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Nociceptin, Phe$^1\psi$-nociceptin$_{1-13}$, nocistatin and prepronociceptin$_{154-181}$ effects on calcium channel currents and a potassium current in rat locus coeruleus *in vitro*

*,[1]Mark Connor, [1]Christopher W. Vaughan, [1]Ernest A. Jennings, [3]Richard G. Allen & [2]Macdonald J. Christie

[1]Department of Pharmacology, The University of Sydney, Sydney 2006, NSW, Australia; [2]The Medical Foundation, The University of Sydney, Sydney 2006, NSW, Australia and [3]The Center for Research on Occupational and Environmental Toxicology, Oregon Health Sciences University, 3181 Sam Jackson Park Road, Portland, Oregon, OR 97201, U.S.A.

1 The actions of the neuropeptide nociceptin, the putative nociceptin receptor antagonist [Phe1ψ(CH_2-NH)Gly^2]-nociceptin-(1–13)NH_2 (Phe$^1\psi$-nociceptin$_{1-13}$) and the putative nociceptin precursor products nocistatin (rat prepronociceptin$_{125-132}$) and rat prepronociceptin$_{154-181}$ were examined on membrane properties of rat locus coeruleus (LC) neurons using whole cell patch clamp techniques.

2 Nociceptin inhibited I_{Ba} in all LC neurons, (pD_2 of 8.9, maximum inhibition 50%). The inhibition of I_{Ba} by nociceptin was associated with slowing of the activation of I_{Ba} and could be significantly reversed by a strong depolarizing prepulse. Phe$^1\psi$-nociceptin$_{1-13}$ also inhibited I_{Ba} in LC neurons (notional pD_2 of 7.6, maximum inhibition 18%). Application of Phe$^1\psi$-nociceptin$_{1-13}$ (1 μM) significantly occluded the subsequent effects of a co-application of nociceptin (3 nM) on I_{Ba}.

3 As previously reported for nociceptin, Phe$^1\psi$-nociceptin$_{1-13}$ caused an outward current in LC neurons voltage clamped at −60 mV (pD_2 of 7.1, maximum current 50% of that of methionine enkephalin, 10 μM). The Phe$^1\psi$-nociceptin$_{1-13}$ induced current reversed polarity at −112 mV and exhibited pronounced inward rectification. Phe$^1\psi$-nociceptin$_{1-13}$ (1 μM) reversibly inhibited the current caused by nociceptin (300 nM) by 30%.

4 Neither nocistatin nor rat prepronociceptin$_{154-181}$ inhibited I_{Ba} in LC neurons, or prevented the subsequent inhibition by nociceptin. Neither nocistatin or prepronociceptin$_{154-181}$ affected the membrane properties of LC neurons.

5 This study demonstrates that nociceptin modulates somatic I_{Ba} in rat LC neurons. The putative ORL1 antagonist Phe$^1\psi$-nociceptin$_{1-13}$ exhibited partial agonist activity at inhibiting I_{Ba} and opening K^+ channels in LC. Other putative nociceptin precursor products were without effect on LC cells.

Keywords: Nociceptin; orphanin FQ; nocistatin; ORL1; locus coeruleus; calcium channels; partial agonist; potassium channels

Abbreviations: ACSF, physiological saline; BSA, bovine serum albumin; CHO, chinese hamster ovary cells; G-protein, heterotrimeric guanine nucleotide-binding protein; I_{Ba}, calcium channel current; I_K, potassium current; LC, locus coeruleus; ME, methionine enkephalin; ORL1, opioid receptor-like protein; Phe$^1\psi$-nociceptin$_{1-13}$, [Phe1ψ(CH_2-NH)Gly^2]-nociceptin-(1–13)NH_2

Introduction

Nociceptin (Meunier *et al*., 1995), also called orphanin FQ (Reinscheid *et al*., 1995), is an endogenous ligand for the opioid-like receptor, ORL1 (Mollereau *et al*., 1994; reviewed in Henderson & McKnight, 1997). To date, nociceptin has been shown to modulate a similar range of ion channels and second messenger cascades as opioids (Henderson & McKnight, 1997) and the nociceptin/ORL1 system has been implicated in a wide variety of physiological processes (Darland *et al*., 1998). Nociceptin is one of three putative peptide products of the prepronociceptin gene (Houtani *et al*., 1996; Okuda-Ashitaka *et al*., 1998) that have been shown to produce behavioural effects in mice (Florin *et al*., 1997; Okuda-Ashitaka *et al*., 1998; Rossi *et al*., 1998). Intriguingly, one of these other prepronociceptin peptides, nocistatin (rat prepronociceptin$_{116-132}$), was reported to reverse the effects of nociceptin both *in vivo* and *in vitro* (Nicol *et al*., 1998; Okuda-Ashitaka *et al*., 1998). The cellular basis for these effects is not established, but nocistatin does not appear to directly interact with ORL1 (Okuda-Ashitaka *et al*., 1998).

Detailed investigations of the role of nociceptin have been hampered by the lack of effective antagonists for the ORL1 receptor. A synthetic analogue of nociceptin, [Phe1ψ(CH_2-NH)Gly^2]-nociceptin-(1–13)NH_2 (Phe$^1\psi$-nociceptin$_{1-13}$), was reported to antagonize the inhibitory effects of nociceptin on contractions of the guinea-pig ileum and mouse vas deferens (Guerrini *et al*., 1998), and to be devoid of significant agonist activity (Calo *et al*., 1998a; Guerrini *et al*., 1998; Meis & Pape, 1998). However, subsequent studies have shown that Phe$^1\psi$-nociceptin$_{1-13}$ is a potent agonist at recombinant ORL1 receptors (Butour *et al*., 1998; Okawa *et al*., 1999) and that it mimics the inhibitory effects of nociceptin on nociceptive responses in rats (Carpenter & Dickenson, 1998; Xu *et al*., 1998), as well as the nociceptin reversal of morphine-induced supra-spinal analgesia in mice (Calo *et al*., 1998b; Grisel *et al*., 1998). Phe$^1\psi$-nociceptin$_{1-13}$ acts as a partial agonist with

*Author for correspondence; E-mail: markc@pharmacol.usyd.edu.au

respect to nociceptin in assays of noradrenaline release in mouse brain (Schlicker *et al*., 1998), substance P release in guinea-pig airway (Shah *et al*., 1998), and activation of K^+ channels (I_K) in suprachiasmatic nucleus neurons (Allen *et al*., 1999).

We have previously shown that nociceptin activates an inwardly rectifying potassium conductance in rat locus coeruleus (LC) neurons (Connor *et al*., 1996). In this study we have compared the effects of nociceptin and $Phe^1\psi$-nociceptin$_{1-13}$ on calcium and potassium channels in locus coeruleus neurons, as well as examining the effects of several other putative prepronociceptin products on LC neurons.

