Nociceptin Inhibits T-Type Ca^{2+} Channel Current in Rat Sensory Neurons by a G-Protein-Independent Mechanism

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Nociceptin (orphanin FQ) is a novel, opioid-like, heptadecapeptide that is an endogenous ligand for the opioid receptor-like (ORL_1) receptor. Unlike classical opioids, nociceptin can produce hyperalgesia when injected intracerebroventricularly into mice. Despite this, nociceptin has been reported to decrease transmitter release, activate an inwardly rectifying K^+ conductance, and suppress high-voltage-activated Ca^{2+} channel conductances (HVA g_{Ca}) in much the same way as μ -, δ -, and κ -opioids. We report an action of nociceptin that is not shared by morphine: the suppression of low-voltage-activated, transient calcium (barium) current ($I_{\text{Ba,T}}$) in acutely dissociated rat dorsal root ganglion (DRG) neurons ($\text{EC}_{50} = 100 \text{ nM}$). This effect was reflected as inhibition of bursts of action potentials that can be evoked in “medium-sized” DRG neurons. Experiments with GTP- γ -S (100 μM), GDP- β -S (2 mM), or aluminum fluoride (AlF_3)

(100 μM) in the patch pipette failed to provide evidence for G-protein involvement in nociceptin-induced $I_{\text{Ba,T}}$ suppression. By contrast, both morphine and nociceptin suppressed HVA g_{Ca} , and the latter response was affected by intracellular GTP- γ -S, GDP- β -S, and AlF_3 in ways that confirmed G-protein involvement. The selective effect of nociceptin on $I_{\text{Ba,T}}$ may therefore be relevant to understanding why its behavioral actions differ from those of other opioids. This G-protein-independent effect of the action of nociceptin may reflect a new general mechanism of action for opioid peptides within the nervous system.

Key words: LC132 receptor; neuropathic pain; sympathetic ganglion; orphan receptor; enkephalin; endorphin; spinal cord; afterdepolarization; anticonvulsant

The heptadecapeptide nociceptin (orphanin FQ) is an endogenous ligand for the opioid receptor-like (ORL_1) receptor (Meunier et al., 1995; Reinscheid et al., 1995; Nothacker et al., 1996). This substance has attracted considerable attention, because unlike conventional opioids it does not produce analgesia when injected intracerebroventricularly into mice; in fact, nociceptin produces hyperalgesia in a tail-flick assay (Meunier et al., 1995; Reinscheid et al., 1995). Despite this, the cellular actions of nociceptin appear similar to those of μ -, δ -, and κ -opioids. Opioids suppress neurotransmitter release (Jessell and Iversen, 1977; MacDonald and Nelson, 1978; Cherubini and North, 1985; Hori et al., 1992; Vaughan and Christie, 1997), and μ -, δ -, or κ -opioids attenuate high-voltage-activated Ca^{2+} channel conductances (HVA g_{Ca}) (Hescheler et al., 1987; Surprenant et al., 1990; Schroeder et al., 1991; Moises et al., 1994a,b; Womack and McCleskey, 1995) and activate an inwardly rectifying K^+ conductance (g_{KIR}) (North et al., 1987; Williams et al., 1988; Grudt and Williams, 1993). Similarly, nociceptin suppresses transmitter release in the periaqueductal gray matter (Vaughan et al., 1997) and in the superficial dorsal horn of neonatal rat spinal cord (Liebel et al., 1997). It also inhibits HVA g_{Ca} in hippocampal neurons (Knoflach et al., 1996) and in a neuroblastoma cell line (Connor et al., 1996b). Nociceptin activates g_{KIR} in dorsal raphe

neurons (Vaughan and Christie, 1996), in midbrain periaqueductal gray neurons (Vaughan et al., 1997), and in locus coeruleus neurons (Connor et al., 1996a).

The molecular mechanisms underlying opioid actions are often studied by examining their effects on g_{Ca} in the cell bodies of sensory neurons that lie in dorsal root ganglia (DRG) (Schroeder et al., 1991; Moises et al., 1994a,b; Wilding et al., 1995; Womack and McCleskey, 1995). We report here an action of nociceptin on rat DRG neurons that is not shared by the classic analgesic opioid morphine. Although both agonists suppress HVA g_{Ca} by a G-protein-dependent mechanism, only nociceptin affects the low-voltage-activated (LVA), transient I_{Ca} (T-current, $I_{\text{Ca,T}}$). This effect, which does not seem to involve G-protein activation, is reflected in the attenuation of action potential afterdepolarization and multiple spike discharge in “medium-sized” DRG cells. Whereas $I_{\text{Ca,N}}$ is normally associated with neurotransmitter release (DeWaard et al., 1996), $I_{\text{Ca,T}}$ is responsible for generating repetitive or bursting discharges, for boosting Ca^{2+} entry, and for generation of low-threshold spikes (Huguenard, 1996). The selective suppression of $I_{\text{Ca,T}}$ by nociceptin may therefore be relevant to understanding why its behavioral actions differ from those of morphine. This novel action supports the idea that certain effects of opioid peptides may be generated without the involvement of G-proteins (Twitchell and Rane, 1994; Brauneis et al., 1996).

