose-response studies are shown in Figure 3 which illustrates the time course cardiovascular and renal responses produced by increasing i.v. bolus doses of HEX 1 in conscious Sprague-Dawley rats. As compared to each group control value, each i.v. bolus dose of HEX 1 (30, 100, 300, and 900 nmol/kg) produced significant changes in mean arterial pressure, urine flow rate, and urinary sodium excretion (for clarity, asterisks denoting statistically significant differences, p<0.05, within each group versus each group’s control value have been omitted). In particular, as compared to respective group control values (C) the i.v. injection of HEX 1 at each doses tested produced a gradual but significant (p<0.05) reduction in mean arterial pressure without altering heart rate. However, even at the highest dose tested (900 nmol/kg, i.v.) the pattern of the hypotensive response produced by HEX 1 (Fig. 3) was substantially different than the immediate but transient decrease in mean arterial pressure elicited by N/OFQ (30 or 100 nmol/kg; Figs, 2A, 2B, 4A and 4B).
Concurrent with the slight (30, 100 or 300 nmol/kg) or moderate (900 nmol/kg) hypotension, increasing i.v. bolus doses of HEX 1 also produced increases in urine flow rate and decreases in urinary sodium excretion (Fig. 3). HEX 1 produced a maximal diuretic response (50 min; Δ 151±6 µl/min) at an i.v. bolus dose of 300 nmol/kg.

Figures 4A and 4B illustrate the changes in heart rate and mean arterial pressure produced by i.v. bolus N/OFQ in conscious rats pretreated i.v. with a NOP receptor partial agonist (same dose of NOP ligands as that used in studies depicted in Figs. 2A-C). Values are peak changes (means ± SE) in mean arterial pressure and heart rate (from control pre-drug baseline values for each animal) produced by N/OFQ (30 nmol/kg, i.v.) in rats pretreated (5-min) with isotonic saline vehicle or [F/G] (900 nmol/kg, i.v.; Fig. 4A), or N/OFQ (100 nmol/kg, i.v.) in rats pretreated (5-min) with isotonic saline vehicle, HEX 1 (100 nmol/kg, i.v.; Fig. 4B) or HEX 2 (100 nmol/kg, i.v.; Fig. 4B). As shown, in isotonic saline vehicle pretreated rats (which itself caused no changes), i.v. bolus N/OFQ (Fig. 4A, 30 nmol/kg; Fig. 4B, 100 nmol/kg) produced marked hypotension and bradycardia. The decreases in mean arterial pressure and heart rate produced by each i.v. bolus dose of N/OFQ were immediate in onset and persisted for approximately 5 to 10 min. In contrast to the N/OFQ-evoked responses, the i.v. bolus injection of [F/G] (Fig. 4A), HEX 1 or HEX 2 (Fig. 4B) did not significantly alter mean arterial pressure or heart rate over the same time period studied. Further, in the same rats pretreated (5-min) i.v. with [F/G] (Fig. 4A), HEX 1 or HEX 2 (Fig 4B), the characteristic and immediate hypotensive and bradycardic responses to an i.v. bolus N/OFQ challenge were either abolished ([F/G] and HEX 1) or attenuated (HEX 2). In other studies, it was observed that the hypotensive and bradycardia responses to i.v. bolus N/OFQ (100 nmol/kg) were not blocked by HEX 2 or [F/G] with a longer
pretreatment time of 30-min (data not shown). However, after a 60-min pretreatment period, HEX 1 still blocked the cardiovascular depressor responses to i.v. N/OFQ.

At all the doses tested (Figs 1-4), the i.v. bolus injection of N/OFQ, [F/G], HEX 1 or HEX 2 did not produce any apparent behavioral/CNS (e.g., agitation, gnawing, licking, exploratory) or sedative/catatonic effects over the course of the experimental protocol.