Methods

Sprague-Dawley rats of either sex (post natal days 19–20, for slice experiments; post natal days 28–35 for dissociated cell experiments) were used for this study. The rats were anaesthetized with halothane and then killed by cervical dislocation. Horizontal slices (250 μm thick for slice experiments; 290–310 μM thick for preparation of dissociated cells) containing the LC were cut with a vibratome in ice cold physiological saline (ACSF) of composition (mM) NaCl 126, KCl 2.5, $MgCl_2$ 1.2, $CaCl_2$ 2.4, NaH_2PO_4 1.2, $NaHCO_3$ 24 and glucose 11; gassed with 95% O_2/5% CO_2. For recordings of I_K, brain slices were placed in a chamber (1.5 ml volume) mounted on the stage of an upright microscope (Olympus BH-2 with a fixed-stage modification) and viewed using a water immersion objective (Zeiss, $\times$40). Slices were continuously superfused (2 ml min^{-1}) with ACSF (32°C). Neurons located in the LC were visualized using infra-red Nomarski optics and recordings of I_K made using standard whole cell voltage clamp techniques. Recordings were made with borosilicate pipettes of resistance 3–7 MΩ when filled with intracellular solution of the following composition (mM): potassium gluconate 140; NaCl 15; $MgCl_2$ 1; HEPES 10; EGTA 11; MgATP 2; Na_2GTP 0.25; adjusted to a pH of 7.3 with KOH. Neurons were voltage clamped at −60 mV (Axopatch 1D, Axon Instruments, Foster City, CA, U.S.A.), series resistance (<12 MΩ) was compensated by 80% and continuously monitored during experiments. Currents were sampled at 50 Hz for subsequent analysis (Axograph 4.0, Axon Instruments). Liquid junction potentials of −11 mV were corrected. Drugs were applied to the slice by changing the perfusion buffer to one that differed only in the content of drug.

For recordings of currents through calcium channels, cells were dissociated using procedures based on those outlined in Ingram *et al*. (1997). After a 30 min incubation in ACSF (35°C) slices were transferred to a dissociation buffer of composition (mM) Na_2SO_4 82, K_2SO4 30, HEPES 10, $MgCl_2$ 5, glucose 10, containing 20 units ml^{-1} papain, pH 7.3 and incubated for 2 min at 35°C. The slices were then placed in fresh dissociation buffer containing 1 mg ml^{-1} bovine serum albumin (BSA) and 1 mg ml^{-1} trypsin inhibitor. The LC region was subdissected from each slice with a fine tungsten wire and the cells dissociated from the slices by very gentle trituration in a pasteur pipette with a fire polished tip. The cells were plated onto plastic culture dishes and kept at room temperature in dissociation buffer. Cells remained viable for up to 6 h after dissociation.

Recordings of currents through Ca^{2+} channels were made using standard whole cell patch clamp techniques (Hamill *et al*., 1981) at room temperature (22–24°C). Immediately prior to recording, cells were superfused with a buffer of composition (mM) NaCl 140, KCl 2.5, $CaCl_2$ 2.5, $MgCl_2$ 1.5, HEPES 10, glucose 10, pH 7.3 in order to wash off the dissociation buffer. For calcium channel current recordings, cells were superfused in solution containing (mM) tetraethylammonium chloride 140, $BaCl_2$ 2, $MgCl_2$ 1, CsCl 2.5, HEPES 10, glucose 10, BSA 0.05%, pH 7.3. Recordings were made with fire polished borosilicate pipettes of resistance approximately 2 MΩ when filled with intracellular solution of the following composition (mM): CsCl 110, MgATP 5, Na_2GTP 0.2, EGTA 10, $CaCl_2$ 2 and HEPES 10, pH 7.3. The peak calcium channel current in each cell was determined by stepping the membrane potential from a holding potential of −90 mV to potentials between −60 and +60 mV, usually for 30 ms, in 10 mV increments. The test current evoking a peak calcium channel current was then evoked every 30 s, and monitored for at least a further 2 min before drugs were applied. The inhibition of drugs was quantified by measuring the current amplitude isochronically with the peak of the control calcium channel current. Cells in which the calcium channel current declined in the absence of drug treatment were discarded. Whole cell capacitance and series resistance were compensated manually by nulling the capacitive transient evoked by a 20 mV pulse from −90 mV. The series resistance was between 1.5 and 5 MΩ; series resistance compensation of at least 80% was used in all experiments. An approximate value of whole cell capacitance was read from the amplifier capacitance compensation circuit (Axopatch 1D). Leak current was subtracted on line using a P/8 protocol, unless otherwise noted, typically the leak conductance was less than 1 nS. Evoked calcium channel currents were sampled at 5–10 kHz and recorded on hard disk for later analysis. Data was collected and analysed off line with the PCLAMP suite of programs (Axon Instruments). Cells were exposed to drugs *via* a series of flow pipes positioned above the cells. Drugs were applied after at least 2 min of control currents were collected, subsequent drug applications were made after the effects of the first drug application had fully reversed, or in the case of drug co-applications, 2 min into the application of the first drug. All data are expressed as mean ± s.e.mean, unless otherwise indicated. Statistical significance was determined by using an unpaired students *t*-test unless otherwise stated.

Drugs and chemicals

Buffer salts were from BDH Australia or Sigma Australia. Papain was from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.). BSA and trypsin inhibitor (Type II-O) were from Sigma Australia. Nociceptin (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser - Ala - Arg - Lys - Leu - Ala-Asn-Gln) was synthesized and purified by Chiron Mimotopes (Clayton, Victoria, Australia). Nocistatin, (rat prepronociceptin$_{125-132}$, mPNP-3-8P, Glu-Val-Glu-Gln-Lyn-Gln-Leu-Gln) was synthesized and purified by Auspep (Parkville, Victoria, Australia). Rat prepronociceptin$_{154-181}$ (mouse prepronociceptin$_{160-187}$) (Phe-Ser-Glu-Phe - Met - Arg - Gln -Tyr-Leu-Val-Leu-Ser-Met-Glu-Ser-Ser-Glu-Arg -Arg -Thr -Leu -His -Gln -Asn -Gly -Asn - Val) was made by solid phase synthesis by Research Genetics, Huntsville, Alabama, U.S.A.). [$Phe1\psi(CH_2$-NH)Gly^2]-nociceptin-(1–13)NH_2 was a kind gift of Dr G. Calo.

Results

Dissociated LC neurons were identified as large (mean membrane capacitance 32 ± 1 pF, n = 86), usually multipolar neurons with somata shapes characteristic of LC neurons described in fixed tissue preparations (e.g. Swanson, 1976). In

our dissociations the only cells of comparable size were large, spherical cells with a single small process, which were presumably MeV neurons. When LC neurons were stepped from a holding potential of −90 mV to potentials between −60 and +60 mV the inward currents in most cells began to activate at about −40 mV and were invariably greatest at membrane potentials between −10 and 0 mV. The peak current density did not differ between cells from male (125±7 pA pF^{-1}, $n=48$) and female rats (116±6 pA pF^{-1}, $n=49$). The peak inward current could be abolished by Cd^{2+} (30 μM, data not shown).