MATERIALS AND METHODS

Male Sprague Dawley rats (180–300 gm) were decapitated in a small animal guillotine, and neurons from L_4 and L_5 DRG were dissociated using a trypsin/collagenase/DNase/trypsin inhibitor procedure as described by White et al. (1989). Dissociated cells were plated into petri dishes, superfused with various extracellular solutions at $\sim 2 \text{ ml/min}$, and used for recording within 2–10 hr. Whole-cell voltage recordings were made using an Axoclamp 1B amplifier in bridge-balance mode, and action potentials (APs) were evoked by passing 2–5 msec depolarizing

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Figure 1. Blockade of $I_{Ba,T}$ by nociceptin. *A₁*, *A₂*, Comparison of the effects of nociceptin and morphine on $I_{Ba,T}$ in the same neuron. Voltage record (step to -40 mV from a holding potential of -90 mV) omitted for clarity. Large capacity current transients accompanying voltage steps have been attenuated or removed by erasing three or more data points (clock speed for data acquisition was 0.05 msec/point). *A₁*, Superimposed records to show almost complete suppression of $I_{Ba,T}$ by $1 \mu\text{M}$ nociceptin. *A₂*, In the same cell, $1 \mu\text{M}$ morphine suppresses the current by $<10\%$. *B*, Superimposed records obtained from another cell to show suppression of $I_{Ba,T}$ tails by $1 \mu\text{M}$ nociceptin. Tails recorded at -60 mV after brief 30 msec step to -40 mV from a holding potential of -90 mV (voltage record omitted for clarity). *C*, I - V relationships for I_{Ba} recorded in the absence and presence of $1 \mu\text{M}$ nociceptin. Asterisk marks characteristic shoulder on I - V relationship that results from $I_{Ba,T}$. Note attenuation of current at -40 mV ($I_{Ba,T}$) and -10 mV (attenuation of HVA I_{Ba}) by nociceptin. *D*, I - V relationships for I_{Ba} recorded in the absence and presence of $1 \mu\text{M}$ morphine. Note lack of attenuation of current at potentials more negative than -40 mV ($I_{Ba,T}$, marked by asterisk) and attenuation of HVA I_{Ba} recorded at potentials positive to -20 mV. *E*, Log-concentration-response relationship for nociceptin. Numbers on graph indicate numbers of cells tested with each concentration; error bars, which indicate SEM, are smaller than data markers in some cases. The concentration of nociceptin required for half-maximum suppression of $I_{Ba,T}$ (EC_{50}) is 100 nM. *F*, Data from *E* replotted as a Hill plot [$\log_{10} R/R_m$ vs $\log_{10}$ nociceptin concentration $\div EC_{50}$; where R is amplitude of response and R_m is the maximum response that can be obtained; K_D , the dissociation equilibrium constant for nociceptin and its receptor is assumed to equal the EC_{50} (100 nM)]. Linear fit to data points yields a line with a gradient of 1 (Hill coefficient of 1.0).

currents through the recording electrode. Single-electrode, discontinuous voltage-clamp mode was used to record I_{Ca} with Ba^{2+} as charge carrier (I_{Ba}). Borosilicate glass patch electrodes had DC resistances of 4–6 M Ω for AP recording or 1–3 M Ω for current recording. Because the dissociated cells were relatively small and spherical, currents of 10 nA or more could be recorded with little or no voltage control problems. I_{Ba} tails recorded at -40 mV were described by a single exponential function. Because Ba^{2+} was used as a charge carrier, the slow tail currents that result from activation of Ca^{2+} -dependent Cl^- conductances (g_{CLCa}) (Mayer, 1985) were not observed. A P/4 leak subtraction paradigm was used when necessary.

Under bridge-balance current-clamp, input capacitance (C_{in}) was calculated from the input resistance (R_{in}) and the membrane time constant (τ_m) using the equation $\tau_m = C_{in}R_{in}$. Under voltage clamp, C_{in} was measured by integrating the area of capacitive current transients that were generated by 10 mV commands (ΔV). This yielded the charge Q that is related to C_{in} by $Q = VC_{in}$. DRG neurons were classified into three groups according to their size and AP shape. “Large” cells were defined as those with AP duration <3 msec and $C_{in} >90$ pF; “medium” cells had an AP duration of 3–5 msec and C_{in} of 70–90 pF; and “small” cells had an AP duration >5 msec and $C_{in} <70$ pF. Both medium and small cells exhibited a “hump” or “shoulder” on the falling phase of their AP (Abdulla and Smith, 1997). Spike duration (AP width) was measured at half-maximum amplitude. Because T-currents ($I_{Ba,T}$) were encountered most frequently in the medium-sized cells (Scroggs and Fox, 1992), all experiments were performed on cells within the 70–90 pF size range, 40% of which had a robust $I_{Ba,T}$.

