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Nociceptin receptor coupling to a potassium conductance in rat locus coeruleus neurones *in vitro*

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1 In this study we have examined the effects of nociceptin, an endogenous ligand for the opioid-like receptor ORL_1, on the membrane properties of rat locus coeruleus (LC) neurones *in vitro*, using intracellular and whole cell patch clamp recording.

2 When locus coeruleus neurones were voltage clamped to −60 mV, application of nociceptin caused an outward current in all cells examined ($n=49$), with an EC_{50} of 90 nM. Neither the potency nor the maximal effect of nociceptin was altered in the presence of the peptidase inhibitors, bestatin (20 μM) or thiorphan (2 μM).

3 The outward currents caused by nociceptin in 2.5 mM extracellular K^+ reversed polarity at −123 mV, more negative than the predicted K^+ reversal potential of −105 mV. Increasing extracellular K^+ to 6.5 mM resulted in a shift of the reversal potential of +25 mV, a shift consistent with a K^+ conductance. The conductance activated by nociceptin showed mild inward rectification.

4 Application of a high concentration of nociceptin (3 μM) occluded the current produced by simultaneous application of high concentrations of Met-enkephalin (10 μM), (3 μM) somatostatin and UK 14304 (3 μM), indicating that nociceptin activated the same conductance as μ-opioid and somatostatin receptors and α_2-adrenoceptors.

5 The actions of nociceptin were weakly antagonized by the opioid antagonist, naloxone, with pK_b's estimated from 2 cells of −4.23 and −4.33. The μ-opioid antagonist, CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Pen-Thr-NH_2, 1 μM), the opioid antagonist, nalorphine (30 μM), or the somatostatin antagonist, CPP (*cyclo*(7-aminoheptanoyl-Phe-D-Trp-Lys-Thr[Bzl]) 3 μM) did not affect the nociceptin-induced current.

6 Dynorphin A (3 μM), another putative endogenous ligand for ORL_1, caused a robust outward current in locus coeruleus neurones that was, however, completely antagonized by moderate concentrations of naloxone (300 nM − 1 μM).

7 Continuous application of nociceptin (3 μM) resulted in a decrease of the outward current to a steady level of 70% of the maximum response with a $t_{1/2}$ of 120s. Desensitization was largely homologous because simultaneous application of Met-enkephalin (30 μM) during the desensitized period of the nociceptin response resulted in an outward current that was 92% of control responses to Met-enkephalin in the same cells. Conversely, continuous application of Met-enkephalin (30 μM) resulted in a decrease of Met-enkephalin current to a steady level that was 54% of the initial current. During this desensitized period application of nociceptin (3 μM) resulted in a current that was 78% of the control responses to nociceptin in the same cells.

8 Thus nociceptin potently activates an inwardly rectifying K^+ conductance in locus coeruleus neurones, with a pharmacological profile consistent with activation of the ORL_1 receptor. Dynorphin A does not appear to be a ligand for ORL_1 in rat locus coeruleus neurones.

Keywords: Nociceptin; ORL_1; orphanin FQ; potassium conductance; locus coeruleus

Introduction

Nociceptin (Meunier *et al.*, 1995), also called orphanin FQ (Reinscheid *et al.*, 1995), is a recently identified ligand for an 'orphan' opioid receptor, ORL_1 (Mollereau *et al.*, 1994). ORL_1 was identified from a human cDNA library on the basis of a close homology to known opioid receptors (Mollereau *et al.*, 1994) and was isolated independently by several groups. However, when ORL_1 and its homologues were transfected into heterologous systems, receptor binding could not be detected with a wide range of radiolabelled opioid and non-opioid ligands (Mollereau *et al.*, 1994; Fukuda *et al.*, 1994; Wang *et al.*, 1994; Lachowicz *et al.*, 1995). In CHO-K1 cells transfected with ORL_1 the opioid agonist, etorphine, modestly inhibited forskolin-stimulated adenylyl cyclase activity in a manner weakly antagonized by diprenorphine, but all other opioid ligands tested were without effect (Mollereau *et al.*, 1994). The endogenous opioid peptide, dynorphin A, also weakly inhibited adenylyl cyclase activity in CHO-K1 and HEK-293 cells transfected with XOR1 (Zhang & Yu, 1995), a rat homologue of ORL_1 (Wang *et al.*, 1994). The inhibition of forskolin-stimulated adenylyl cyclase activity in cells transfected with ORL_1 (or its rat homologue) was used as a bioassay to facilitate the detection and purification of endogenous ligands for the receptor. Two groups independently isolated the same heptadecapeptide ligand and named it nociceptin (Meunier *et al.*, 1995), and orphanin FQ (Reinscheid *et al.*, 1995), respectively. Nociceptin most closely resembles the dynorphin family of peptides, but it lacks the N-terminal tyrosine essential for peptides to be active at opioid receptors (Chavkin & Goldstein, 1981).