Discussion

The findings of these studies demonstrate that in conscious rats, NOP receptor partial agonists are functionally selective ligands which affect cardiovascular and renal function differently depending on the route of administration of the compound. When administered into the brain (i.c.v.), all NOP receptor ligands tested (e.g., [F/G], HEX 1, HEX 2) behaved as full agonists, highly mimicking the effects of N/OFQ and producing marked cardiovascular (bradycardia, hypotension) and renal (diuresis, antinatriuresis, renal sympathoinhibition) responses. In contrast, following peripheral (i.v. bolus) injection, these same NOP receptor ligands elicited a complex pharmacological and physiological profile that was different from that of N/OFQ and which ranged from full agonist (water diuresis), partial agonist (sub maximal hypotension without altering heart rate) to antagonist (blockade of N/OFQ-evoked bradycardia and hypotension) behavior.

The compounds [F/G], HEX 1 and HEX 2 are recognized to behave functionally as NOP receptor partial agonists with varying degrees of efficacy in different biological model systems. However, there is now considerable evidence that each of these ligands can each behave as pure antagonists (Guerrini et al., 1998; Bigoni et al., 1999; Madeddu et al., 1999, Berger et al., 2000; Malinowska et al., 2000b), partial agonists (Dooley et al., 1997; Bigoni et al., 1999; Berger et al.,
2000; Calo’ et al., 2000) or full agonists (Butour et al., 1998; Kapusta et al., 1999; Berger et al., 2000; Malinowska et al., 2000a; Olszewski et al., 2000) across different in vitro and in vivo systems (see Calo’ et al, 2000 for review). The results of the present studies extend these observations and clearly demonstrate that these same NOP receptor ligands can display mixed pharmacological behavior from full/partial agonist to antagonist activity within a given biological system, in this case involving processes regulating cardiovascular and renal function in vivo.

As we previously reported for [F/G] (Kapusta et al., 1999), the i.c.v. injection of all 3 NOP receptor ligands in conscious rats produced cardiovascular depressor and water diuretic responses that were similar in pattern and magnitude to those elicited by N/OFQ, thus behaving as full agonists. Although not tested, it is likely that these NOP receptor ligands acted centrally to affect cardiovascular and renal function in a manner similar to N/OFQ through modulation of central neural (sympathetic inhibitory and parasympathetic stimulatory) outflow (Giuliani et al., 1997; Kapusta et al., 1999; Kapusta et al., 2002 Shirasaka et al., 1999) and inhibition of ADH release (Kapusta, 2000; Kakiya et al., 2000). In contrast to their responses in the brain, when [F/G], HEX 1 and HEX 2 were administered into the periphery as an i.v. bolus injection these compounds did not alter heart rate and only gradually and slightly reduced mean arterial pressure. These observations are of interest considering that N/OFQ (30 or 100 nmol/kg) characteristically produces an immediate and marked bradycardia and hypotension following i.v. bolus injection in conscious or anesthetized rats (Bigoni et al., 1999; Champion et al., 1997a,b; Madeddu et al., 1999; Kapusta, 2000; Malinowska et al., 2000a, 2002). However, as partial agonists it might be expected that these ligands are required to bind to and activate more receptors to elicit an equal response. This possibility was excluded though, since in dose-
response studies even the highest dose of the NOP receptor partial agonist tested (HEX 1; 900 nmol/kg, i.v.) did not affect mean arterial pressure or heart rate in a fashion similar to that elicited by i.v. bolus N/OFQ. While the mechanism(s) by which N/OFQ and NOP receptor partial agonists produce different cardiovascular responses remains to be determined, it is possible that anesthesia may influence this pathway. This is suggested since, in urethane-anesthetized rats, [F/G] and HEX 1 produced a significant bradycardia and hypotension (Malinowska et al., 2000a).