Only cells with “giant” $I_{Ba,T}$ (>1 nA) (Rusin and Moises, 1995) were selected for analysis.

For AP recording, external solution contained (in mM): 150 NaCl, 5 KCl, 2.5 CaCl_2 , 1 MgCl_2 , 10 HEPES-NaOH, pH 7.4, and 10 D-glucose; osmolarity, 330–340 mOsm. Internal solution contained (in mM): 130 potassium gluconate, 2 Mg-ATP, 0.3 Na-GTP, 11 EGTA, 10 HEPES-KOH, pH 7.2, and 1 CaCl_2 ; osmolarity, 310–320 mOsm. For recording LVA and HVA I_{Ba} , external solution contained (in mM): 160 TEA-Cl, 10 HEPES, 2 BaCl_2 , 10 glucose, and 200 nM TTX, adjusted to pH 7.4 with TEA-OH; internal solution contained (in mM): 120 CsCl₂, 5 Mg-ATP, 0.4 Na-GTP, 10 EGTA, 20 HEPES-CsOH, pH 7.2. Except where stated otherwise, $I_{Ba,T}$ was recorded in response to a command to -40 mV, and HVA currents (N, L, P, and presumptive Q types) (Rusin and Moises, 1995, DeWaard et al., 1996) were activated by commands to -10 mV. The holding potential (V_h) for voltage-clamp experiments was -90 mV.

Drugs were applied by bath superfusion using a tap system that had an exchange time of ~ 90 sec. Nociceptin (rat or human; Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln) was from Peptide Institute Inc. (Louisville, KY); naloxone hydrochloride, naloxone benzoylhydrazone (nalbuphine), and guanosine-5'-O-(3-thiodiphosphate) (GTP- γ -S; trithium salt) were from Research Biochemical International (Natick, MA), and guanosine-5'-O-(2-thiodiphosphate) (GDP- β -S; trithium salt) was from Calbiochem (San Diego, CA) or from RBI. All other chemicals were from Sigma (St. Louis, MO).

Data were acquired and analyzed using Pclamp software (version

Figure 2. Effects of nociceptin and morphine on HVA g_{Ba} . A_1 , B_1 , Currents recorded in response to a step from -90 to -10 mV in the presence and absence of $1 \mu\text{M}$ nociceptin or $1 \mu\text{M}$ morphine. Note suppression of current and slowed rate of activation in the presence of agonists. A_2 , B_2 , Removal of suppression of HVA g_{Ba} induced by $1 \mu\text{M}$ nociceptin or $1 \mu\text{M}$ morphine with depolarizing prepulses to $+100$ mV. Current responses to depolarizing commands are off scale; $2 \text{ nA}/20 \text{ msec}$ calibration in A_1 refers also to records in B_1 , and $2 \text{ nA}/40 \text{ msec}$ calibration in A_2 refers also to records in B_2 . Records in A_1 and A_2 from the same cell and records in B_1 and B_2 from another cell.

5.5.1), and final figures, except for Figure 6A, were produced using Origin 4.10' (Microcal).

RESULTS

Suppression of T-current by nociceptin

Nociceptin (0.01 – $1.0 \mu\text{M}$) suppressed $I_{Ba,T}$ in all cells tested ($n > 100$). Figure 1A₁ illustrates almost complete suppression of $I_{Ba,T}$ by $1 \mu\text{M}$ nociceptin in a medium-sized DRG neuron. An equal concentration of morphine produced only a small ($<10\%$) suppression of $I_{Ba,T}$ in the same cell (Fig. 1A₂). Morphine (up to $10 \mu\text{M}$) produced no more than 20% suppression of $I_{Ba,T}$ in 18 of 67 cells tested, and the remainder (49) were unaffected. Nociceptin-induced inhibition was reflected in attenuation of $I_{Ba,T}$ tails recorded at -60 mV (Fig. 1B).

The preferential blockade of $I_{Ba,T}$ by nociceptin is illustrated further by the current–voltage (I – V) curves shown in Figure 1C,D. The presence of $I_{Ba,T}$ is seen as an inflection on the descending part of the curve between -50 and -25 mV (Fox et al., 1987). This inflection is attenuated in the presence of nociceptin (Fig. 1C) but not in the presence of morphine (Fig. 1D). At more positive voltages, N-, L-, P-, and presumptive Q-type conductances are activated (Fox et al., 1987; Rusin and Moises, 1995). Components of these HVA conductances were attenuated by both nociceptin and morphine. This is clear from the I – V curves shown in Figure 1C,D and from the data records in Figure 2A₁,B₁.

