Pre- and postsynaptic inhibition by nociceptin in guinea pig small intestinal myenteric plexus in vitro

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Liu, Sumei, Hong-Zhen Hu, Jun Ren, Chuanyun Gao, Na Gao, Zhong Lin, Yun Xia, and Jackie D. Wood. Pre- and postsynaptic inhibition by nociceptin in guinea pig small intestinal myenteric plexus in vitro. Am J Physiol Gastrointest Liver Physiol 281: G237–G246, 2001.—Actions of nociceptin on electrical and synaptic behavior of morphologically and neurochemically identified neurons in the guinea pig duodenal myenteric plexus were studied with conventional techniques. Nociceptin hyperpolarized the membrane potential in 104 of 121 AH-type and 28 of 51 S-type neurons with an EC50 of 22.5 ± 4.4 nM and slow synaptic excitation in 38 of 45 neurons (EC50 = 15.1 ± 1.6 nM). Presynaptic inhibitory action of nociceptin was unaffected by naloxone and was antagonized by [Phe¹-psi(CH₂-NH)-Gly²]nociceptin(1–13)-NH₂. Nociceptin-evoked responses were blocked by pertussis toxin and unaffected by naloxone. The selective opioid receptor-like (ORL)₁ receptor antagonist [Phe¹-psi(CH₂-NH)-Gly²]nociceptin(1–13)-NH₂ suppressed the nociceptin-evoked responses while behaving like a partial agonist. The nonselective ORL₁ antagonist naloxone benzoylhydrazone competitively suppressed nociceptin actions with a pA2 value of 5.8. Nociceptin acted at presynaptic inhibitory receptors to suppress fast excitatory nicotinic postsynaptic potentials in 25 of 30 neurons (EC50 = 22.5 ± 4.4 nM) and slow synaptic excitation in 38 of 45 neurons (EC50 = 15.1 ± 1.6 nM). Presynaptic inhibitory action of nociceptin was unaffected by naloxone and was antagonized by [Phe¹-psi(CH₂-NH)-Gly²]nociceptin(1–13)-NH₂ or naloxone benzoylhydrazone. The results suggest that nociceptin acts both pre- and postsynaptically by activating an ORL₁ receptor that is distinct from typical naloxone-sensitive opioid receptors.

autonomic nervous system; enteric nervous system; intestine; orphanin FQ; opioid receptor-like₁ receptor

THE HEPTADECAPEPTIDE NOCICEPTIN (20), also known as orphanin FQ (29), is a recently discovered neuropeptide that has been identified as the endogenous ligand for the “orphan” opioid receptor-like (ORL)₁ receptor (3, 4, 11, 21, 33, 35). Despite evidence for certain structural analogies between nociceptin and opioids as well as between the ORL₁ receptor and opioid receptors, nociceptin selectively binds to the ORL₁ receptor but not to µ-, δ-, or κ-opioid receptor subtypes, and opioid peptides do not bind to the ORL₁ receptor (20, 21, 29). Like other opioid receptors, the ORL₁ receptor is coupled to G proteins (20, 29) that, when activated, result in inhibition of forskolin-stimulated adenylyl cyclase activity (20, 29), suppression of Ca²⁺ channels (7, 16), activation of inward rectifying K⁺ channels (6, 31, 32), and modulation of neurotransmitter release (10, 17, 28, 34).

Both nociceptin and ORL₁ receptors are widely expressed in discrete areas of the central nervous system and are thought to serve a number of functional roles including processing of nociceptive stimuli, control of neuroendocrine functions, and regulation of blood pressure and water balance (8). The presence of ORL₁ receptors has also been reported in several peripheral organs including the intestine, vas deferens, and spleen, and biological effects of nociceptin have been shown at these sites (21, 27, 33). Nociceptin evokes TTX-sensitive contractions in the isolated colon of rats and mice, which suggests that enteric neurons are involved (26, 30, 38). Nociceptin suppresses the electrically stimulated contractions of the guinea pig ileum (39) and suppresses acetylcholine release in response to electrical field stimulation in rat stomach and small intestine (38).