Nociceptin inhibits adenylyl cyclase activity in cells transfected with ORL_1 (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995) and nociceptin was recently shown to increase K^+ conductance in rat dorsal raphe neurones *in vitro* (Vaughan & Christie, 1996), and to inhibit voltage-dependent Ca^{2+} channels and mobilize intracellular Ca^{2+} in the human neuroblastoma cell line, SH-SY5Y (Connor *et al.*, 1996).

In situ hybridization studies have indicated that mRNA for ORL_1 is expressed in many brain regions (Mollereau *et al.*, 1994; Bunzow *et al.*, 1994; Lachowicz *et al.*, 1995), including

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the locus coeruleus (LC; Fukuda *et al.*, 1994), a brainstem nucleus that is an important site of action for opioids (Pepper & Henderson 1980; Williams *et al.*, 1982). In the present study we sought to define the actions of nociceptin on LC neurones and to compare the responses elicited by nociceptin to those obtained with ligands for the μ-opioid receptors found on LC neurones.

Methods

Electrophysiological recordings

Male Sprague-Dawley rats (150–300 g) were anaesthetized with halothane and then killed by cervical dislocation. Horizontal slices (between 300–350 μm thick) containing the LC were cut and maintained at 35°C in physiological saline of the following 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 recording, slices were hemisected, fully submerged and perfused at rate of 1.5 ml min^{-1}. Drugs were applied to the slice by changing the perfusion buffer to one that differed only in its content of drug. Experiments involving dynorphin A or high concentrations of Met-enkephalin (30 μM) were conducted in the continuous presence of the peptidase inhibitors, bestatin (20 μM) and thiorphan (2 μM).

Intracellular recordings were made with microelectrodes of resistance 35–55 MΩ, filled with 2 M KCl and dipped in 'Sigmacote' (Sigma, St Louis MO, U.S.A.). Membrane current and voltage was measured with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA, U.S.A.); the recordings were plotted directly onto chart paper and also digitized for later analyses (PCLAMP and AXOTAPE software, Axon Instruments). Membrane potential was clamped at −60 mV using discontinuous voltage-clamp with switching frequencies of 3.5–4.5 kHz. The potential at the headstage was continuously monitored on a separate oscilloscope. Some recordings were made by the whole cell patch clamp technique, in order to obtain optimal voltage control for the determination of steady state current/voltage relationships in the cells. For these experiments the electrodes (resistance 3–6 MΩ) contained (mM): K-gluconate 125, NaCl 15, $MgCl_2$ 2, HEPES 10, EGTA 11, MgATP 2, NaGTP 0.25, pH 7.3. Recordings were made as described elsewhere (Osborne *et al.*, 1996). Cells were clamped at −60 mV and the membrane potential was stepped between −50 and −140 mV for 250 ms. All data are expressed as mean ± s.e. mean.

Drugs and chemicals

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). Buffer salts were from BDH Australia. Met-enkephalin, bestatin and thiorphan were purchased from Sigma Australia. CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Pen-Thr-NH_2), idazoxan hydrochloride, naloxone hydrochloride and UK14304 (5-Bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6 quinoxalinamine) were purchased from Researched Biochemicals International (Natick, MA, U.S.A.). Somatostatin$_{1-14}$ was purchased from Auspep (Melbourne, Australia). CPP (*cyclo*(7-aminoheptanoyl-Phe-D-Trp-Lys-Thr[Bzl])) was purchased from Bachem (Bubendorf, Switzerland). Nalorphine hydrochloride was a kind gift of Dr Laurie Mather. Dynorphine A was a kind gift from the National Institute on Drug Abuse, U.S.A.