The EC_{50} for nociceptin-induced suppression of $I_{Ba,T}$ from the log-concentration–response relationship (Fig. 1E) was 100 nM with a Hill coefficient of 1.0 (Fig. 1F). Nociceptin ($1 \mu\text{M}$) completely blocked $I_{Ba,T}$ in 22 of 77 cells tested. This contrasted with the actions of both nociceptin and morphine on HVA conductances, in which suppression rarely exceeded 70% .

Figure 3. Pharmacology of nociceptin attenuation of T-current. Superimposed data records show (A_1) suppression of $I_{Ba,T}$ by $1 \mu\text{M}$ nociceptin, (A_2) a second response to nociceptin recorded in the same cell in the presence of $1 \mu\text{M}$ naloxone, (B_1) suppression of $I_{Ba,T}$ in another cell by $1 \mu\text{M}$ nociceptin, and (B_2) attenuation of nociceptin-induced inhibition in the second cell by $1 \mu\text{M}$ nalbuzh. $I_{Ba,T}$ evoked by a step to -40 mV from holding potential of -90 mV (voltage record omitted for clarity).

Pharmacology of DRG T-current

The current that was inhibited by nociceptin was clearly $I_{Ba,T}$, because it was almost completely blocked by Ni^{2+} ($100 \mu\text{M}$) (DeWaard et al., 1996) yet was reduced by $<25\%$ by Cd^{2+} ($100 \mu\text{M}$; $n = 4$). By contrast, HVA conductances were selectively blocked by Cd^{2+} ($100 \mu\text{M}$) and were reduced by $<25\%$ by Ni^{2+} ($100 \mu\text{M}$; $n = 4$). In another experiment, we found that $20 \mu\text{M}$ Ni^{2+} produced clear suppression of $I_{Ba,T}$, with negligible effect on HVA I_{Ba} .

$I_{Ba,T}$ was not affected by ω -conotoxin GVIA ($1 \mu\text{M}$; $n = 3$), and nifedipine ($2 \mu\text{M}$; $n = 3$) produced $<10\%$ suppression.

Ethosuximide, a drug that is used in the management of absence seizures, has been reported to inhibit $I_{Ba,T}$ in thalamic relay (Coulter et al., 1990; Huguenard and Prince, 1994) and reticular neurons (Huguenard and Prince, 1994). This substance was without effect on $I_{Ba,T}$ in DRG neurons ($500 \mu\text{M}$; $n = 2$).

Pharmacology of nociceptin-induced T-current suppression

Effects mediated via ORL_1 receptors are unaffected or relatively insensitive to inhibition by the broad spectrum μ -, δ -, and κ -opioid antagonist naloxone (Connor et al., 1996a,b; Faber et al., 1996). In agreement with this, naloxone (up to $1 \mu\text{M}$) failed to antagonize the suppression of $I_{Ba,T}$ by nociceptin ($n = 3$) (Fig. 3A₁,A₂). On the other hand, the κ_3 -agonist nalbuzh has been reported to exert an antagonist action at ORL_1 receptors (Dunnill et al., 1996). Figure 3B₂ shows that $1 \mu\text{M}$ nalbuzh almost completely prevented suppression of $I_{Ba,T}$ by $1 \mu\text{M}$ nociceptin ($n = 3$) (Fig. 3B₁).

Nalbuzh also has significant antagonist action at μ -opioid receptors (Dunnill et al., 1996), and we found that it antagonized the actions of both morphine and nociceptin on HVA I_{Ba} ($n = 4$).

Role of G-proteins

Agonist-modulation of N-type Ca^{2+} channel currents via G-proteins is associated with slowing of the activation kinetics of the

Figure 4. Evidence against G-protein involvement in the effect of nociceptin on T-currents. **A₁**, Superimposed data records to show suppression of $I_{Ba,T}$ by 100 nM nociceptin. **A₂**, Superimposed records from **A₁** normalized and replotted. Note that the two records superimpose exactly, and the presence of nociceptin does not slow the onset or the rate of inactivation of the current. **B₁**, Graph of time course of an experiment done with a pipette containing 100 μ M GTP- γ -S. Points represent amplitudes of $I_{Ba,T}$ or HVA I_{Ba} evoked by a double-pulse protocol applied once every 20 sec. Both $I_{Ba,T}$ and HVA I_{Ba} are suppressed by 1 μ M nociceptin. Although $I_{Ba,T}$ returns to control amplitude after nociceptin washout, HVA I_{Ba} remains suppressed throughout the course of the experiment. The cell had been left to stabilize with intracellularly applied GTP- γ -S for 15 min before the application of nociceptin. **B₂**, Original superimposed data record collected before, during, and after application of nociceptin in the experiment plotted in **B₁**. The cell was stepped from -90 to -40 mV to activate $g_{Ba,T}$ and then to -10 mV to activate HVA g_{Ba} . Nociceptin invokes reversible suppression of the former and irreversible suppression of the latter.