Several reports describe the occurrence of nociceptin-like immunoreactivity in the enteric nervous system of the rat and guinea pig (2, 38). It is localized to cell bodies and dense fiber networks in the myenteric plexuses of the duodenum, ileum, and colon. Aside from this, the physiological role of nociceptin in the enteric nervous system is unknown. The aim of the present study was to examine the effects of nociceptin on electrical and synaptic behavior of myenteric neurons in the guinea pig duodenum. A preliminary report of the results has appeared in abstract form (18).

MATERIALS AND METHODS

Adult male Hartley strain guinea pigs (400–600 g) were stunned by a blow to the head and exsanguinated from the cervical vessels according to procedures approved by the Ohio State University Laboratory Animal Care and Use Committee. A 2- to 5-cm segment of duodenum was removed proximal to the pyloric region. Preparations of the myenteric
plexus to be used for electrophysiological recording were microdissected as described earlier (37). The preparation was mounted in a 2.0-ml recording chamber that was superfused at a rate of 10–15 ml/min with Krebs solution warmed to 37°C and gassed with 95% O2–5% CO2 to buffer pH to 7.3–7.4. The composition of the Krebs solution was (in mM) 120.9 NaCl, 5.9 KCl, 1.2 MgCl2, 1.2 NaH2PO4, 14.4 NaHCO3, 2.5 CaCl2, and 11.5 glucose. The Krebs solution contained nifedipine (1 μM) and scopolamine (1 μM) to prevent smooth muscle movements from dislodging the intracellular electrode.

The myenteric ganglia were visualized with differential interference contrast optics and epilumination. Ganglia selected for study were immobilized with 100-μm-diameter L-shaped stainless steel wires placed on either side of the ganglion. Transmembrane electrical potentials were recorded with conventional intracellular microelectrodes filled with 2% biocytin in 2 M KCl containing 0.05 M Tris buffer (pH 7.4). Resistances of the electrodes were 80–120 MΩ. The same electrodes were used to inject the neuronal tracer biocytin intraneuronally. Passage of hyperpolarizing current into the impaled neurons. The preamplifier (M767, World Precision Instruments, Sarasota, FL) was equipped with a bridge circuit for intraneuronal current injection. Fast and slow excitatory postsynaptic potentials (EPSPs) were evoked by electrical shocks (0.1–20 Hz) applied focally to interganglionic connectives with 20-μm-diameter Teflon-insulated Pt wire electrodes connected through stimulus-isolation units (Grass S48) to Grass S48 stimulators. Chart records were made on Astro-Med thermal recorders. The amplitude of the spikes in some of the recordings was blunted by the low-frequency response of the recorder. All data were recorded on videotape for later analysis.

At the end of each recording session, the marker dye biocytin was injected into the impaled neurons from the recording electrodes by the passage of hyperpolarizing current (0.5 nA for 10–30 min). The anal end of the preparations was marked, and the tissue was transferred to a disposable chamber filled with fixative that contained 4% formaldehyde plus 15% of a saturated solution of picric acid and was kept at 4°C overnight. The preparations were cleared in three changes of dimethyl sulfoxide and three 10-min washes with PBS, reacted with avidin coupled to horseradish peroxidase, carried through a diaminobenzidine color-developing reaction, and dehydrated in alcohol. They were then mounted in Canada balsam and examined microscopically.

Neurochemical coding of the neurons that responded to nociceptin was determined by first reacting the preparations with streptavidin coupled to fluorescein to reveal biocytin fluorescence. They were then processed for immunohistochemical demonstration of calbindin, calretinin, or nitric oxide synthase (NOS) immunoreactivity. For calbindin localization, mouse anti-calbindin antiserum at a dilution of 1:2,000 was used; for calretinin, goat anti-calretinin at 1:1,500; and for NOS, rabbit anti-NOS at 1:500. The preparations were then incubated with secondary antibodies labeled with Texas red. Fluorescent labeling was examined under a Nikon Eclipse E600 fluorescent microscope that was equipped with appropriate filters and a SPOT-2 chilled color and B/W digital camera (Diagnostic Instruments, Sterling Heights, MI).