In the current studies the i.v. bolus pretreatment (5-min) of rats with [F/G], HEX 1 and HEX 2 prevented/attenuated the hypotension and bradycardia typically evoked by an i.v. bolus N/OFQ challenge. These findings demonstrate that these NOP receptor ligands also display antagonist activity and can block the peripheral mechanisms by which N/OFQ affects heart rate and mean arterial pressure. Similarly, Madeddu and colleagues (1999) demonstrated that, in conscious mice, i.v. bolus [F/G] did not alter mean arterial pressure or heart rate but prevented the hypotension, bradycardia and increase in aortic blood flow evoked by N/OFQ. Of interest, we demonstrated that at a time in which i.v. bolus pre-treatment of rats with HEX 1 completely blocked the cardiovascular responses to i.v. bolus N/OFQ, the cardiovascular depressor responses to central (i.c.v.) injection of N/OFQ remained intact. While it remains to be tested, this observation provides support for separate central versus peripheral NOP receptor systems that control cardiovascular function.

In contrast to their partial agonist (sub maximal hypotension) and antagonist effects on cardiovascular function, when administered alone i.v. bolus [F/G], HEX 1 and HEX 2 produced significant diuretic and antinatriuretic responses (i.e., water diuresis). The renal responses to these ligands were unexpected and of merit considering that at all i.v. bolus doses of N/OFQ (30,
100 or 300 nmol/kg) tested did not itself affect urine flow rate. Of interest though, in previous studies we showed that N/OFQ is effective in producing a water diuresis when administered as a continuous i.v. infusion (Kapusta et al., 1997; Kapusta, 2000). Moreover, during i.v. infusion N/OFQ (low or high dose) does not elicit any marked cardiovascular responses and instead produces only a subtle reduction in mean arterial pressure with no change in heart rate (Kapusta et al., 1997; Kapusta, 2000), these being cardiovascular responses similar to those elicited by i.v. bolus injection of NOP receptor partial agonists (present study). While the mechanisms have yet to be explored, it is apparent that by altering the method of i.v. drug administration (infusion versus bolus injection) the pattern of cardiovascular and renal excretory responses produced by N/OFQ can change and can highly mimic that elicited by the i.v. bolus injection of the NOP receptor partial agonists.

At present, the mechanisms by which purported NOP receptor partial agonists produce functionally selective effects on cardiovascular and renal function remain unknown. Different subtypes of NOP receptors in central versus peripheral tissues have been speculated, but not reported to exist. Alternatively, the NOP receptor density and stimulus/response coupling efficiency in various tissues (e.g., potentially high in the CNS and low in peripheral tissues) may influence the degree of agonism of the three low efficacy agonists (i.e., partial agonists) used in this study (Berger et al., 2000; McDonald et al., 2003). This premise is supported by studies which have used an ecdysone inducible expression system showing that partial agonist behavior of a ligand is dependent upon the level of NOP receptor expression (McDonald et al., 2003). Together, our data strongly indicates that there exists agonist-specific regulation of the NOP receptor. Structure-activity studies have suggested that the mode of binding between the hexapeptide HEX 1 and the NOP receptor is different than that between N/OFQ and NOP.
receptors (Kawano et al., 2002). In fact, using photo-affinity labeling, Bes and Meunier (2003) have identified a hexapeptide binding site within the NOP receptor that is physically remote and distinct from the region in which N/OFQ binds. As suggested by these investigators, it is likely that the ability of the hexapeptides and N/OFQ to interact with the NOP receptor in different ways (i.e., affects on G-protein coupling and second messenger pathways) is consistent with their distinct pharmacological activities (Bes and Meunier, 2003). Of interest, Corbani and colleagues (2004) demonstrated that the full agonists N/OFQ and Ro64-6198 induced rapid internalization of the hNOP receptor, while antagonists and the partial agonist Ac-RYYRWR-NH₂ had little effect on internalization. These findings are of merit in that N/OFQ failed to alter urine flow rate at any i.v. bolus dose tested. Based on the observations by Corbani et al. (2004) the initiation/onset of the renal excretory responses to NOP receptor partial agonists may be related to sustained ligand receptor binding/coupling and less potential to induce desensitization than full NOP receptor agonists such as N/OFQ. Instead, the duration of the renal excretory responses may be related to the dose and/or metabolic stability of the NOP receptor ligand (see Kapusta et al., companion paper). Finally, it remains to be determined whether different NOP receptor partial agonists undergo tissue specific metabolism similar to N/OFQ (Terenius et al., 2000) and whether potential active fragments affect cardiovascular and renal function by NOP receptor dependent or independent pathways.