current as the channels are changed from a “willing” to a “reluctant” state (Bean, 1989; Dolphin, 1996). This type of kinetic slowing has been reported for the action of nociceptin on HVA I_{Ca} in hippocampal neurons (Knoflach et al., 1996). An obvious increase in the time constant for activation of HVA I_{Ba} is seen in the presence of morphine or nociceptin in Figure 2A₁, B₁. Suppression of HVA g_{Ba} was relieved by 75 msec depolarizing prepulses to $+100$ mV (Fig. 2A₂, B₂). This type of facilitation, which is also characteristic of G-protein-coupled responses (Dolphin, 1996), was reported previously by Womack and McCleskey (1995) for the action of the μ -opioid selective agonist PLO 17 (Tyr-Pro-N-Me-Phe-D-Pro-NH₂) on HVA I_{Ba} in DRG.

The inactivation kinetics of $g_{Ba,T}$ prevented us from testing whether nociceptin suppression was relieved by a large depolarizing prepulse. There was no alteration, however, in the rate of activation of $g_{Ba,T}$ when it was recorded in the presence of nociceptin. Figure 4A₁ illustrates moderate suppression of $I_{Ba,T}$ by 100 nM nociceptin. The data records were normalized to the same amplitude and replotted in Figure 4A₂. The two records superimpose exactly, indicating that the rate of onset of the current, as well as the rate of inactivation at -40 mV, is unchanged by nociceptin. This raised the possibility that its actions involve a novel, G-protein-independent mechanism.

This possibility was tested by substituting 100 μ M GTP- γ -S for the GTP that was normally included in the pipette solution. If the action of nociceptin on $I_{Ba,T}$ did not involve a G-protein, it should produce reversible suppression of the current in the presence of GTP- γ -S (Moises et al., 1994a). This seemed to be the case ($n = 5$). We took advantage of the use of HVA I_{Ba} as an internal

positive control to demonstrate inhibition of G-protein inactivation by GTP- γ -S. A double-step protocol from -90 mV was used to activate $g_{Ba,T}$ at -40 mV and then to activate HVA g_{Ba} at -10 mV. Figure 4B₁ shows data from a cell studied with 100 μ M GTP- γ -S. The amplitudes of $I_{Ba,T}$ responses and HVA I_{Ba} responses evoked once every 20 sec are plotted against time. Although the nociceptin-induced suppression of HVA g_{Ba} was irreversible, as would be expected for a G-protein-coupled response, complete recovery of nociceptin suppression of $I_{Ba,T}$ was observed. Superimposed data records obtained before, during, and after nociceptin application are shown in Figure 4B₂. $I_{Ba,T}$ recovers after nociceptin has been washed out, but HVA I_{Ba} does not. It may be noticed that nociceptin does not seem to invoke kinetic slowing of HVA I_{Ba} in Figure 4B₂. This may reflect the presence of GTP- γ -S in the patch pipette.

GDP- β -S irreversibly inactivates G-proteins (Burch and Axelrod, 1987). As might be expected, replacement of GTP in the patch pipette with 2 mM GDP- β -S prevented modulation of HVA g_{Ba} by nociceptin. $I_{Ba,T}$ suppression in the same cell, at the same time, was unaffected ($n = 4$). A typical experiment, using the double-pulse protocol to sequentially activate $g_{Ba,T}$ and HVA g_{Ba} , is illustrated in Figure 5A. The superimposed records show that nociceptin suppresses $I_{Ba,T}$ but has no effect on HVA conductances.

G-protein function can also be modified radically by AIF₃. This substance acts as the AIF₄⁻ anion, which is an analog of the γ -phosphate of GTP (Bigay et al., 1987). AIF₃ therefore strongly activates G-proteins and thereby occludes the response of their downstream targets to agonists. In our experiments, HVA g_{Ba}

Figure 5. Further evidence against G-protein involvement in the effect of nociceptin on T-currents. *A*, Effect of 1 μ M nociceptin on $I_{Ba,T}$ and HVA I_{Ba} in the same cell studied with 2 mM GDP- β -S in the pipette. The cell had been allowed to equilibrate with intracellularly applied GDP- β -S for 15 min before the application of nociceptin. Superimposed records acquired before and during nociceptin application show that $I_{Ba,T}$ was suppressed when HVA I_{Ba} was unaffected. *B*, An experiment similar to that of *A* but using AlF_3 (100 μ M) instead of GDP- β -S in the pipette. The cell had been allowed to equilibrate with intracellularly applied AlF_3 for 20 min before the application of 1 μ M nociceptin. Superimposed records acquired before, during, and after nociceptin application show that $I_{Ba,T}$ was reversibly suppressed when HVA I_{Ba} was unaffected. Voltage protocol in *B* applies also to experiment illustrated in *A*.