The actions of nociceptin and related pharmacological agents were studied by either pressure microejection or application in the superfusion solution. Micropipettes (10-μm diameter) manipulated with the tip close to the impaled neurons were used to microeject the substances. Pressure pulses of nitrogen with predetermined force and duration were applied to the micropipettes through electronically controlled solenoid valves. The pharmacological agents used in this study and their sources were as follows. Nociceptin (orphanin FQ), naloxone, acetylcholine, substance P, 5-hydroxytryptamine (5-HT), TTX, pertussis toxin (PTX), bestatin, D-thiorphan, and barium chloride (BaCl2) were obtained from Sigma (St. Louis, MO). [Phe1-psi(CH2-NH)-Gly2]nociceptin(1–13)-NH2 (NC-NH2), nocistatin, and naloxone benzoylhydrazide (NBH) were from RBI (Natick, MA). Nociceptin(1–7) was from Toceis Cookson (Ballwin, MO). Fluorescein streptavidin was from Vector (Burlingame, CA). Calbindin, calretinin, and NOS antiserum were from Chemicon (Temecula, CA).

Data are expressed as means ± SE; n values refer to the number of neurons. The concentration-response curves for drug-induced responses were constructed with the following least-squares fitting routine: 

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V = V_{\text{max}}/[1 + (EC_{50}/C)^n_{H}]
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where \(V\) is the observed response, \(V_{\text{max}}\) is maximum response, \(EC_{50}\) is the concentration that induces the half-maximal response, \(C\) is the concentration, and \(n_{H}\) is the apparent Hill coefficient. Antagonist potency was assessed by constructing Schild plots for three different concentrations of the antagonist and the calculation of pA2 values (1).

RESULTS

Results were obtained from 172 myenteric neurons with impalements lasting from 20 min to 8 h. All neurons had resting membrane potentials greater than −45 mV. The neurons were classified into AH and S type according to the criteria described previously by Hirst et al. (15) and Wood (36). Of all the neurons examined, 121 were identified as AH type and 51 as S type.

Nociceptin-induced hyperpolarization. Application of nociceptin either by addition to the superfusion solution or by pressure microejection hyperpolarized the membrane potential, decreased the input resistance, and suppressed excitability in 104 of 121 AH-type and 28 of 51 S-type neurons. The average maximal membrane hyperpolarization during application of 300 nM nociceptin was 19.8 ± 4.5 mV (n = 11). The input resistance with this concentration of nociceptin was reduced by 34.5 ± 9.9% (n = 8). Suppression of excitability was reflected by the failure of depolarizing current pulses to evoke action potentials (data not shown), blockade of anodal-break excitation at the offset of intraneuronally injected hyperpolarizing current pulses (Figs. 1A and 2B), and inhibition of the ongoing discharge of action potentials in spontaneously active neurons (Figs. 2A and 4A). These effects began within 10–30 s after entry of nociceptin into the tissue chamber and developed gradually over a period of 1–2 min. Recovery of membrane potential and input resistance to control levels required 7–15 min after washout. In 43.3% (13 of 30) of the neurons, subsequent applications of nociceptin at the same concentration evoked weaker responses, presumably because of receptor desensitization phenomena. In neurons without apparent desensitization, the effects of nociceptin were concentration dependent, with an EC50 of 11.9 ± 1.2 nM (Fig. 1B). The inhibitory effects of 100 nM nociceptin were unaffected by the addition of 300 nM TTX. The mean hyperpolarizing response was 15.0 ± 3.0 mV in con-
controls (n = 6) and 14.5 ± 3.1 mV in TTX (n = 6), indicating a direct action of nociceptin rather than activation of neurons synaptically connected with the impaled ganglion cell.

