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Nociceptin receptor-mediated Ca^{2+} channel inhibition and its desensitization in NG108-15 cells

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Abstract

It has been shown that the membrane of hybrid NG108-15 neuroblastoma × glioma cells contains a high-affinity binding site for nociceptin. In the present study, we first demonstrated the expression of nociceptin receptor mRNA in NG108-15 cells. Application of nociceptin to NG108-15 cells produced a concentration-dependent ($EC_{50} = 29$ nM) inhibition of Ca^{2+} channel currents in a pertussis toxin-sensitive fashion. This nociceptin-induced inhibition of Ca^{2+} channel currents was prevented in the presence of ω-conotoxin GVIA, a blocker of the N-type Ca^{2+} channel, and had both voltage-dependent and -independent components. Prolonged application of nociceptin elicited homologous desensitization of the inhibition with a time constant of 5.3 min. These results indicate that the nociceptin receptor is coupled to the N-type Ca^{2+} channel via pertussis toxin-sensitive G proteins in NG108-15 cells and that this coupling is associated with rapid and homologous desensitization.

Keywords: Nociceptin receptor; Ca^{2+} channel, N-type; Desensitization; NG108-15 cells; Patch clamp

1. Introduction

During the course of attempts to clone the cDNAs of three major types of opioid receptors (μ, δ and κ), our laboratory and others have identified a novel member of the opioid receptor family, termed ROR-C (Fukuda et al., 1994), ORL1 (Mollereau et al., 1994) or LC132 (Bunzow et al., 1994). Although it has $>50\%$ amino acid sequence homology with μ, δ and κ opioid receptors, none of the opioid receptor ligands tested bind to it with high affinity. A heptadecapeptide has been isolated from mammalian brain as an endogenous ligand for this 'orphan' receptor and was named nociceptin (Meunier et al., 1995) or orphanin FQ (Reinscheid et al., 1995). In contrast to the analgesic effect produced by opioid receptor agonists, nociceptin is reported to induce hyperalgesia when injected intracerebroventricularly (Meunier et al., 1995; Reinscheid et al., 1995). At the cellular level, however, application of nociceptin elicits similar effects as those induced by activation of opioid receptors, such as inhibition of adenylate cyclase (Meunier et al., 1995; Reinscheid et al., 1995) and voltage-gated Ca^{2+} channels (Connor et al., 1996a; Knoflach et al., 1996) or activation of inwardly rectifying K^+ channels (Vaughan and Christie, 1996; Connor et al., 1996b; Vaughan et al., 1997) and mitogen-activated protein kinase (MAPK; Fukuda et al., 1997).

NG108-15 mouse neuroblastoma × rat glioma hybrid cells possess a homogeneous population of δ-opioid receptors (Evans et al., 1992) and have been used extensively to study the cellular effects of opioids. The δ-opioid receptor is negatively coupled to adenylate cyclase and the N-type Ca^{2+} channel via pertussis toxin-sensitive G proteins in NG108-15 cells (Morikawa et al., 1995). It has been shown recently that nociceptin binds to the NG108-15 cell membrane with high affinity and inhibits adenylate cyclase in NG108-15 cells (Ma et al., 1997). In the present investigation, we demonstrate the expression of nociceptin receptor mRNA, using the rat nociceptin receptor (ROR-C) cDNA probe, and the coupling of the nociceptin receptor to the N-type Ca^{2+} channel in NG108-15 cells. We further show that this nociceptin receptor-mediated Ca^{2+} channel inhibition undergoes rapid and homologous desensitization.

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2. Materials and methods

2.1. Drugs

Nociceptin was obtained from TOCRIS COOKSON (St. Louis, MO, USA). [D-Ala^2, D-Leu^5]enkephalin (DADLE) was purchased from Peptide Institute (Osaka, Japan). [α-^{32}P]dCTP was from Amersham (Tokyo, Japan). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan), Nacalai Tesque (Kyoto, Japan) and Sigma (St. Louis, MO, USA).