Results

Nociceptin produced a concentration-dependent outward current when applied to LC neurones (Figure 1). All LC neurones responded to nociceptin applied at a concentration of 1 nM or more ($n=49$). The effects of nociceptin reversed on washout. Maximally effective concentrations of nociceptin (3–10 μM) caused an outward current of 451 ± 39 pA ($n=16$), significantly greater than that produced by high concentrations of Met-enkephalin (10 μM) (394 ± 28 pA, $P<0.001$, paired t test) in the same cells (Figure 1a). A concentration - response relationship for nociceptin activation of the outward current was determined by application of one or more concentrations of nociceptin to single neurones and expressing the resulting currents as a percentage of the current caused by 10 μM Met-enkephalin in the same cells (Figure 1b). The concentration - response relationship for nociceptin was fitted with a logistic function to produce an EC_{50} for nociceptin activation of the outward current of 90 ± 25 nM. The Hill slope of the curve was 0.7 ± 0.1.

Application of the peptidase inhibitors, bestatin (2 μM) and thiorphan (20 μM), did not alter the magnitude of the outward current produced either by submaximally effective concentrations of nociceptin (10–100 nM, $n=4$) or by a maximally effective concentration of nociceptin (3 μM, 409 ± 69 pA in control $n=8$, versus 414 ± 62 pA in bestatin/thiorphan, $n=7$).

We examined the conductance activated by nociceptin by determining the steady state current-voltage relationships for LC neurones in the presence and absence of nociceptin. These experiments were performed by the whole cell patch clamp

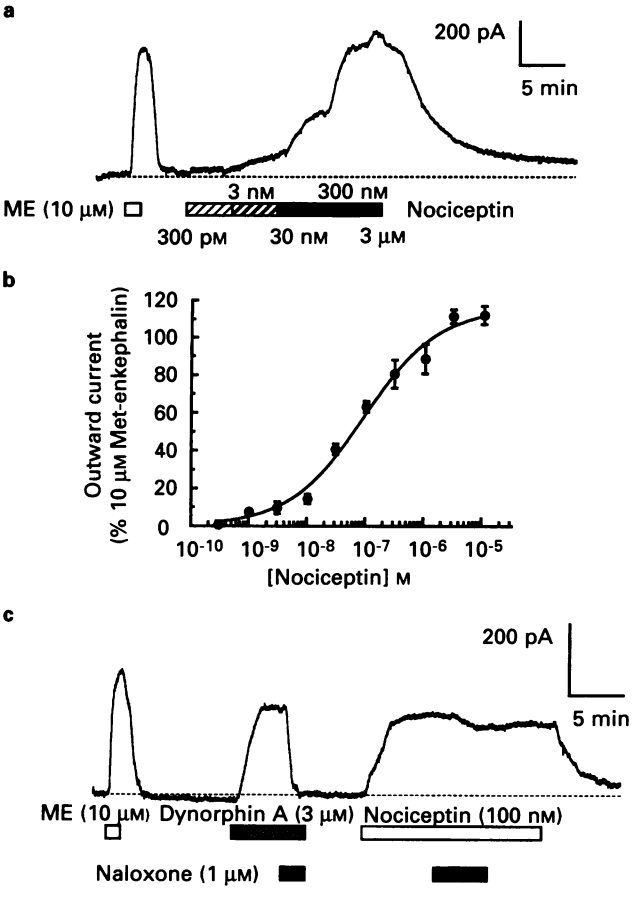

Figure 1 The effect of nociceptin on membrane currents of LC neurones. (a) Membrane currents induced by Met-enkephalin (ME, 10 μM) and increasing concentrations of nociceptin (3 nM–3 μM) in a single neurone voltage clamped at −60 mV. Drugs were superfused for the duration shown by the horizontal bars. (b) Concentration-response relationship of outward currents induced by nociceptin in LC neurones. Each point represents between 3 and 12 individual cells tested at each concentration of nociceptin. The points were fit with a logistic function yielding an EC_{50} of 90 ± 25 nM. (c) The membrane currents induced by the putative ORL_1 ligand, dynorphin A (3 μM), were blocked by a moderate concentration of naloxone, those caused by an equi-effective concentration of nociceptin were essentially unaffected.