Nociceptin inhibited the peak inward I_{Ba} in all LC neurones when applied at a concentration of 300 pM or more ($n=89$). The effects of nociceptin reversed on washout (Figure 1a). A concentration response relationship for nociceptin inhibition of I_{Ba} was determined by application of one or more concentrations of nociceptin to cells stepped repetitively from −90 mV to the membrane potential that evoked the largest I_{Ba} in each neuron (either −10 or 0 mV). A logistic function fitted to the concentration-response relationship for nociceptin inhibition of I_{Ba}, gave a pD_2 for nociceptin of 8.9±0.1 with a slope factor the curve of 0.8±0.1 (Figure 1b). The maximum inhibition of I_{Ba} by nociceptin was about 50% (Figure 1b).

The inhibition of I_{Ba} by nociceptin was evident at a wide range of membrane potentials (Figure 2a) and was associated with a pronounced slowing of the activation of I_{Ba} (Figures 1a and 2b). When applied at a concentration of 3 nM, nociceptin increased the 0–95% risetime of I_{Ba} from 2.9±0.2 ms to 5.9±0.8 ms ($P<0.003$, paired t-test, $n=9$), the slowing reversed on washout of nociceptin (risetime was 2.8±0.2 ms after wash $P>0.7$, paired t-test versus predrug risetime).

The inhibition of I_{Ba} by nociceptin could be attenuated by a strong positive depolarizing step shortly before the test step. In the experiments illustrated in Figure 3a, LC steps were stepped twice to −10 mV, with an 80 ms depolarizing step to +80 mV between the test steps. These experiments were performed without leak subtraction. In control conditions, the amplitudes of the first (T1) and second (T2) test step did not differ from each other ($P>0.8$, unpaired t-test), the ratio of T2 : T1 was 0.97±0.02 ($n=9$). However, in the presence of nociceptin (100 nM), the amplitudes of the first step and the second step were significantly different from each other ($P<0.006$, unpaired t-test), the ratio of T2 : T1 was 1.6±0.05 ($n=9$). In the presence of nociceptin the amplitude of the first step was reduced by 52±2% ($P<0.0002$, paired t-test) compared to the first test step in the absence of drug, while the amplitude of the second test step was reduced by only 21±1% ($P<0.0002$, paired t-test) compared to second test step in the absence of nociceptin. The inhibition of the first test pulse (T1) by nociceptin was significantly greater ($P<0.002$) than the inhibition of the test pulse (T2) following the 80 ms depolarization to +80 mV.

While the step to +80 mV partly reversed the nociceptin inhibition of the amplitude of I_{Ba}, it completely reversed the kinetic slowing of I_{Ba} activation caused by nociceptin. The 0–95% risetime of the first step to −10 mV (T1) was 2.7±0.1 ms, in the presence of nociceptin (100 nM) the risetime was 4.9±0.3 ms ($P<0.0002$, paired t-test, $n=9$). The 0–95% risetime of the test step (T2) after the depolarizing step was 2.9±0.1 ms, in the presence of nociceptin the risetime was also 2.9±0.1 ms, which is not different from that in the absence of drug ($P>0.8$, paired t-test, $n=9$).

When there was no depolarizing step between the two test pulses, there was not relief from the effect of nociceptin on the amplitude or kinetics of I_{Ba} (Figure 3b). When cells were stepped twice to −10 mV, separated by 90 ms at the holding potential of −90 mV, the amplitudes of the first (T1) and second (T2) test step did not differ from each other ($P>0.8$, unpaired t-test), the ratio of T2 : T1 was 0.95±0.01 ($n=5$). In the presence of nociceptin (100 nM), the amplitude of the first

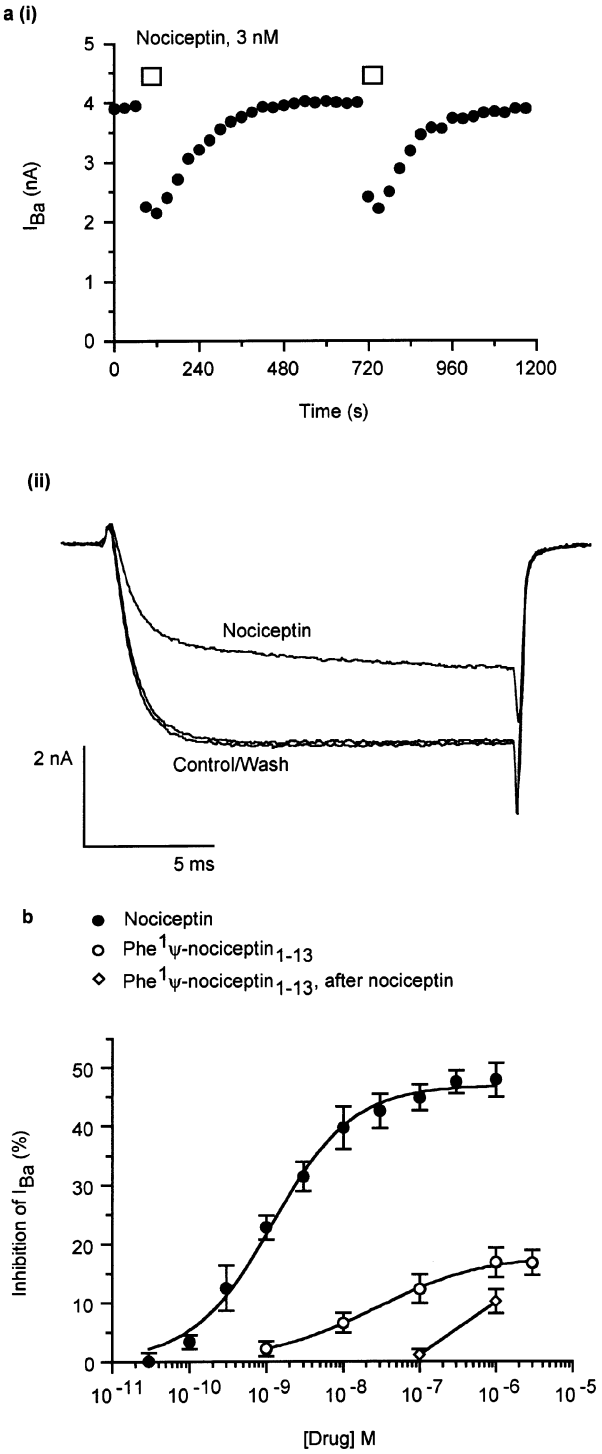

Figure 1 Modulation of LC calcium channel currents by nociceptin. I_{Ba} was elicited by repetitively stepping the membrane potential from −90 mV to −10 mV. (a) (i) A time plot of the peak amplitude of I_{Ba} illustrating the effects of repeated applications of nociceptin. (ii) Selected traces from the same experiment, showing the inhibition of I_{Ba} by nociceptin. (b), Concentration-response relationship for nociceptin (EC_{50}, 2 nM), and Phe$^1\psi$-nociceptin$_{1-13}$ (EC_{50}, 30 nM) inhibition of I_{Ba} in LC neurons. Each point represents at least five cells tested. Also shown is the response of LC neurons to Phe$^1\psi$-nociceptin$_{1-13}$ applied after application and washout of nociceptin (1–30 nM) ($n=5$ for 100 nM, $n=9$ for 1 μM Phe$^1\psi$-nociceptin$_{1-13}$).