2.2. Cell culture

NG108-15 cells were cultured as described previously (Morikawa et al., 1998). For electrophysiological recordings, cells were plated onto 15-mm-diameter round glass coverslips coated with 0.01% (w/v) poly-L-lysine (mol. wt. > 300 000) and differentiation was induced by incubation with 1 mM dibutyryl cyclic AMP for 5–14 days.

2.3. RNA blot hybridization analysis

Poly(A)$^+$ RNAs from NG108-15 cells and rat brain were analyzed as described previously (Fukuda et al., 1994). The hybridization probe used was a 1.2-kb *Eco*RI/*Sac*I fragment from pROR30 (Fukuda et al., 1994). The probe was labelled with [α-^{32}P]dCTP by the random primer method. Autoradiography was performed at −80°C for 6 days with an intensifying screen.

2.4. Electrophysiological recordings

A glass coverslip on which differentiated cells were grown was transferred to the recording chamber (~200 μl) and continuously perfused at 1–2 ml/min. Ba^{2+} currents (I_{Ba}) through voltage-gated Ca^{2+} channels were recorded at room temperature (22–25°C) by the whole-cell variation of the patch clamp technique, using a single-electrode voltage-clamp amplifier (Axoclamp-2B; Axon Instruments, Foster City, CA, USA). Patch pipettes had resistances of 2–4 MΩ when filled with the internal solution of the following composition: 120 mM CsCl, 20 mM tetraethylammonium chloride, 10 mM EGTA-CsOH, 2 mM Mg-ATP, 0.2 mM Na-GTP, 50 U/ml creatine phosphokinase, 20 mM Na_2 creatine phosphate and 10 mM Hepes; pH was adjusted to 7.2 with CsOH. The external solution contained 10 mM $BaCl_2$, 145 mM NaCl, 5.5 mM CsCl, 2 mM $MgCl_2$, 10 mM glucose, 0.25 μM tetrodotoxin and 10 mM Hepes; pH was adjusted to 7.4 with NaOH. Currents were low-pass filtered at 1 kHz (−3 dB) and digitized at 5 kHz. Data were stored and analysed by using pCLAMP software (Axon Instruments). Leakage and capacitative currents were subtracted by an on-line P/5 protocol.

I_{Ba} was elicited every 20 s by applying 100-ms voltage steps to 0 mV from a holding potential of −80 mV, unless otherwise stated. Correction for rundown of I_{Ba} was made as follows, assuming that the current amplitude declines linearly with time (Morikawa et al., 1998). First, I_{Ba} amplitude was plotted against time. Then, the portion of the plot before application of drugs and the portion in which the recovery after washout had reached a steady-state phase were fitted to a straight line and extrapolated to the whole plot. Finally, the original plot was divided by this straight line to obtain a plot of normalized I_{Ba} amplitude. After stabilization of I_{Ba}, which usually took ~10 min after breaking into the cell, we routinely waited another 10 min. Cells in which I_{Ba} amplitude declined > 10% during this 10-min period were not used for experiments.

2.5. Data analysis

The concentration–response relationship was fitted to a logistic function, using the Levenberg–Marquardt algorithm implemented in the ORIGIN software (Microcal Software, Northampton, MA, USA). The time course of desensitization was fitted to a single exponential function, using the Chebyshev fit method supported by the data analysis program Clampfit in pCLAMP. All data are expressed as means ± S.E.M.

3. Results

3.1. Expression of nociceptin receptor mRNA in NG108-15 cells

In order to examine the expression of nociceptin receptor mRNA in NG108-15 cells, poly(A)$^+$ RNA preparations from NG108-15 cells were subjected to blot hybridization analysis, using the ROR-C cDNA probe (Fig. 1). NG108-15 cells contained an RNA species of ~3.7 kb that hybridized with the ROR-C cDNA probe and corresponded in size to the major RNA species detected in rat cerebrum.