decreased to $\sim 20\%$ of control after ~ 20 min when 100 μ M AlF_3 was included in the patch pipette. $I_{Ba,T}$ was relatively stable under these conditions and was still susceptible to reversible suppression by nociceptin ($n = 4$). A typical experiment is illustrated in Figure 5*B*. The superimposed records show that nociceptin reversibly suppresses $I_{Ba,T}$ when HVA conductances in the same cell are refractory to modulation by G-protein-coupled agonists.

Biophysical aspects of the action of nociceptin

Inhibition of $I_{Ba,T}$ by nociceptin did not exhibit "use-dependence." Because the time constant for removal of $I_{Ba,T}$ inactivation at -90 mV is ~ 1 sec (Carbone and Lux, 1987), we were unable to repeatedly activate the current, even with 30 msec pulses, at frequencies above 0.4 Hz. These brief pulses were used to limit the amount of inactivation that developed at -40 mV, so that less inactivation had to be removed between the pulses at -90 mV. When nociceptin was applied while $I_{Ba,T}$ was repeatedly evoked at 0.4 Hz, the current attained a reduced steady-state amplitude within 3 min of initiating nociceptin superfusion.

When nociceptin was washed out and reapplied in the absence of channel opening, a similar rate and extent of depression of $I_{Ba,T}$ was seen ($n = 4$). The block was therefore not use-dependent. In the experiment illustrated in Figure 6*A*, the downward deflections are low-speed chart recordings of successive current responses ($I_{Ba,T}$) to 30 msec pulses to -40 mV (from $V_h = -90$ mV) delivered at 0.4 Hz. The brief $I_{Ba,T}$ responses and the associated tail and capacitive currents cannot be distinguished on the chart. The progressive decrease in amplitude of the deflections reflects suppression of the current by nociceptin. When nociceptin is reapplied in the absence of repeated $I_{Ba,T}$ responses, the same amount of block still occurs at about the same rate.

In other experiments, in which both $I_{Ba,T}$ and HVA I_{Ba} were recorded, there seemed to be a slight delay in the onset of block of HVA I_{Ba} compared with $I_{Ba,T}$.

We were also able to exclude an alteration in steady-state inactivation as the mechanism for a nociceptin-induced decrease in $I_{Ba,T}$. Figure 6*B* shows the steady-state inactivation curve for $I_{Ba,T}$; the current was evoked at -40 mV following steps from various prepulse potentials. The voltage protocol is shown as an inset to the graph. Maximal $I_{Ba,T}$ was evoked from holding potentials exceeding -105 mV, and currents evoked from different prepulse potentials were normalized to this maximal value. In the presence of 100 nM nociceptin, which reduced $I_{Ba,T}$ recorded at 40 mV by 36%, there was no obvious change in the normalized steady-state inactivation curve. Original data records are shown in Figure 5*C*₁, *C*₂. Similar effects were seen in a total of five cells.

Effect of nociceptin on action potentials and burst discharges

$I_{Ca,T}$ plays a role in the production of burst discharges in various neuronal types (Huguenard, 1996), and it has been reported to underlie an afterdepolarization that follows a single AP in some DRG neurons (White et al., 1989). This afterdepolarization gives rise to a burst of two or more successive APs in response to a single brief stimulus. Typical current-clamp recordings from a cell of this type are shown in Figure 7. The afterdepolarization and associated second and third spikes were attenuated by 1 μ M nociceptin ($n = 4$) (Fig. 7*A*) but not by 1 μ M morphine ($n = 4$) (Fig. 7*B*). Figure 7*C* illustrates suppression of the afterdepolarization by Ni^{2+} (100 μ M; $n = 4$), a result consistent with the involvement of $I_{Ca,T}$ in its generation (White et al., 1989).

DISCUSSION

We find that the novel opioid peptide nociceptin selectively inhibits $I_{Ba,T}$ in medium-sized DRG neurons by a G-protein-independent mechanism. This effect is reflected in impairment of the ability of the neurons to generate bursts of APs in response to a brief excitatory stimulus.