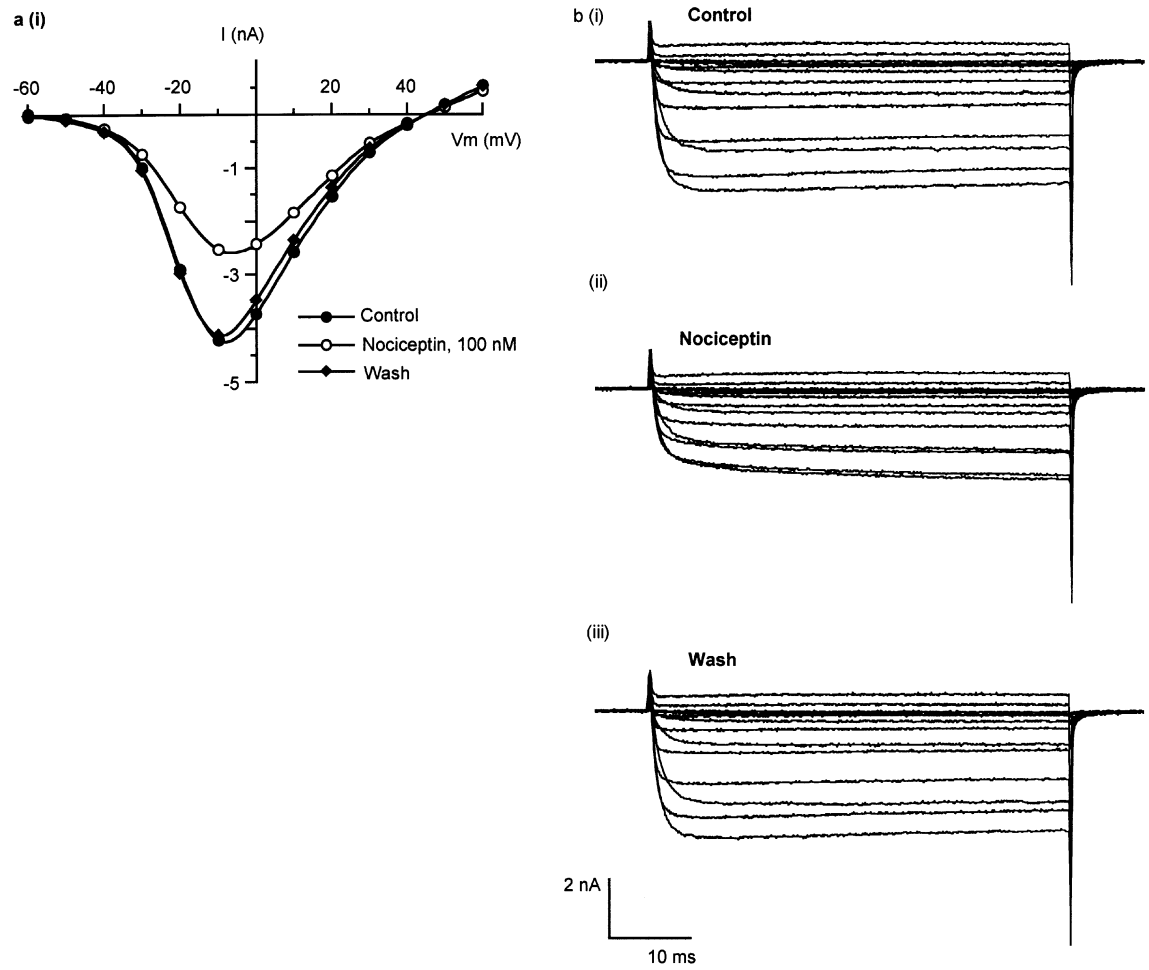

Figure 2 Characteristics of nociceptin modulation of I_{Ba}. I_{Ba} were elicited by stepping the membrane potential from −90 mV to potentials between −60 and +60 mV in 10 mV increments. Nociceptin inhibited I_{Ba} over a range of membrane potentials. (a) A plot of the peak inward current at each test potential, before, during and after an application of nociceptin, 100 nM. This cell is a typical example of six experiments. (b) Families of traces from the same experiment, showing the I_{Ba} elicited by the steps from a holding potential of −90 mV to a test potential between −60 and +60 (i) in the absence of drug, (ii) in the presence of nociceptin and (iii) following wash of nociceptin.

test pulse was reduced by 47±4% ($P<0.03$, paired t-test, $n=5$) and the amplitude of the second test pulse was reduced by 45±4% ($P<0.04$, paired t-test). In the presence of nociceptin the ratio of T2 : T1 was 0.98±0.1 ($n=5$). The 0–95% risetime of the first test step to −10 mV was 2.6±0.1 ms, in the presence of nociceptin (100 nM) the risetime was 4.2±0.5 ms ($P<0.02$, paired t-test, $n=5$). The 0–95% risetime of the second test step was 2.7±0.1 ms, in the presence of nociceptin the risetime was 4.9± 0.5 ms ($P<0.002$, $n=5$).

When applied to LC neurons before nociceptin, Phe$^1\psi$-nociceptin$_{1-13}$ inhibited I_{Ba} in a concentration dependent manner, however, unlike the effects of nociceptin, the inhibition of I_{Ba} by Phe$^1\psi$-nociceptin$_{1-13}$ did not readily reverse on washout (Figure 4b). Equations describing concentration response relationships for agonists assume agonist/effector reactions are freely reversible, and the interaction of Phe$^1\psi$-nociceptin$_{1-13}$ with I_{Ba} is apparently not. However, a logistic function was fitted to the concentration response data from cells in which Phe$^1\psi$-nociceptin$_{1-13}$ was applied first, to obtain a notional pD_2 of 7.6±0.2, with a maximum inhibition of about 18% (Figure 1b). In cells where nociceptin (1–30 nM) had been applied before Phe$^1\psi$-nociceptin$_{1-13}$, the agonist effects of Phe$^1\psi$-nociceptin$_{1-13}$ on I_{Ba} were strongly attenuated (Figure 1b).

When LC cells were stepped twice to −10 mV, with an 80 ms depolarizing step to +80 mV between the test steps, application of Phe$^1\psi$-nociceptin$_{1-13}$ (3 μM) inhibited the amplitude of first step (T1) by 24±9%, ($P<0.05$, paired t-test, $n=7$) but did not significantly inhibit the amplitude of the second step (T2, inhibition was 10±5%, $P=0.08$, paired t-test, $n=7$). In these cells the ratio of T2 : T1 before the application of Phe$^1\psi$-nociceptin$_{1-13}$ (3 μM) was 1.01±0.04, during the application of Phe$^1\psi$-nociceptin$_{1-13}$ (3 μM) the ratio was 1.25±0.09 ($P=0.06$ paired t-test, $n=7$).