3.2. Nociceptin receptor-mediated inhibition of Ca^{2+} channel currents in NG108-15 cells

We next tested whether the nociceptin receptor expressed in NG108-15 cells was functionally coupled to Ca^{2+} channels. Application of nociceptin to NG108-15 cells elicited a rapid and reversible inhibition of I_{Ba} in a concentration-dependent manner (Fig. 2). Out of 98 cells tested, 71 cells responded to nociceptin. The mean inhibition of I_{Ba} produced by a maximally effective concentration of nociceptin (1 μM) was 24 ± 1% (n = 71). When the concentration–response relationship for nociceptin-induced inhibition of I_{Ba} was fitted to a logistic function, the EC_{50} value was estimated to be 29 nM (Fig. 2B).

A maximally effective concentration of DADLE (100 nM), a peptide opioid agonist, inhibited I_{Ba} in virtually all cells tested (77 out of 80 cells) with a mean inhibition of $37 \pm 2\%$. The inhibition elicited by DADLE is mediated by the δ-opioid receptor in NG108-15 cells (Morikawa et al., 1995). In the 77 cells that responded to DADLE, 1 μM nociceptin inhibited I_{Ba} in 58 cells by $23 \pm 2\%$. The ratio of the maximal inhibition produced by nociceptin and DADLE was 0.73 ± 0.06 ($n = 58$).

To confirm that the response to nociceptin was not due to activation of the δ-opioid receptor, the effect of naloxone, a nonselective opioid receptor antagonist, on nociceptin-induced inhibition of I_{Ba} was examined. The ratio of the responses to 1 μM nociceptin in the presence and absence of 100 μM naloxone in single cells was 1.00 ± 0.04 ($n = 8$), while the ratio of the responses to 100 nM DADLE in the presence and absence of 10 μM naloxone in single cells was 0.14 ± 0.07 ($n = 6$). Thus, the response to nociceptin was not affected by a 100 times higher concentration of naloxone, whereas the response to DADLE was nearly abolished by a 100 times higher concentration of naloxone.

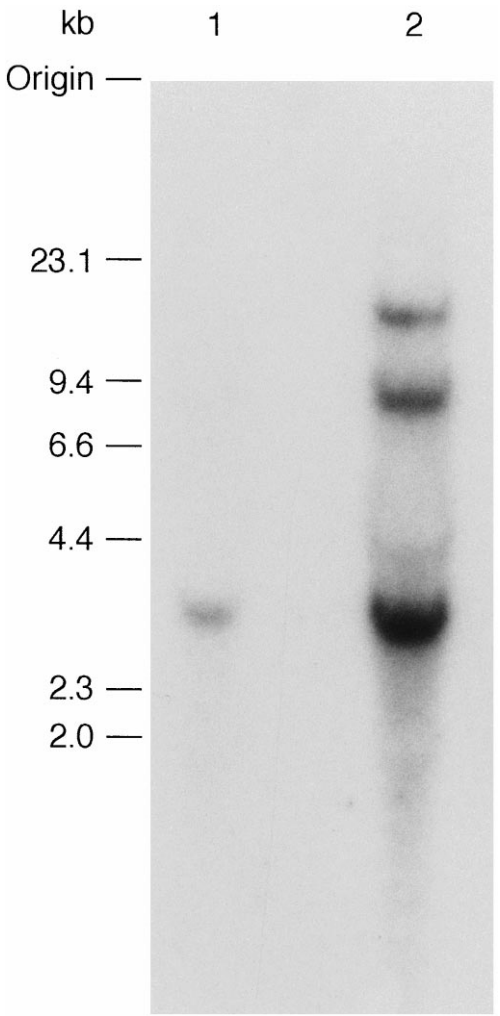

Fig. 1. RNA blot hybridization analysis of nociceptin receptor mRNA in NG108-15 cells and rat cerebrum. Poly(A)$^+$ RNA preparations (30 μg each) from NG108-15 cells (lane 1) and rat cerebrum (lane 2) were electrophoresed, blotted and hybridized with the radiolabelled ROR-C cDNA probe. The size markers are indicated on the left.