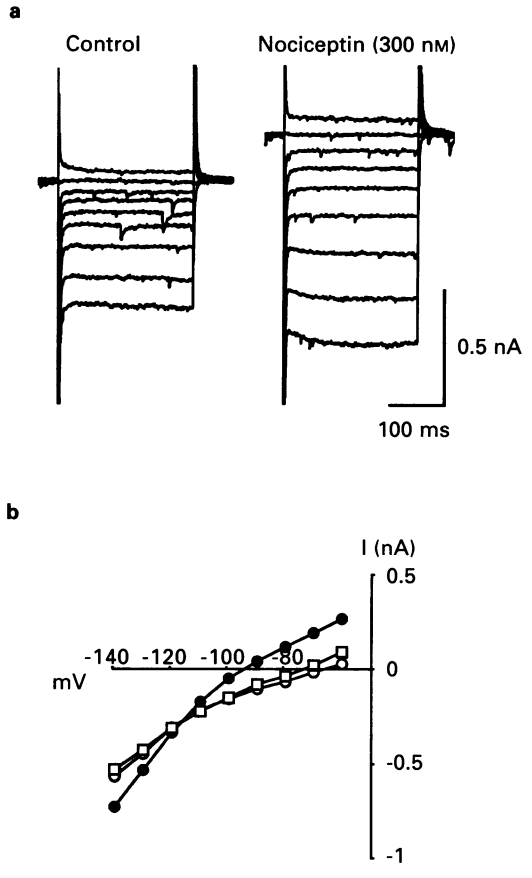

Figure 2 Nociceptin increases inwardly rectifying K^+ conductance of LC neurones. These recordings were made by the whole cell patch clamp technique, as outlined in the methods. (a) Voltage command steps 250 ms in duration were in 10 mV increments from −50 to −140 mV from a holding potential of −60 mV. The resulting currents in the absence (left) and presence (right) of (300 nM) nociceptin in a single neurone are shown. (b) The current-voltage relationships (control (○), in nociceptin (●) and wash (□)) are plotted from the amplitude of evoked currents shown in (a).

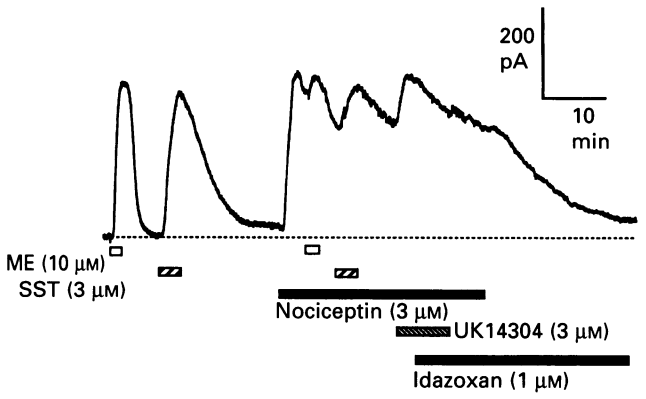

Figure 3 Nociceptin activates the same population of K^+ channels as μ-opioid, somatostatin and α_2 receptors in LC neurones. The trace shows the continuous record of the membrane currents induced by maximally effective concentrations of various agonists in a single LC neurone. Drugs were superfused for the duration shown by the horizontal bars. Addition of a maximally effective concentration of nociceptin occluded the subsequent increases in K^+ current by Met-enkephalin (ME, 10 μM), somatostatin (SST, 3 μM) and UK 14304 (3 μM).

technique, in order to optimize voltage control of the neurones. In 7 out of 9 cells a clear reversal of the nociceptin-induced current was obtained, (e.g. Figure 2). Where it could be determined, the reversal potential for the potential for the nociceptin-induced current was -123 ± 4 mV ($n=7$). The calculated reversal potential for a pure K^+ conductance with 2.5 mM extracellular K^+ is −106 mV. When the nociceptin-induced current was re-examined in 6.5 mM external K^+ the reversal potential shifted to -101 ± 5 mV ($n=4$), a shift of +22 mV. The predicted shift in reversal potential for a K^+ conductance is +25 mV under these conditions. The current activated by nociceptin also showed mild inward rectification. When measured in 2.5 mM extracellular K^+, the slope conductance of the nociceptin-activated current at potentials between −60 and −90 mV was 3.2 ± 0.6 nS, and was 4.6 ± 1 nS at potentials between −110 and −130 mV.