Superfusion of Phe$^1\psi$-nociceptin$_{1-13}$ occluded the effects of a co-application of nociceptin (3–30 nM nociceptin, $n=9$, Figure 4a). In the presence of Phe$^1\psi$-nociceptin$_{1-13}$ (1 μM), I_{Ba} was inhibited by 7±2%, application of nociceptin (3 nM) in the continued presence of Phe$^1\psi$-nociceptin$_{1-13}$ (1 μM) did not inhibit I_{Ba} any further (inhibition was 1±1%, $n=5$, Figure 4a). In parallel control experiments nociceptin inhibited I_{Ba} by 26±4% ($P<0.01$ vs total inhibition of I_{Ba} by Phe$^1\psi$-nociceptin$_{1-13}$ (1 μM) + nociceptin (3 nM), which was 8±3%). Because of the partial agonist-like actions of Phe$^1\psi$-nociceptin$_{1-13}$ in LC cells, we did not systematically examine the potency of Phe$^1\psi$-nociceptin$_{1-13}$ to inhibit the actions of nociceptin. Application of Phe$^1\psi$-nociceptin$_{1-13}$ also partly occluded the inhibition of I_{Ba} by a maximally effective concentration of the opioid agonist methionine-enkephalin

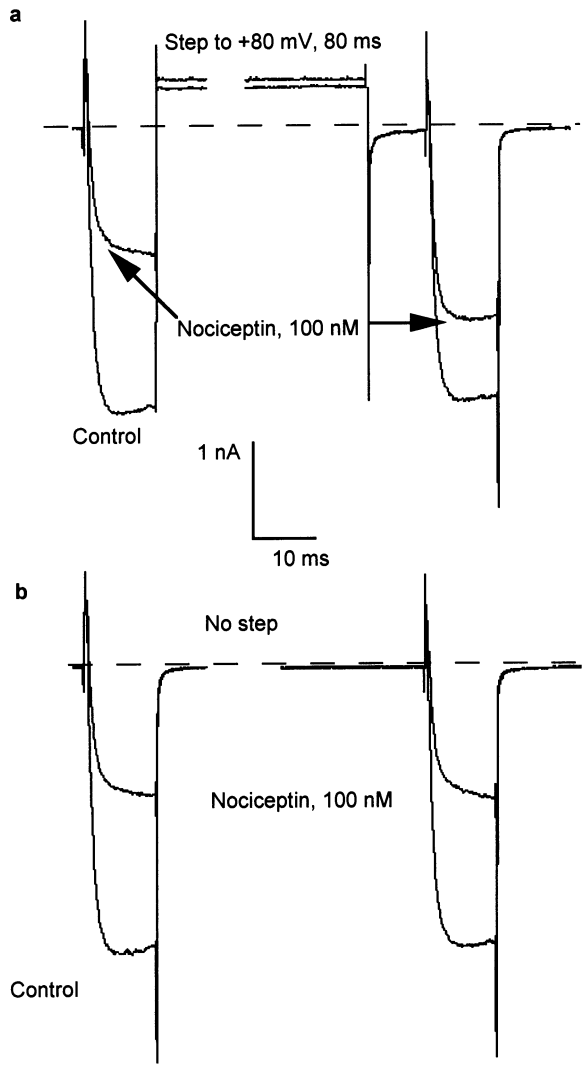

Figure 3 Nociceptin inhibition of I_{Ba} is relieved by a positive prepulse. An LC neuron was voltage clamped at −90 mV and stepped twice to a test potential of −10 mV, with 90 ms between the test pulses. In (a), and 80 ms positive step to +80 mV was applied to the cell immediately after the first test pulse. In (b), the cell was held at −90 mV for the 90 ms between test pulses. The resulting raw current traces for steps in the absence of drug and in the presence of nociceptin are shown. Traces in (a) and (b) are from two separate applications of nociceptin about 10 min apart. The dashed line represents the zero current line, because of the complex step paradigm leak subtraction was not used. The break in the current traces represent a section of about 50 ms that has been omitted for clarity. Note that in (a), the amplitude of the second step is facilitated compared with the first, while in (b) there is no facilitation. Note also that in (a), nociceptin inhibits the outward current through the calcium channels at +80 mV. The experiment in (a) is typical of nine cells, the experiment in (b) of five cells.

(ME, Figure 4b). ME alone (10 μM) inhibited I_{Ba} by 47±2% ($n=7$); in the presence of Phe$^1\psi$-nociceptin$_{1-13}$ (1 μM) and ME (10 μM) the inhibition of I_{Ba} was also 47±2% ($n=6$). In these experiments Phe$^1\psi$-nociceptin$_{1-13}$ (1 μM) alone inhibited I_{Ba} by 12±2% and ME inhibited the remaining current by 39±2% (significantly less than control, $P<0.02$).

Nociceptin increases I_K in LC neurons in brain slices (Connor *et al*., 1996). Phe$^1\psi$-nociceptin$_{1-13}$ also produced a reversible, concentration-dependent outward current when applied to LC neurons voltage clamped at −60 mV in slices (Figure 5). The current activated by Phe$^1\psi$-nociceptin$_{1-13}$ was examined by determining the steady state current-voltage relationships for LC neurons in the presence and absence of Phe$^1\psi$-nociceptin$_{1-13}$. The current activated by Phe$^1\psi$-nociceptin$_{1-13}$ reversed polarity at −112±4 mV ($n=5$), and was accompanied by an increase in membrane conductance. The current activated by Phe$^1\psi$-nociceptin$_{1-13}$ showed pronounced inward rectification. The cord conductance of the Phe$^1\psi$-nociceptin$_{1-13}$-activated current measured at −60 mV was 0.8±0.3 nS, the cord conductance measured at −130 mV was 2.1±0.5 nS ($n=5$). The conductance activated by a high concentration of ME (10 μM) in the same cells reversed polarity at −116±7 mV ($n=5$). The cord conductance of the ME-activated current measured at −60 mV was 2.1±0.3 nS, and 4.7±0.6 nS when measured at −130 mV ($n=5$).

A logistic function fitted to the concentration-response relationship for Phe$^1\psi$-nociceptin$_{1-13}$ activation of I_K gave a pD_2 of 7.10±0.05 with a slope factor for the curve of 1.8±0.05 (Figure 5b). The highest concentrations of Phe$^1\psi$-nociceptin$_{1-13}$ examined caused an outward current that was about 50% of that caused by application of a high concentration of ME (10 μM, Figure 5). We have previously shown that nociceptin produces a maximal current similar to that caused by high concentrations of ME.

Application of Phe$^1\psi$-nociceptin$_{1-13}$ (1 μM) in the continued presence of a submaximally effective concentration of nociceptin (300 nM) reversibly reduced the outward current caused by nociceptin to 70±7% of the pre-Phe$^1\psi$-nociceptin$_{1-13}$ value ($n=7$, Figure 5c).