The morphology of the neurons exposed to nociceptin was identified in 89 of 172 injected cells, and these were classified according to their shape (12, 36). Nociceptin hyperpolarized multipolar Dogiel type II neurons (92%), uniaxial Dogiel type I neurons (45%), and uniaxial neurons with numerous filamentous dendrites (53%). All the nociceptin-responsive Dogiel type II neurons had AH-type electrophysiological behavior, and the Dogiel type I neurons had S-type electrophysiological behavior. Neurons with filamentous dendrites belonged to either AH or S cell types.

Data on the immunoreactivity for calbindin, calretinin, and NOS were obtained from 34 neurons that were hyperpolarized by nociceptin. Thirteen AH-type neurons with Dogiel type II morphology were tested for calbindin immunoreactivity; of these, 10 neurons (77%) were immunoreactive (Fig. 3, A–C). Eleven S-type neurons with Dogiel type I morphology were tested for NOS immunoreactivity; of these, six neurons (54%) were immunopositive (Fig. 3, D–F). Ten S-type neurons with Dogiel type I morphology were tested for calretinin immunoreactivity; none was immunoreactive (Fig. 3, G–I).

Mediation of nociceptin responses by ORL1 receptors. To determine whether the responses evoked by nociceptin were mediated by the ORL1 receptor or by one of the classic opioid receptors, the nonselective opioid receptor antagonist naloxone, the selective ORL1 receptor antagonist NC-NH2, and the nonselective ORL1 receptor antagonist NBH were used. Bath application of nociceptin (30 nM) elicited a membrane hyperpolarization of 10.4 ± 3.3 mV (n = 5; Fig. 2A) that was unaltered by the addition of 10 μM naloxone (10.0 ± 2.8 mV; n = 5; Fig. 2A). On the other hand, the hyperpolarization evoked by 1 μM [Met]enkephalin in the same neurons was suppressed significantly by 10 μM naloxone (8.8 ± 2.0 vs. 1.6 ± 0.7 mV; n = 5; P < 0.05; Fig. 2A). The putative ORL1 antagonist NC-NH2 (1 μM) suppressed the hyperpolarization evoked by 30 nM nociceptin by 60% when coapplied with nociceptin in the superfusion solution (13.1 ± 1.1 vs. 5.2 ± 0.8 mV; n = 5; P < 0.05; Fig. 2B). Application of the putative ORL1 antagonist (1 μM) alone produced a small hyperpolarizing response in 12 of 17 cells (Fig. 2B). The K3-receptor agonist NBH has been reported to exert antagonist actions at ORL1 receptors (9, 23). Figure 4A shows concentration-dependent suppression of the effects of nociceptin (30 nM) by NBH. The concentration-response curve for nociceptin was shifted in a rightward direction by NBH (Fig. 4B). Schild analysis confirmed that the antagonism was competitive with a pA2 value of 5.8 (Fig. 4C). NBH (1, 3, or 10 μM) did not change the membrane potential when applied alone.

Pharmacological analysis of the hyperpolarizing action of NC-NH2 showed the threshold concentration for measurable membrane hyperpolarization to be 30–100 nM. The EC50 was 165.4 ± 20.96 nM, and the maximal hyperpolarization was 8.0 ± 1.1 mV (n = 5), evoked by 3 μM NC-NH2. NC-NH2 appeared to be a partial agonist at the ORL1 receptor because the maximum hyperpolarizing effect obtained for NC-NH2 (3 μM) was only ~40% of that for 300 nM nociceptin. The hyperpolarizing action of 1 μM NC-NH2 was also decreased or abolished by 3 μM NBH (n = 5; data not shown).

Evidence for increased K+ conductance. Plots of current-voltage relations revealed decreased input resistance during the hyperpolarizing action of nociceptin that was reflected by a decreased slope relative to control values. The current-voltage curves obtained in the presence and absence of nociceptin intersected at membrane potentials between −80 and −105 mV, with an average of −90.5 ± 1.2 mV (n = 7; Fig. 5A).