We further examined whether the conductance increased by nociceptin was similar to that activated by other inhibitory agonists in the LC. During continuous application of a maximally effective concentration of nociceptin (3 μM), co-application of high concentrations of Met-enkephalin (10 μM), somatostatin (3 μM) and the α_2-adrenoceptor agonist, UK-14304 (3 μM) did not produce an outward current greater than that caused by nociceptin alone ($n=3$) (Figure 3).

Dynorphin A, a putative endogenous ligand for ORL_1 receptors (Zhang & Yu, 1995), when applied at a concentration of 3 μM, induced an outward current in LC neurones that was $65 \pm 4\%$ ($n=3$) of that induced by 10 μM Met-enkephalin. However, the actions of dynorphin A at this concentration were completely blocked by the opioid receptor antagonist, naloxone (300 nM–1 μM, $n=3$) (Figure 1c). In contrast, naloxone (1–10 μM, $n=4$) only weakly inhibited the nociceptin-induced outward currents. In two cells, we estimated the affinity of naloxone at receptors for nociceptin by examining cumulative dose-response curves for nociceptin in the absence and presence of various concentrations of naloxone. The pK_Bs for naloxone in these two cells were −4.33 and −4.23. Furthermore, the outward current induced by a sub-maximally effective concentration of nociceptin (100 nM) was not affected by the μ-opioid receptor antagonist, CTAP (1 μM), the opioid antagonist, nalorphine (30 μM), or the somatostatin receptor antagonist, CPP (3 μM)($n=3$ for each).

Application of μ-opioid or α_2 adrenoceptor agonists to LC neurones for several minutes results in a decrease of the response to the agonist. Continuous application of a maximally effective concentration of nociceptin (3 μM) resulted in a decline in the outward current from maximum of 355 ± 45 pA to steady level of 254 ± 36 pA, a decline of $29 \pm 2\%$ ($n=7$) (Figure 4a). The $t_{1/2}$ for this desensitization was 120 ± 17s. We tested whether this desensitization was homologous to the nociceptin receptor by applying a high concentration of Met-enkephalin when the nociceptin current had reached a new steady level (Figure 4b). Application of Met-enkephalin (30 μM) to 3 neurones caused an outward current of 410 ± 110 pA. When the Met-enkephalin was re-applied in the presence of nociceptin the total outward current was 381 ± 110 pA, $92 \pm 4\%$ of the original Met-enkephalin current. Continuous application of a high concentration of Met-enkephalin (30 μM) induced an outward current of 440 ± 174 pA, which decreased to a steady level of 235 ± 30 pA, a decline of $46 \pm 3\%$ ($n=4$). In these neurones a maximally effective concentration of nociceptin (3 μM) alone caused an outward current of 490 ± 101pA, when the nociceptin was reapplied during the steady phase of the Met-enkephalin (30 μM) response it induced an outward current of 370 ± 60 pA, $78 \pm 5\%$ of the original nociceptin current (Figure 5c).

Discussion

This study demonstrates that nociceptin, the endogenous ligand for ORL_1, potently and efficaciously increases an inwardly rectifying K^+ conductance in rat LC neurones. These results are consistent with the reported presence of mRNA for