Application of nocistatin (10 μM) did not affect I_{Ba} in any LC neuron tested (Figure 4c, $n=8$), nor did it affect the inhibition of I_{Ba} by a subsequent co-application of nociceptin. The inhibition of I_{Ba} produced by nociceptin (3 nM) in the presence of nocistatin (10 μM) was similar to that produced in the absence of nocistatin (29±3%, $n=7$, versus 27±4%, $n=6$, respectively). Application of nocistatin (1 μM) did not change the membrane current or conductance of LC neurons voltage clamped at −60 mV in slices ($n=5$).

Application of rat prepronociceptin$_{154-181}$ (1 μM) did not affect I_{Ba} in any LC neuron tested (I_{Ba} was 100±1% of control after 2 min in prepronociceptin$_{154-181}$, $n=8$), nor did it affect the inhibition of I_{Ba} by a subsequent co-application of nociceptin. The inhibition of I_{Ba} produced by nociceptin (3 nM) in the presence of prepronociceptin$_{154-181}$ (1 μM) was similar to that produced in the absence of prepronociceptin$_{154-181}$ (20±3%, $n=5$, versus 22±5%, $n=4$, respectively). Application of prepronociceptin$_{154-181}$ (1 μM) did not change the membrane current or conductance of LC neurons voltage clamped at −60 mV in slices ($n=5$).

Discussion

This study demonstrates that nociceptin, the endogenous ligand for the ORL1 receptor, inhibits I_{Ba} in all rat locus coeruleus neurons. Nociceptin has been previously shown to increase I_K in the same neurons (Connor *et al*., 1996). The functional significance of modulation of I_{Ba} in LC neurons is not established, however, nociceptin is known to reduce noradrenaline release from mouse brain cortex *in vitro*, which presumably reflects inhibitory actions of nociceptin on the nerve terminals of LC cells (Schlicker *et al*., 1998). It is possible that this inhibition of noradrenaline release occurs *via* inhibition of LC nerve terminal calcium channels, although opening of nerve terminal (or somatic) K^+ channels would be expected to have a similar inhibitory effect. The potency for nociceptin inhibition of noradrenaline release (about 30 nM, Schlicker *et al*., 1998) is between that for nociceptin inhibition of I_{Ba} (2 nM), and activation of I_K (90 nM, Connor *et al*., 1996).

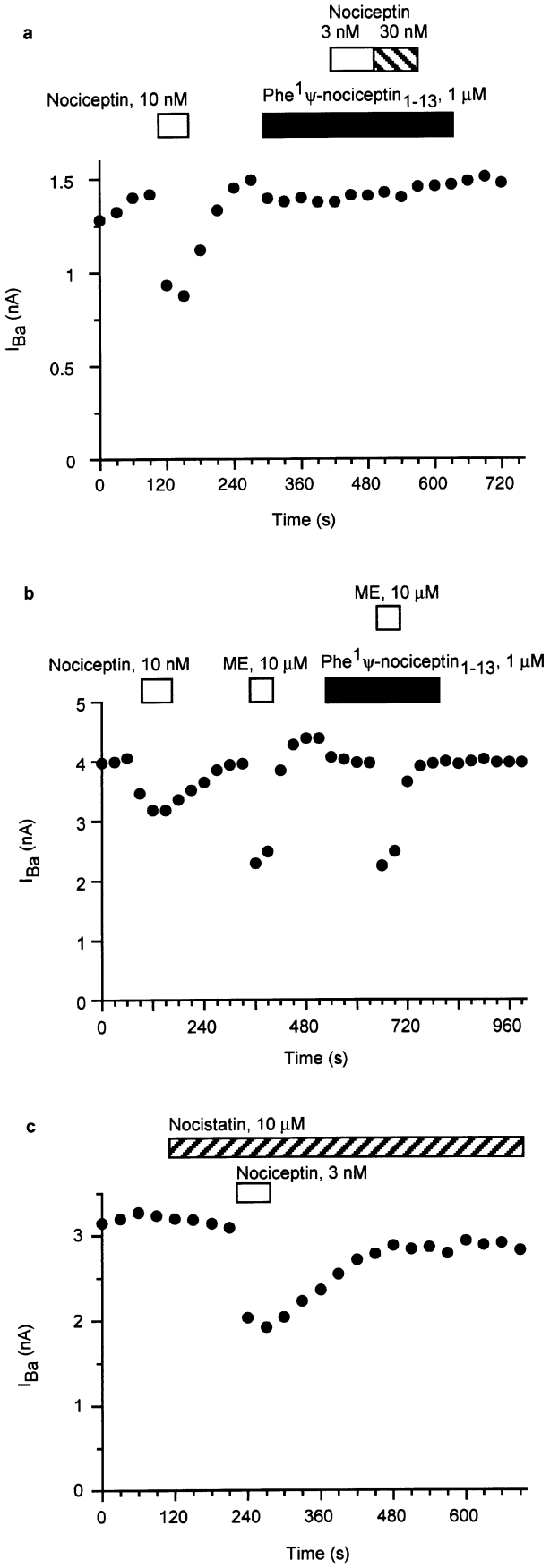

Figure 4 Phe$^1\psi$-nociceptin$_{1-13}$ but not nocistatin blocks the effects of nociceptin on I_{Ba}. I_{Ba} was elicited by repetitively stepping the membrane potential from −90 mV to 0 mV in each cell. (a) A time plot of the peak amplitude of I_{Ba} illustrating the effects of nociceptin, followed by the application of the Phe$^1\psi$-nociceptin$_{1-13}$, which has little effect on I_{Ba} itself but antagonizes the effects of a subsequent co-application of nociceptin (3 and 30 nM). (b) A time plot of the peak amplitude of I_{Ba} illustrating the effects of nociceptin and ME, followed by an application of Phe$^1\psi$-nociceptin$_{1-13}$, which has little effect on a subsequent co-application of ME. (c) A time plot of the peak amplitude of I_{Ba} illustrating the effect of nocistatin, followed by a co-application of nociceptin and nocistatin.