**Fig. 1.** Inhibitory action of nociceptin on myenteric neurons in the guinea pig duodenum. **A:** nociceptin (1–300 nM) evoked concentration-dependent membrane hyperpolarization associated with a rightward change in input resistance and a decrease in neuronal excitability in an AH neuron. Decreased input resistance was reflected by reduced amplitude of electronic potentials produced by repetitive injection of constant-current hyperpolarizing pulses. Decreased neuronal excitability was reflected by the disappearance of anodal-break excitation at the offset of the hyperpolarizing current pulses. **B:** morphology of the neuron from which data were obtained. **C:** concentration-response relation for maximal membrane hyperpolarization produced by nociceptin. Data were fitted with the logistic equation (see MATERIALS AND METHODS). Each point represents 5–12 neurons. **EC50** = 11.9 ± 1.2 nM; Hill coefficient = 0.99.
suggested that the reversal potential for the conductance change was near the estimated K_\text{1} equilibrium potential (36). This suggestion was reinforced by observations that manual current clamp of the membrane potential to progressively greater levels of hyperpolarization was accompanied by a progressive decrease in the amplitude of the hyperpolarizing response to nociceptin (Fig. 5C). Current clamp to membrane potentials more positive than the resting potential resulted in an increase in the amplitude of the hyperpolarizing responses to nociceptin (Fig. 5C). The hyperpolarizing responses were nullified when the membrane potential was clamped between -90 and -105 mV. The ionic nature of the nociceptin effects was investigated further by the application of 300 \mu M BaCl_2 to nonspecifically block K^- channels. Nociceptin (100 nM) did not hyperpolarize the neurons in the presence of 300 \mu M BaCl_2 (n = 5; Fig. 5C). This was consistent with the hypothesis that increased K^- conductance was involved in the hyperpolarizing action.

PTX, which inhibits the activity of the G_\alpha/Go G protein family, was used to test the hypothesis that the receptors for nociceptin were G protein coupled. Longitudinal muscle-myenteric plexus preparations were incubated for 14–16 h at 37°C in culture medium containing 400 ng/ml of PTX in an incubator with a 5% CO_2 atmosphere. The hyperpolarizing action of 100 nM nociceptin did not occur in any of 11 neurons after incubation with PTX. Nociceptin (100 nM) evoked characteristic hyperpolarizing responses (mean = 17.07 ± 1.71 mV) in 15 neurons from longitudinal muscle-myenteric plexus preparations incubated in the same way without PTX.

Effects of protease inhibitors nociceptin(1–7) and nocistatin. To exclude the possibility that the inhibitory actions of nociceptin were due to products resulting from proteolytic degradation of the peptide, the protease inhibitors bestatin (20 \mu M) and DL-thiorphan (2 \mu M) were applied together with nociceptin (100 nM). The inhibitory effects of nociceptin were unaffected by the presence of the protease inhibitors (n = 5). Application of 10 \mu M nociceptin(1–7), a major metabolite that is derived from the NH_2-terminal region of nociceptin, had no effect on the membrane potential and
did not interfere with the actions of nociceptin (100 nM). These findings suggest that the inhibitory effects observed for nociceptin were a result of the actions of the intact peptide.

Nocistatin is another novel heptadecapeptide derived from the same precursor as nociceptin (24, 25). Application of nocistatin (10 μM) did not change the membrane potential in any of the myenteric neurons tested (n = 6), nor did it affect the inhibitory actions of nociceptin.

Nociceptin-evoked presynaptic inhibition. Focal electrical stimulation applied to interganglionic connectives evoked fast EPSPs characteristic of well-documented nicotinic EPSPs known to occur in enteric neurons (36). Nociceptin (1–300 nM) suppressed, in a concentration-dependent manner, the stimulus-evoked fast EPSPs in most of the neurons examined (25 of 30), with an EC50 of 22.5 ± 4.4 nM (Figs. 6 and 7). Suppression of the fast EPSPs by nociceptin was unaffected by naloxone but was abolished by the selective ORL1 receptor antagonist NC-NH2 and the nonselective ORL1 receptor antagonist NBH (Fig. 6A). NC-NH2 (1 μM) suppressed fast EPSPs by 17.0 ± 1.0% in 9 of 12 neurons. This was suggestive of partial agonist activity. Nociceptin did not reduce the depolarizing responses evoked by exogenously applied acetylcholine (control, 15.0 ± 1.5 mV vs. nociceptin, 14.7 ± 1.6 mV; n = 6; P > 0.05; Fig. 6B). In four myenteric neurons, nociceptin suppressed the fast EPSPs without producing any effects on the membrane potential. This and the failure to suppress responses to applied acetylcholine suggested that the site of action of nociceptin was at presynaptic inhibitory ORL1 receptors on the nicotinic nerve terminals and that suppression of the fast EPSPs resulted from inhibition of acetylcholine release from the terminals.