Unlike HVA g_{Ba} , which is subject to regulation by a wide variety of neuromodulators that act through heptahelical, G-protein-coupled receptors (Dolphin, 1996), very few agonists have been reported to modulate $I_{Ba,T}$ (Kobrin et al., 1994; Huguenard, 1996). Although $I_{Ba,T}$ in sensory neurons is decreased when G-proteins are activated with GTP- γ -S (Gross et al., 1990), this mechanism does not seem to be involved in the action of nociceptin. Because the G-protein-mediated effects of opioid agonists on HVA g_{Ba} are well characterized in DRG neurons (Moises et al., 1994a,b; Wilding et al., 1995; Womack and McCleskey, 1995), each cell that was studied served as its own control. This allowed us to convincingly exclude a role for G-proteins, because nociceptin attenuated $I_{Ba,T}$ after its effects on

Figure 6. Biophysical aspects of nociceptin-induced T-current suppression. **A**, Slow speed chart record to show rate of onset and the effect of nociceptin with and without repeated activation of $g_{\text{Ba,T}}$. **Top record** is current, and **bottom record** is voltage. Deflections result from brief (30 msec) voltage commands to -40 mV from the holding potential of -90 mV applied once every 2.5 sec (0.4 Hz). Nociceptin ($1 \mu\text{M}$) was first applied (at *left*) while $g_{\text{Ba,T}}$ was repeatedly activated. The current deflections attain a smaller steady-state amplitude 3 min after initiating nociceptin superfusion. Nociceptin was reapplied (at *right*) in the absence of $g_{\text{Ba,T}}$ activation. Voltage commands were recommenced 3 min after initiating nociceptin superfusion, and $I_{\text{Ba,T}}$ responses were attenuated to the same extent as those recorded when the conductance had been activated repetitively in the presence of nociceptin. **B**, Normalized steady-state inactivation plots for $g_{\text{Ba,T}}$ acquired before and during application of 100 nM nociceptin. Cell was held at a series of prepulse potentials ($+5 \text{ mV}$ increments from -115 mV) for 200 msec before activating $g_{\text{Ba,T}}$ at -40 mV . Voltage protocol is shown as *inset*. **C₁**, **C₂**, Original data records used to produce the data in **B**. A split-clock protocol was used so that 100 msec calibration refers to prepulse and 25 msec calibration refers to recordings of $I_{\text{Ba,T}}$.

HVA g_{Ba} were compromised with GDP- β -S or AlF_3 (Fig. 5). Similarly, in cells in which G-protein-coupled effects of nociceptin on HVA g_{Ba} were rendered irreversible by the inclusion of GTP- γ -S in the pipette, nociceptin invoked a reversible suppression of $I_{\text{Ba,T}}$ (Fig. 4B). The fact that nociceptin clearly altered the activation kinetics of HVA g_{Ba} (Fig. 2A) yet failed to alter that of $g_{\text{Ba,T}}$ (Fig. 4A) may also be consistent with modulation of T-type Ca^{2+} channels by a G-protein-independent mechanism (but see Kobrinsky et al., 1994).

Nociceptin was identified as an endogenous opioid agonist for the heptahelical ORL₁ receptor (Meunier et al., 1995; Reinscheid et al., 1995; Nothacker et al., 1996), and Connor et al. (1996b) showed that its effects on Ca^{2+} channel currents and intracellular Ca^{2+} accumulation in neuroblastoma cells are blocked by pertussis toxin. Because the effects of nociceptin on $I_{\text{Ba,T}}$ in DRG neurons do not seem to involve G-proteins, this raises the possibility that they may not be mediated via the heptahelical ORL₁ receptor. Although the effect of nociceptin is antagonized by nalbuzoh (Fig. 2B) (Dunnill et al., 1996), the selectivity of this antagonist is yet to be established. On the other hand, the EC_{50} for the action of nociceptin on $I_{\text{Ba,T}}$ of 100 nM is comparable with that for its presumed actions at ORL₁ receptors ($22\text{--}90 \text{ nM}$) (Connor et al., 1996a,b; Vaughan and Christie, 1996; Vaughan et al., 1997). In our opinion, neither the effect of nalbuzoh nor the similarities in EC_{50} values allow the unequivocal implication or exclusion of a role for ORL₁ in the actions of nociceptin on $I_{\text{Ba,T}}$. One remote possibility is that the activated ORL₁ receptor interacts directly with the T-type Ca^{2+} channel without the intervention of a G-protein.