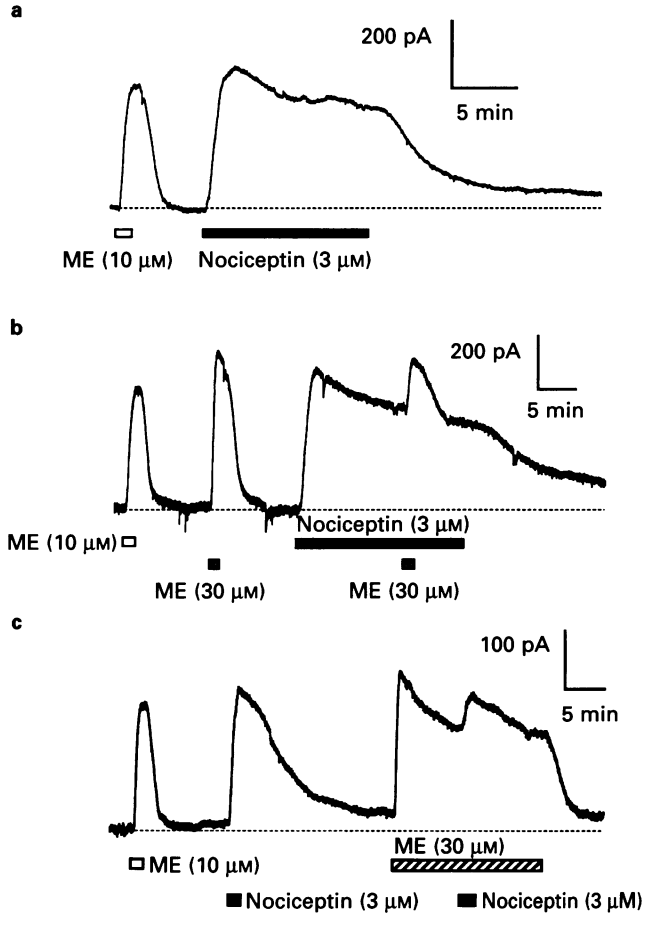

Figure 4 Nociceptin responses desensitize in a largely homologous manner. Drugs were superfused for the duration shown by the horizontal bars. (a) Application of a high concentration of nociceptin (3 μM) results in a large outward current that declines to a plateau over the duration of the nociceptin application. (b) Application of a high concentration of Met-enkephalin (30 μM) during the period of nociceptin desensitization results in a response that is not different from the earlier response to Met-enkephalin alone. (c) Application of a high concentration of nociceptin during the period of Met-enkephalin densitization results in a moderately reduced nociceptin response.

ORL_1 (Fukuda *et al.*, 1994; Bunzow *et al.*, 1994) in LC neurones. We have reported previously that nociceptin couples to an inwardly rectifying K^+ channel in rat dorsal raphe neurones (Vaughan & Christie, 1996), and have also observed a nociceptin-induced increase in K^+ conductance in the periaqueductal gray (Vaughan & Christie, unpublished observations). Both of these areas also contain mRNA encoding ORL_1 receptors (Mollereau *et al.*, 1994; Bunzow *et al.*, 1994; Lachowicz *et al.*, 1995). It thus appears that inhibition of neuronal activity is a common consequence of activation of the nociceptin/ORL_1 system.

It is likely that nociceptin acts via ORL_1 to increase the K^+ current in LC, although this cannot be demonstrated directly in the absence of selective antagonists for ORL_1. Nociceptin did not act via μ-, δ- or κ-opioid receptors to increase the K^+ current in LC because high concentrations of general opioid antagonists, naloxone and nalorphine, and the selective μ-opioid receptor antagonist CTAP, did not inhibit the actions of nociceptin. Furthermore, there was no significant cross-desensitization between the actions of nociceptin and Met-enkephalin. Finally, nociceptin activates K^+ currents in dorsal raphe neurones (Vaughan & Christie, 1996), cells that are not thought to contain μ-opioid receptors (Williams *et al.*, 1988).

The potency of nociceptin observed in LC (90±25 nM) is somewhat weaker than that reported for nociceptin inhibition of adenylyl cyclase in CHO-K1 cells expressing ORL_1 (IC_{50} about 1 nM, Meunier *et al.*, 1995; Reinscheid *et al.*, 1995), for increasing K^+ conductance in dorsal raphe neurones (12±2 nM, Vaughan & Christie 1996), and for inhibiting Ca^{2+} currents and mobilizing intracellular Ca^{2+} in SH-SY5Y cells (42±13 nM and 60±22 nM respectively, Connor *et al.*, 1996). There are a number of possible reasons for the moderately reduced potency of nociceptin in the LC when compared with these other systems. Firstly, in the cells where ORL_1 was heterologously expressed and shown to inhibit adenylyl cyclase there is likely to be a large receptor reserve for coupling to any second messenger system. Secondly, an important factor when considering peptide potencies in brain slices versus cells in culture is degradation of the agonist (Williams *et al.*, 1987). However, we found no evidence that the peptidase inhibitors, bestatin and thiorphan, enhanced the potency of nociceptin in LC, despite the fact that peptidases sensitive to these inhibitors are important mediators of enkephalin degradation in the LC (Williams *et al.*, 1987). Similarly, bestatin and thiorphan also had no effect on the potency of nociceptin in rat dorsal raphe neurones (Vaughan & Christie, unpublished observations). It is possible that peptidases insensitive to bestatin or thiorphan are important in the degredation of nociceptin in the LC, because in dorsal raphe neurones nociceptin was approximately 7 times more potent than in LC. This difference could reflect the fact that the whole cell patch clamp recording technique used in the dorsal raphe selects cells near the surface of slices, but it could also be indicative of a moderate difference in the receptor reserve for ORL_1 activation of K^+ currents between the two types of neurone.