In summary, we demonstrated that in conscious rats NOP receptor partial agonists produced functionally selective effects on cardiovascular and renal function ranging from full agonist (i.c.v., cardiovascular depressor; i.c.v. and i.v., water diuresis), partial agonist (i.v., sub maximal hypotension without altering heart rate) to antagonist (i.v., blockade of N/OFQ-evoked bradycardia and hypotension) behavior. Based on their ability to produce a selective water
diuresis following i.v. bolus injection without apparent adverse cardiovascular or CNS effects, we propose that metabolically stable NOP receptor partial agonists (e.g., ZP120; Kapusta et al., companion paper) may be useful therapeutically as novel peripherally-acting aquaretics for the acute management of severe water retention and/or hyponatremia.
References

Butour JL, Moisand C, Mollereau C and Meunier JC (1998) [Phe\(^{1}\)ψ(CH\(_{2}\)-NH)Gly\(^{2}\)]nociceptin(1-13)-NH\(_{2}\) is an agonist of the nociceptin (ORL1) receptor. *Eur J Pharmacol* 349:R5-R6.


Champion HC and Kadowitz PJ (1997b) Nociceptin, an endogenous ligand for the ORL1 receptor, has novel hypotensive activity in the rat. Life Sciences 60:PL241-245.


Footnotes

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**Figure Legends**

**Fig. 1.** Peak changes in cardiovascular and renal function produced by i.c.v. injection of N/OFQ and NOP receptor partial agonists in conscious Sprague-Dawley rats. Values are means ± S.E. and illustrate the peak (maximum) changes from control (pre-drug baseline values) in cardiovascular and renal excretory function produced by the i.c.v. injection of isotonic saline vehicle (5 µl; n=8), N/OFQ (5.5 nmol; n=10) or the NOP receptor partial agonists [F/G] ([F/G]N/OFQ(1-13)-NH₂; 7.3 nmol; n=9), HEX 1 (Ac-RYYRIK-NH₂; 1.0 nmol; n=5) or HEX 2 (Ac-RYYRWK-NH₂; 0.73 nmol; n=6). Cardiovascular and renal function was measured in each rat during control (20-min) and immediately following i.c.v. drug injection for 80-min (consecutive 10-min experimental periods). HR, heart rate; MAP, mean arterial pressure; V, urine flow rate; UNaV, urinary sodium excretion. *P<0.05, significantly different from saline-treated group. Basal (predrug/vehicle) control values for each parameter were not significantly different between groups. Basal control values for the saline-treated group were: HR, 412±12 bpm; MAP, 121±4 mmHg; V, 46±7 µl/min; UNaV, 7.9±0.6 µeq/min.
Fig. 2. Peak changes in cardiovascular (Fig. 2A and 2B) and renal excretory (Fig. 2C) function produced by the i.v. bolus injection of N/OFQ and NOP receptor partial agonists in conscious Sprague-Dawley rats. Values are means ± S.E. and illustrate the peak (maximum) changes from control (pre-drug baseline values) in cardiovascular and renal excretory function produced by the i.v. bolus injection of isotonic saline vehicle (200 µl; n=8), N/OFQ (100 nmol/kg; n=6) or the NOP receptor partial agonists [F/G] (900 nmol/kg; n=6), HEX 1 (100 nmol/kg; n=6) or HEX 2 (100 nmol/kg; n=6). Cardiovascular and renal function was measured in each rat during control (20-min) and immediately following i.v. bolus drug injection for 80-min (consecutive 10-min experimental periods). Due to differences in time of onset and peak effects between N/OFQ (rapid onset and short duration) and other NOP receptor ligands (slow onset and long duration), cardiovascular data are presented as peak changes (mean ± SE) from pre-drug control values that occurred within the first 10 minutes immediately following i.v. bolus drug/saline injection (Fig. 2A) and the peak changes that were observed over the remaining 70-min protocol (Fig. 2B; time points 20-80 min). Fig. 2C depicts the peak changes in urine flow rate and urinary sodium excretion from pre-drug baseline control that occurred over the entire 80-min study (consecutive 10-min periods). Abbreviations as in Fig. 1. *P<0.05, significantly different from saline-treated group. Basal (predrug/vehicle) control values for each parameter were not significantly different between groups. Basal control values for the saline-treated group depicted in Figs. 2A-2C were: HR, 417±16 bpm; MAP, 117±5 mmHg; V, 54±8 µl/min; UNaV, 8.3±0.6 µeq/min.
Fig. 3. Dose-response study of the cardiovascular and renal responses produced by i.v. bolus injection of the NOP receptor partial agonist, HEX 1, in conscious rats. Values are means ± SE illustrating the systemic cardiovascular and renal excretory responses produced by i.v. bolus injection of isotonic saline vehicle (200 µl; n=8) or HEX 1 at doses of 30 nmol/kg (▲; n=4), 100 nmol/kg (◊; n=6), 300 nmol/kg (Δ; n=5), or 900 nmol/kg (●; n=5) per rat. Urine samples were collected during control (C, 20-min) and immediately after drug/vehicle injection for 80-min (time points 10-80 min). Abbreviations as in Fig. 1. For clarity, asterisks denoting statistically significant differences within each group have been omitted and are discussed in the text.