Slow EPSPs in the present study were similar to those previously described (36). Focal repetitive stimulation of interganglionic nerve fibers evoked slow EPSPs in most (>80%) myenteric neurons. Nociceptin...
(1–300 nM) reversibly suppressed the amplitude of the slow EPSPs in 84% of the neurons (38 of 45). This occurred in neurons that were hyperpolarized by nociceptin (n = 31) and also in neurons in which nociceptin did not change the membrane potential (n = 7). In the neurons hyperpolarized by nociceptin, the slow EPSPs were evoked after current clamping the membrane potential back to the resting level. The effect of nociceptin was concentration dependent, with an EC50 of 15.1 ± 1.6 nM (Fig. 7). Suppression of the slow EPSPs was unaffected by naloxone but was reduced or prevented by concomitant superfusion with NC-NH2 or NBH (Fig. 8A). Application of NC-NH2 (1 μM) alone caused 21.0 ± 3.0% inhibition of slow EPSPs in five of eight neurons, indicative of partial agonist activity. Application of substance P or 5-HT, both of which are putative neurotransmitters for the slow EPSPs (36), evoked characteristic slow EPSP-like responses consisting of slowly activating depolarization, increased input resistance, and enhanced excitability. Slow responses to substance P (16.0 ± 2.4 mV; n = 6) or 5-HT (15.7 ± 1.2 mV; n = 6) were unaffected by 100 nM nociceptin (15.7 ± 1.9 and 16.3 ± 1.4 mV, respectively; P > 0.05) in the same neurons where slow EPSPs were suppressed or abolished by nociceptin (Fig. 8B). This suggested a presynaptic inhibitory action of nociceptin to suppress the release of the neurotransmitter for the slow EPSPs.

**DISCUSSION**

We found that nociceptin, an endogenous ORL1 receptor ligand, evoked concentration-dependent and reversible hyperpolarization of the membrane potential in myenteric neurons of the guinea pig duodenum. The hyperpolarizing responses were associated with decreased input resistance and suppression of excitability that appeared to reflect increased K+ conductance. Persistence of the inhibitory effect after blockade of synaptic transmission by TTX was indicative of a direct action at receptors on the neuronal cell bodies. We also found that nociceptin has a presynaptic inhibitory action on fast nicotinic excitatory synaptic transmission and non-cholinergic slow excitatory synaptic transmission in the duodenal myenteric plexus.

Three lines of evidence suggest that the nociceptin-induced membrane hyperpolarization was mediated by ORL1 receptors. First, the responses to nociceptin were reversibly blocked by the selective ORL1 receptor antagonist NC-NH2 (14), whereas naloxone, a prototypical antagonist to the μ-, δ-, and κ-subtypes of opioid receptors, was ineffective even at high concentrations. NC-NH2 alone had direct membrane hyperpolarizing effects similar to those of nociceptin, suggesting that this peptidergic antagonist might be a partial agonist. Second, NBH, a putative nonselective ORL1 receptor
antagonist, also blocked the effect of nociceptin. This naloxone derivative was first described as an agonist at $\kappa_3$- and as an antagonist at both $\mu$- and $\delta$-receptors (13). It has been recently recognized as an ORL1 receptor antagonist in rat vas deferens (9, 23) and in mouse brain (34). Third, the concentration-response relationships for nociceptin (i.e., EC$_{50}$ = 11.9 nM) were comparable to those reported earlier for various types of