We found no evidence that the endogenous κ-opioid receptor preferring agonist dynorphin A (Chavkin *et al.*, 1982; Goldstein & Naidu, 1989) acted at ORL_1 receptors in LC neurones. It has been suggested that dynorphin A is an endogenous agonist for ORL_1 on the basis of experiments showing that dynorphin A could both activate K^+ channels in *Xenopus* oocytes transfected with ORL_1 and inhibit the adenylyl cyclase activity of ORL_1-transfected CHO-K1 and HEK-293 cells (Zhang & Yu, 1995). Dynorphin A (3 μM) did induce a robust outward current in LC neurones; however, this effect was rapidly reversed by application of moderate concentrations of naloxone, consistent with dynorphin A activation of μ-opioid receptors. Although dynorphin A has at least a 30 fold selectivity for κ-opioid receptors over μ-opioid receptors, it still has an affinity of considerably less than 1 μM for μ-opioid receptors *in vitro* (e.g. Goldstein & Naidu, 1989). The finding that a high concentration of dynorphin A did not induce a significant naloxone-insensitive outward current in LC is consistent with our observation that high concentrations of the peptide induced only small outward currents in dorsal raphe neurones (Vaughan & Christie, 1996). Taken together these findings provide evidence that dynorphin A does not act potently at ORL_1 receptors in neurones, making it unlikely that it is an endogenous agonist for the receptor.

Activation of many G-protein coupled receptors increases an inwardly rectifying K^+ conductance in LC neurones including μ-opioid (North & Williams, 1985) and somatostatin receptors, (Inoue *et al.*, 1988), α_2-adrenoceptors (North & Williams, 1985), and galanin receptors (Pieribone *et al.*, 1995). It appears that nociceptin activates the same population of K^+ channels as μ-opioid-, somatostatin-receptors and α-adrenoceptors because the application of a high concentration of nociceptin occluded the effects of simultaneous applications of Met-enkephalin, somatostatin and UK14304. This occlusion was unlikely to be due to a heterologous desensitization of the K^+ channels, as we showed that there was virtually no cross-desensitization between nociceptin and Met-enkephalin. Previous studies demonstrated that there was also little cross-desensitization between μ-opioid receptor, α_2-adrenoceptor and somatostatin receptor activation of the inward rectifier in LC neurones (Harris & Williams, 1991; Fiorello & Williams, 1996). The desensitization produced by a maximally effective concentration of nociceptin (29%) was less than that produced

by a maximally effective concentration of Met-enkephalin (46%). It has been demonstrated previously that receptors coupled to inward rectifier K^+ channels in the LC exhibit different degrees of desensitization, with α_2 receptors showing much less desensitization than μ-opioid or somatostatin receptors (Harris & Williams, 1991; Fiorello & Williams, 1996). The desensitization at the receptor for nociceptin is more than that observed for the α_2-adrenoceptor but less than that seen at μ-opioid or somatostatin receptors in the LC.

We have shown that the nociceptin/ORL_1 system inhibits the activity of neurones by opening K channels (this paper, Vaughan & Christie 1996), and nociceptin has also been shown to inhibit adenylyl cyclase in cells transfected with ORL_1 (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995) as well as to inhibit voltage-dependent Ca currents and mobilize intracellular Ca^{2+} in SH-SY5Y neuroblastoma cells (Connor *et al.*, 1996). Inhibitory actions of nociceptin acting on ORL_1 receptors are likely to be functionally important in diverse regions of the nervous system.

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