Figs. 4A and 4B. Effects of i.v. bolus NOP receptor partial agonist pretreatment on the cardiovascular responses to i.v. bolus N/OFQ. Values are means ± SE illustrating the peak (maximum) changes in mean arterial pressure and heart rate (from control pre-drug baseline values) produced by i.v. bolus N/OFQ alone (Fig. 4A, 30 nmol/kg; Fig. 4B, 100 nmol/kg) or by i.v. bolus N/OFQ in rats pretreated (+ N/OFQ; 5-min i.v. Pre-Tx) with one of the NOP receptor partial agonists [F/G] (Fig. 4A, 900 nmol/kg; n=6), HEX 1 (Fig. 4B, 100 nmol/kg; n=8) or HEX 2 (Fig. 4B, 100 nmol/kg; n=6). MAP, mean arterial pressure; HR, heart rate. See Fig. 1 for abbreviations for NOP receptor ligands. *P<0.05, significantly different from respective pre-drug baseline control value. †P<0.05, significantly different from the N/OFQ alone group value. Basal control values for each parameter prior to vehicle/drug pretreatment were not significantly different between groups.
Fig. 1

△ HR (bpm)

△ MAP (mmHg)

△ V (μl/min)

△ UNaV (μeq/min)

ICV:

- **Saline** (5 μl; n = 8)
- **N/OFQ** (5.5 nmol; n = 10)
- **[F/G]** (7.3 nmol; n = 9)
- **HEX 1 = Ac-RYYRIK-NH₂** (1.0 nmol; n = 5)
- **HEX 2 = Ac-RYYRWK-NH₂** (0.73 nmol; n = 6)
Fig. 2C

Peak changes - 0 to 80 min

$\Delta V$ (μl/min)

$\Delta U$NaV (μeq/min)

IV Bolus:

- Saline
- N/OFQ
- [F]G
- HEX 1
- HEX 2

* indicates significant difference compared to control.
Fig. 4A

**MAP**

- Peak Δ MAP (mmHg)
  - Saline Pt
  - NOFQ
  - [F/G] Pt
  - NOFQ

**HR**

- Peak Δ HR (bpm)
  - Saline Pt
  - NOFQ
  - [F/G] Pt
  - NOFQ

**Pre-Tx (pt)**
- Saline (200 µl; n = 6)
- [F/G] (900 nmol/kg; n = 9)

**Treatment**
- + N/OFQ (30 nmol/kg; i.v., 5-min after Pre-Tx)
Fig. 4B

MAP

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HR

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Tx:

- + N/OFQ (100 nmol/kg, i.v., 5-min. after Pre-Tx)

* Significant difference from Pre-Tx
* Significant difference from HEX 1 Pt
* Significant difference from HEX 2 Pt