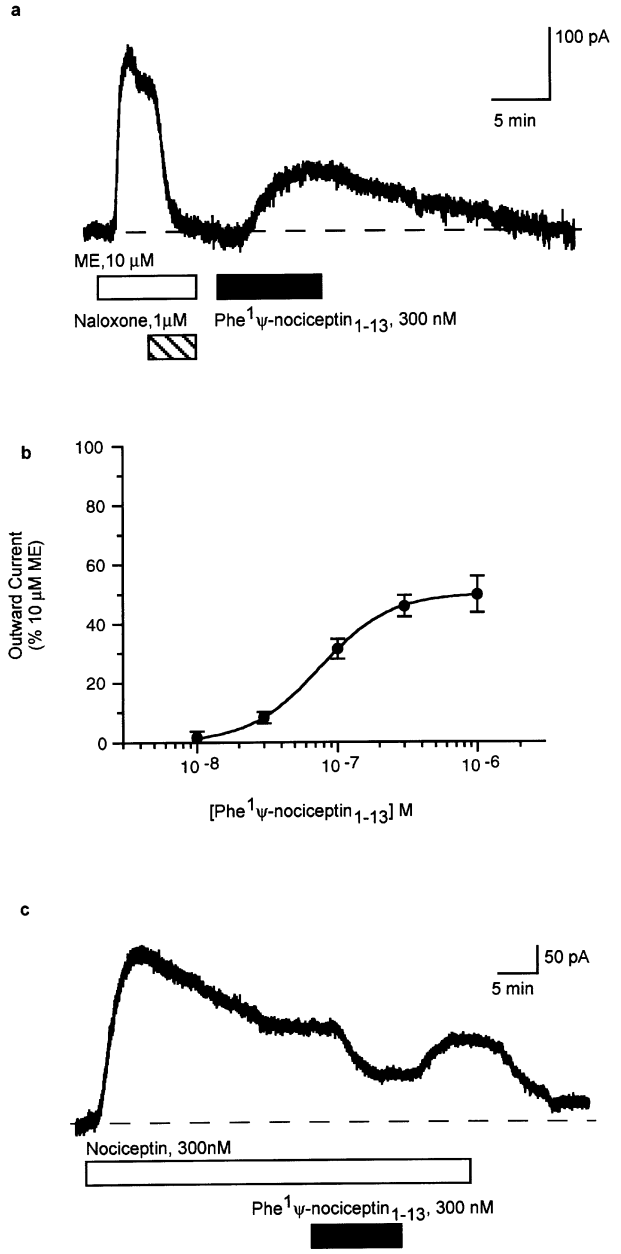

Figure 5 Modulation of LC I_K by Phe$^1\psi$-nociceptin$_{1-13}$. LC neurons in slices were voltage clamped at −60 mV. (a) A continuous record of the membrane currents caused by superfusion of ME and Phe$^1\psi$-nociceptin$_{1-13}$. (b) Concentration-response relationship for Phe$^1\psi$-nociceptin$_{1-13}$ (EC_{50} 75 nM) activation of I_K in LC neurons, plotted as a percentage of the current resulting from the application of a high concentration of ME (10 μM) in each cell. Each point represents between four and eight cells tested. (c) A continuous record of the membrane currents caused by nociceptin, including a co-application of Phe$^1\psi$-nociceptin$_{1-13}$, which partially antagonizes the effects of nociceptin.

The finding that nociceptin was considerably more potent at inhibiting I_{Ba} in dissociated LC cells than in activating I_K in LC cells in slices is similar to previous findings in PAG neurons, where nociceptin inhibited I_{Ba} in dissociated cells with an EC_{50} of 5 nM (Connor & Christie, 1998), but activated I_K with an EC_{50} of 42 nM (Vaughan *et al*., 1997). These differences in potency may reflect stronger coupling of ORL1 receptor to I_{Ba} than I_K, as previously demonstrated for the μ-opioid receptor in acutely isolated neonatal LC neurons (Ingram *et al*., 1997). Alternatively, these differences could be due to degradation of nociceptin in slice preparations. It would have been of interest to determine the effects of nociceptin on I_{Ba} in LC slices, however the size and geometry of LC neurons make it impossible to achieve a semblance of a voltage clamp of I_{Ba} in these cells (M. Connor and M.J. Christie unpublished observations 1987–1999).

The inhibition of I_{Ba} in LC by nociceptin was probably mediated by activation of heterotrimeric guanine nucleotide binding proteins (G proteins), as has been demonstrated for nociceptin inhibition of high voltage activated I_{Ba} in hippocampus (Knoflach *et al*., 1996), periaqueductal grey (Connor & Christie, 1998) and sensory neurons (Abdulla & Smith, 1997). In the present study the inhibition of I_{Ba} by nociceptin was rapid, reversible, evident across a range of membrane potentials and was associated with a pronounced slowing of the activation of the currents, all characteristic features of the ubiquitous G protein $\beta\gamma$-subunit mediated pathway for inhibition of I_{Ba} (Herlitze *et al*., 1996; Ikeda, 1996). Further, the nociceptin-induced inhibition of the amplitude of I_{Ba} could be significantly reversed by a depolarizing prepulse to +80 mV. The relief of inhibition by the depolarizing prepulse is thought to reflect a voltage-dependent dissociation of G protein $\beta\gamma$ subunits from the calcium channels (Herlitze *et al*., 1996; Ikeda, 1996; Zamponi & Snutch, 1998). Intriguingly, in contrast to the incomplete reversal of the nociceptin-induced inhibition of I_{Ba} amplitude, the depolarizing prepulse completely reversed the nociceptin-induced kinetic slowing of I_{Ba}. Although the reason for this is not known, the observation suggests that there may be a voltage-independent component of nociceptin modulation of I_{Ba} in LC neurons. It should be noted that the present study utilized Ba^{2+} as a charge carrier and strong intracellular Ca^{2+} buffering to maintain stable calcium channel currents. Thus, any modulation of I_{Ba} by Ca^{2+}-dependent process would be likely to be suppressed in the present experiments, and it is possible that nociceptin may act *via* such additional mechanisms to modulate I_{Ba} *in vivo*.

The peptide analogue of nociceptin, Phe$^1\psi$-nociceptin$_{1-13}$, appears to act as a partial agonist both at inhibiting I_{Ba} and activating I_K. The maximal effect of Phe$^1\psi$-nociceptin$_{1-13}$ was less than that of nociceptin on both conductances (see Connor *et al*., 1996, for comparison of nociceptin and ME on I_K) while high concentrations of Phe$^1\psi$-nociceptin$_{1-13}$ occluded the effects of a co-application of nociceptin. Previous studies have reported Phe$^1\psi$-nociceptin$_{1-13}$ to be either a pure antagonist (Bigoni *et al*., 1999; Guerrini *et al*., 1998; Meis & Pape, 1998), a partial agonist (Allen *et al*., 1999; Bigoni *et al*., 1999; Okawa *et al*., 1999; Schlicker *et al*., 1998; Shah *et al*., 1998) or a full agonist (Butour *et al*., 1998; Calo *et al*., 1998b; Grisel *et al*., 1998; Okawa *et al*., 1999; Xu *et al*., 1998) in various assays of putative ORL1 function. The agonist activity of Phe$^1\psi$-nociceptin$_{1-13}$ appears to be strongest in whole animals experiments (Calo *et al*., 1998b; Grisel *et al*., 1998; Kapusta *et al*., 1999; Xu *et al*., 1998) or cells overexpressing ORL1 (Butour *et al*., 1998; Okawa *et al*., 1999).