Fig. 5. Increased $K^+$ conductance was associated with nociceptin-evoked membrane hyperpolarization. A: current-voltage ($I$-$V$) relations in the presence and absence of nociceptin. Decreased slope with nociceptin present in the bathing solution reflects decreased input resistance. The $I$-$V$ curves intersect at $-90$ mV, which is close to the value for the estimated $K^+$ equilibrium potential. Inset, electrophysiological record showing the response to nociceptin for the neuron from which the $I$-$V$ curves were obtained. B: morphology of the neuron from which data in A were obtained. C: relations between the holding potential in current-clamp mode and hyperpolarizing responses to nociceptin. Hyperpolarizing responses to nociceptin were increased as the membrane potential was current clamped to membrane potentials more positive than the resting potential of $-68$ mV and were decreased or abolished as the membrane potential was current clamped to membrane potentials more negative than the resting potential. D: graph of data in C showing a reversal potential of $-103$ mV. E: morphology of the neuron from which data in C and D were obtained. F: BaCl$_2$ reversibly suppressed the hyperpolarizing action of nociceptin. G: morphology of the neuron from which data in F were obtained.

Fig. 6. Nociceptin suppressed fast excitatory postsynaptic potentials (EPSPs). A: bath application of nociceptin suppressed fast EPSPs evoked by focal electrical stimulation of interganglionic connectives. Inhibitory action was unaffected by naloxone and was offset by coapplication of either NC-NH$_2$ or NBH with nociceptin. The nicotinic antagonist hexamethonium reduced fast EPSPs. B: nociceptin did not reduce the amplitude of nicotinic responses evoked by microejection of ACh in the same neuron. C: morphology of the neuron from which the results were obtained.
cellular responses to nociceptin, including increase in K^+ conductance in dorsal raphe nuclei (EC50 = 22.5 ± 4.4 nM; Ref. 31) and the locus coeruleus (EC50 = 90 nM; Ref. 6), reduction of Ca^{2+} currents (EC50 = 42 nM; Ref. 7), and inhibition of adenylate cyclase in CHO cells expressing cloned ORL1 receptors (EC_{50} = 1 nM; Refs. 20, 29).

Furthermore, the mRNA for the ORL1 receptor (27, 33) and immunoreactivity against nociceptin peptide (2, 38) were found to be expressed in the myenteric plexus. Therefore, it seems reasonable to postulate a functional role for the ORL1 receptor-nociceptin peptide system in the enteric nervous system.

Recent studies on other neuronal types have indicated that nociceptin acts to increase K^+ conductance via G protein coupling to the ORL1 receptor (6, 31, 32). In the present study, the membrane hyperpolarization and decreased input resistance in response to nociceptin suggested an increase in K^+ conductance. Findings that the hyperpolarizing action was suppressed by Ba^{2+} and that the reversal potential was near the estimated K^+ equilibrium potential support this suggestion (36). Nociceptin has been reported to act by opening K^+ channels in other neuronal types (6, 31, 32), and this appears to be the case also for enteric neurons. In this respect, the action of nociceptin is reminiscent of the hyperpolarizing action of opioid peptides on enteric neurons (22).

The hypothesis that the nociceptin receptors were G protein coupled was based on the primary structure of

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**Fig. 7.** Concentration-response relations for nociceptin-induced suppression of fast and slow EPSPs. Each point represents 4–16 neurons for fast EPSPs and 5–18 neurons for slow EPSPs. EC_{50} = 22.5 ± 4.4 nM and Hill coefficient = 0.98 for suppression of fast EPSPs. EC_{50} = 15.1 ± 1.6 nM and Hill coefficient = 1.00 for suppression of slow EPSPs.