Schroeder et al. (1991) reported that $g_{\text{Ca,T}}$ in sensory neurons is attenuated by the μ -receptor selective agonist D-Ala², N-Me-Phe⁴-Gly⁵-ol enkephalin (DAMGO). It is therefore possible that

only certain opioid peptides attenuate $I_{\text{Ba,T}}$ and that this action is not shared by other opioids, including PLO17 (Moises et al., 1994b) and morphine. It therefore would be of interest to determine whether the action of DAMGO on T-channel currents described by Schroeder et al. (1991) is G-protein-mediated and whether it is blocked by naloxone. This antagonist was ineffective in antagonizing the action of nociceptin on $I_{\text{Ba,T}}$ (Fig. 2A). Two other reports of effects of opioid peptides that do not seem to involve G-proteins have already appeared. Twitchell and Rane (1994) were unable to implicate G-proteins in the potentiation of Ca^{2+} -sensitive K^+ current by the μ -selective agonist DAMGO, and Brauneis et al. (1996) showed that dynorphin inhibited currents through recombinant NMDA channels expressed in *Xenopus* oocytes that did not express opioid receptors. Perhaps G-protein-independent actions contribute to behavioral effects seen with opioid peptides in whole animals. Thus, suppression of $I_{\text{Ca,T}}$ by nociceptin may contribute to the hyperalgesic effect seen in tail-flick tests when it is administered intracerebroventricularly to mice (Meunier et al., 1995; Reinscheid et al., 1995).

Little can be said, at this stage, about the molecular mechanism of the action of nociceptin. Because effects on steady-state inactivation and effects mediated via G-proteins have been excluded, nociceptin may interact directly with the T-type Ca^{2+} channel. Because the EC_{50} is 100 nM , this would predict a residency time (T) of $\sim 100 \text{ msec}$ on its receptor site. This was estimated by assuming a K_D of 10^{-7} that is equal to the EC_{50} and using the approximation that $T = 10^{-8}/K_D$ (Hille, 1992). A relatively long residency time suggests that nociceptin would not produce a "flickering" type of open-channel block, and this is consistent with the observation that it did not affect the rate of activation or inactivation of $I_{\text{Ba,T}}$ (Fig. 4A₂). Direct interaction of nociceptin with T-type Ca^{2+} channels could therefore involve "closed-

Figure 7. Effects of nociceptin, morphine, and Ni^{2+} on APs, bursting activity, and afterdepolarizations. All records were obtained from the same cell. APs were evoked from a 5 msec/0.3 nA depolarizing current command applied via the recording electrode. Superimposed records of bursts of APs were recorded before and during superfusion of (A) 1 μM nociceptin, (B) 1 μM morphine, and (C) 100 μM Ni^{2+} . Note that afterdepolarization and burst of spikes are attenuated by nociceptin and Ni^{2+} but not by morphine. Calibration (40 mV/40 msec) in B and current trace in C apply to all records.

channel block," as has been proposed for ethosuximide block of thalamic channels (Coulter et al., 1991), or a very rapidly developing block of open channels in which the peptide would enter each channel as soon as it opens and prevent the passage of ions. In either case, no use-dependence would be observed.

Activation of a receptor tyrosine kinase is another plausible, G-protein-independent mechanism for the action of a peptide such as nociceptin. The possible relevance of this mechanism derives from the observation that HVA I_{Ca} in molluscan neurons is enhanced within minutes of application of mammalian nerve growth factor (Wilderling et al., 1995). Also, peptides such as angiotensin II and vasopressin can act via tyrosine kinase pathways to cause contraction of certain types of smooth muscle (Hollenberg, 1994). A third possibility is that nociceptin exerts its effects via activation of membrane-bound guanylyl cyclase in the same way as atrial natriuretic factor (Fenrick et al., 1994). These and other possible mechanisms could be readily examined in future work; it may be that the preservation of a nociceptin

response in the presence of GTP analogs and AlF_3 precludes a role for the direct activation of guanylyl cyclase.

The observation that $I_{\text{Ba,T}}$ in DRG is blocked by nociceptin but not by ethosuximide may reflect differences in the properties of the T-type Ca^{2+} channels in thalamic neurons compared with sensory neurons. This goes along with the idea that several subtypes of T-channels exist in neurons (Kobrin et al., 1994; Huguenard, 1996).

Because $I_{\text{Ca,T}}$ is responsible for generating repetitive or bursting discharges, for boosting Ca^{2+} entry, and for low-threshold spike generation (Huguenard, 1996), it can contribute to convulsant activity in central neurons. The medium-sized DRG neurons used in the present study also tend to exhibit short bursts of 2–4 APs in response to a brief depolarizing stimulus. This burst of APs originates from an afterdepolarization that follows the first AP. Our results (Fig. 7C) with the T-channel blocker Ni^{2+} (DeWaard et al., 1996) support the hypothesis that $I_{\text{Ca,T}}$ underlies the generation of this afterdepolarization (White et al., 1989). Because nociceptin binding sites have been detected in various CNS regions, including cortex and thalamus (Sim et al., 1996), the observation that nociceptin blocks both $I_{\text{Ba,T}}$ and bursting activity (Fig. 7) raises the possibility that it may be an endogenous anticonvulsant. Because nociceptin blocks $I_{\text{Ba,T}}$ in a cell type in which ethosuximide is ineffective, the development of appropriate nociceptin analogs may provide a logical route for the development of new anticonvulsant therapies.

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