The most parsimonious explanation of the different actions of Phe$^1\psi$-nociceptin$_{1-13}$ in various bioassays is that the receptor/effector coupling for nociceptin/ORL1 differs in various parts of the nervous system. In a cell line where ORL1 is a heterologously expressed to a high level, Phe$^1\psi$-nociceptin$_{1-13}$ is full agonist, as may be expected in a situation when coupling efficiency is artificially high. Further, agents that are partial agonists in *in vitro* bioassays, such as morphine, (e.g. Alt *et al*., 1998; Lemaire *et al*., 1978; Ingram *et al*., 1997) can demonstrate full agonist activity in *in vivo* assays of more complex functions such as analgesia, presumably because of the larger receptor reserve apparently available *in vivo* (e.g. Adams *et al*., 1990; Mjanger & Yaksh, 1990).

The perceived lack of agonist activity of Phe$^1\psi$-nociceptin$_{1-13}$ in peripheral assays of nociceptin action has also been suggested to reflect a heterogeneity of nociceptin receptors, with Phe$^1\psi$-nociceptin$_{1-13}$ only being an agonist at those in the central nervous system (e.g. Butour *et al*., 1998; Calo *et al*., 1998b). Although a potential molecular basis for ORL1 receptor heterogeneity has been established with the identification of a number of splice variants of the receptor, there is no evidence that these receptors are functionally different (Pan *et al*., 1998; Wang *et al*., 1994). Studies of nociceptin receptor binding in rodent brain have generally found only one high affinity nociceptin binding site (Foddi & Mennini, 1997; Makman *et al*., 1997; Albrecht *et al*., 1998; Varani *et al*., 1998, but see Mathis *et al*., 1997), although direct comparisons between central and peripheral nervous system nociceptin binding have not been performed.

The high activity of Phe$^1\psi$-nociceptin$_{1-13}$ in whole animal studies has led to the suggestion that it may be converted into an active compound *in vivo* (Kapusta *et al*., 1999). The studies reported here on dissociated cells demonstrate that Phe$^1\psi$-nociceptin$_{1-13}$ retains agonist activity in a situation where metabolism is unlikely.

The effects of Phe$^1\psi$-nociceptin$_{1-13}$ were reversible in slices, but did not reverse within the timecourse of experiments on I_{Ba}. The reason for this is unknown, but it could reflect either a very slow dissociation rate of Phe$^1\psi$-nociceptin$_{1-13}$ from ORL1 receptors under conditions used to record I_{Ba}, or a non-specific interaction of Phe$^1\psi$-nociceptin$_{1-13}$ with either I_{Ba} or the G-proteins which presumably transduce the signal between ORL1 and I_{Ba}. The effects of Phe$^1\psi$-nociceptin$_{1-13}$ on I_{Ba} were probably mediated *via* a G protein dependent mechanism, because Phe$^1\psi$-nociceptin$_{1-13}$ inhibition of I_{Ba} could be partially relieved by a depolarizing step to +80 mV, similar to the effects of nociceptin itself. Phe$^1\psi$-nociceptin$_{1-13}$ did not prevent the inhibition of I_{Ba} by maximally effective concentration of ME (M. Connor, unpublished observations 1999), although the inhibition of I_{Ba} by ME was partly occluded by Phe$^1\psi$-nociceptin$_{1-13}$. The lack of additivity between ME and Phe$^1\psi$-nociceptin$_{1-13}$ likely reflects the fact that I_{Ba} in LC neurons can only be inhibited to a maximum of about 50% (e.g. by high concentrations of nociceptin, as reported above).

The precursor polypeptide for nociceptin has been suggested to encode up to four additional potential peptides (Houtani *et al*., 1996; Mollereau *et al*., 1996; Nothacker *et al*., 1996). The 28 amino acids carboxy-terminal to the nociceptin sequence in the precursor are strictly conserved across mouse (prepronociceptin$_{160-187}$), rat (prepronociceptin$_{154-181}$), human and cow (Houtani *et al*., 1996; Mollereau *et al*., 1996; Nothacker *et al*., 1996; Okuda-Ashitaka *et al*., 1998). The carboxy-terminal peptide appears to be a necessary product of nociceptin production. Immunoreactivity for the octacosapeptide has been detected in mouse hypothalamus and amygdala (R.G. Allen, manuscript in preparation) and a fragment

comprising the first 17 amino acids of this peptide has been shown to both stimulate locomotion (*Noc*II, Florin *et al*., 1997) and produce naloxone-sensitive analgesia (orphanin FQ2, Rossi *et al*., 1998) in mice. There is no direct evidence that the 17 amino fragment of the C-terminal octacosapeptide is produced *in vivo*, so we examined the effects of the complete peptide on LC neurons. The peptide had no direct effects on LC I_{Ba} or the membrane properties of LC neurons in slices, and it did not occlude the effects of nociceptin. These results are consistent with previously reported lack of effects of the octacosapeptide or its 17 amino acid fragment on nociceptin binding to ORL1 (Nothacker *et al*., 1996). It is not known if rat prepronociceptin$_{154-181}$ is released in the region of the LC, nor is there any information as to the location or nature of the receptors with which it interacts.

The other product of the nociceptin precursor that has been shown to have biological activity has been named nocistatin, because it prevents or reverses the activity of nociceptin in several *in vivo* and *in vitro* assays (e.g. Minami *et al*., 1998; Nicol *et al*., 1998; Okuda-Ashitaka *et al*., 1998). The nocistatin sequence is not as clearly conserved across species as nociceptin or the C-terminal octacosapeptide, however, a minimal core sequence required for nocistatin activity has been identified, Glu-Gln-Lys-Gln-Leu-Gln. In this study we used an extended octapeptide sequence conserved in rat and mouse, Glu-Val-Glu-Gln-Lys-Gln-Leu-Gln (rat prepronociceptin$_{125-132}$, mouse prepronociceptin$_{131-138}$, Okuda-Ashitaka *et al*., 1998). Nocistatin alone had no effect on LC neurons, nor did it prevent the effects of a subsequent co-application of nociceptin. These findings suggest that, perhaps not unexpectedly, the interactions of nociceptin and nocistatin may occur at an inter-neuronal level, rather than at the level of a single cell. Although there appears to be a high affinity binding site for nocistatin in brain, nothing is known about its localization, pharmacology or molecular identity (Okuda-Ashitaka *et al*., 1998).

This study demonstrates that nociceptin potently inhibits I_{Ba} in all LC neurons, which provides a further possible mechanisms for nociceptin modulation of noradrenaline release in brain (Connor *et al*., 1996; Schlicker *et al*., 1998). The putative nociceptin receptor antagonist Phe$^1\psi$-nociceptin$_{1-13}$ is a partial agonist at inhibiting I_{Ba} and opening I_K in the LC, which makes it of limited use in investigating the function of ORL1 in this brain region. Nocistatin and rat prepronociceptin$_{154-181}$ had no direct effects on LC neurons, suggesting that the receptors for these putative neuropeptides are either not located on LC neurons, or that the receptors couple to changes in cellular excitability *via* mechanisms not readily detectable using conventional electrophysiological techniques. The ORL1/nociceptin system is likely to be an important regulator of a range of functions within the LC and its projection fields.

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