**Fig. 8.** Nociceptin suppressed stimulus-evoked slow EPSPs. A: bath application of nociceptin suppressed slow EPSPs. Inhibitory action was unaffected by naloxone and was offset by coapplication of either NC-NH$_2$ or NBH with nociceptin. B: application of substance P or 5-hydroxytryptamine evoked a slow EPSP-like response in the neuron in A. Nociceptin did not reduce the amplitude of responses evoked by microejection of substance P or 5-hydroxytryptamine in the same neuron. C: morphology of the neuron from which data were obtained.
the ORL₁ receptor, which displays the seven membrane-spanning domains of typical G protein-coupled receptors (3, 11, 21, 35). Nociceptin is known to suppress forskolin-stimulated cAMP accumulation in transfected cells (20, 29) and to increase inwardly rectifying K⁺ conductance in amygdaloid neurons (19) via PTX-sensitive G proteins. In the present study, incubation with PTX prevented responses to nociceptin, indicative of coupling of the ORL₁ receptor to the G_{i/G}_{o} class of G proteins.

Both AH- and S-type neurons were hyperpolarized by nociceptin. Nevertheless, responses to nociceptin were quantitatively different for the two types of neurons, with the greatest proportion of responses occurring in AH neurons. Most nociceptin-responsive AH neurons had Dogiel type II morphology, whereas the nociceptin-responsive S-type neurons had either filamentous or Dogiel type I morphology. The available evidence suggests that S-type neurons with Dogiel type I morphology and NOS immunoreactivity are inhibitory motor neurons that project to circular muscle layers (5), whereas the AH neurons with Dogiel type II morphology are interneurons responsible for excitatory drive and coordination of the discharge of motor neurons to intestinal effector systems (12, 36). The intestinal circular muscle coat is under tonic influence stemming from the firing of inhibitory motor neurons, with cell bodies located in the myenteric plexus (36). AH interneurons are thought to be synthetically coupled into networks that supply excitatory drive to the inhibitory motor neurons. By suppressing firing in AH-type interneurons and/or inhibitory motor neurons, nociceptin removes inhibition from the muscle and unmasks myogenic contractile activity (36). This may be the neural basis for the elevated contractile activity of the intestinal musculature that has been reported as another action of nociceptin (26, 30, 38). Enteric neuronal involvement in nociceptin-evoked contractile responses is further suggested by susceptibility to blockade by TTX (26, 38).

Nociceptin behaved like opiates and opioid peptides (36) in its action to suppress stimulus-evoked nicotinic fast EPSPs. This appeared to be a direct action at presynaptic inhibitory receptors on cholinergic nerve terminals because nociceptin did not suppress the depolarizing action of exogenously applied acetylcholine, and the suppression of fast nicotinic EPSPs occurred in neurons without any nociceptin-induced hyperpolarization.

Nociceptin also suppressed slow EPSPs. Stimulus-evoked slow EPSPs were assumed to reflect the release of excitatory neurotransmitters from the terminals of noncholinergic neurons because muscarinic receptors were blocked by the presence of 1 μM scopolamine in the bathing solution. Substance P and 5-HT are among the putative mediators of slow EPSPs in the enteric nervous system and evoke slow EPSP-like responses when applied experimentally to enteric neurons (36). Nociceptin did not reduce the slow EPSP-like responses to exogenously applied substance P or 5-HT. This implicates presynaptic inhibition of neurotransmitter release as the likely explanation for the inhibitory action on slow synaptic excitation. Suppression of both fast and slow EPSPs by nociceptin was blocked by NC-NH₂ or NBH but not by naloxone. The finding that the membrane hyperpolarization evoked by nociceptin was also reversed by NC-NH₂ and NBH suggests that ORL₁ receptors mediate both the pre- and postsynaptic inhibitory actions of nociceptin.

In summary, we found two distinct actions of nociceptin on guinea pig myenteric neurons. One action was direct membrane hyperpolarization and suppression of neuronal excitability at the level of the ganglion cell somas. The second action was presynaptic inhibition of neurotransmitter release at fast and slow excitatory synapses. These actions involve activation of G protein-coupled ORL₁ receptors that are distinct from naltrexone-sensitive opioid receptors. The widespread pre- and postsynaptic actions of nociceptin that were observed in this study suggest that this peptidergic system could play an important role as a neuromodulator in the enteric nervous system.

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