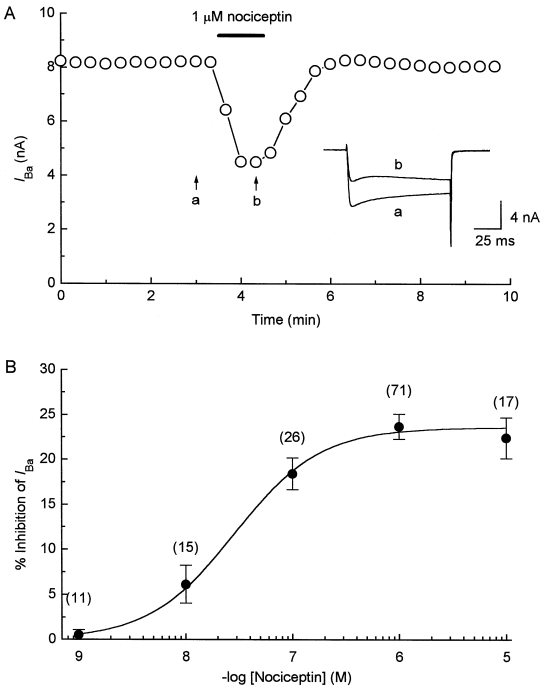

Fig. 2. Inhibition of I_{Ba} by nociceptin in NG108-15 cells. (A) Time plot of I_{Ba} amplitude vs. time. Nociceptin (1 μM) was perfused at the time indicated by the horizontal bar. Current traces at the times indicated by the arrows are shown in the inset. (B) Concentration–response relationship for the inhibition of I_{Ba} by nociceptin. Data are expressed as means ± S.E.M. for the number of cells indicated in parentheses. The smooth line represents a fit to a logistic function.

3.3. G protein involvement

To examine whether G proteins were involved in the response to nociceptin, the cells were treated overnight with 100 ng/ml pertussis toxin. In these cells, 1 μM nociceptin elicited no measurable inhibition of I_{Ba} ($n = 12$). This suggests that pertussis toxin-sensitive G proteins (G_i or G_o) mediate the effect of nociceptin.

3.4. Inhibition of the N-type Ca^{2+} channel current

While differentiated NG108-15 cells possess both L-type and N-type Ca^{2+} channels, the endogenous δ-opioid receptor couples exclusively to the N-type Ca^{2+} channel (Morikawa et al., 1995). To examine whether the nociceptin receptor couples exclusively to the N-type Ca^{2+} channel as well, we tested the effect of nifedipine, a blocker of the L-type Ca^{2+} channel, and ω-conotoxin GVIA, a blocker of the N-type Ca^{2+} channel, on nociceptin-induced inhibition of I_{Ba}. Nifedipine (10 μM) blocked I_{Ba} by $13 \pm 1\%$ in four cells. In these four cells,

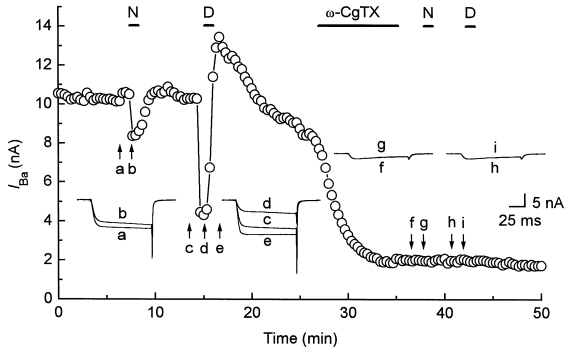

Fig. 3. Inhibition of the N-type Ca^{2+} channel current by nociceptin. I_{Ba} amplitude is plotted vs. time. Nociceptin (1 μM; N), DADLE (100 nM; D), and ω-conotoxin GVIA (1 μM; ω-CgTX) were perfused at the times indicated by the horizontal bars. Current traces at the times indicated by the arrows are shown in the inset. In this cell, a large facilitation of I_{Ba} was observed after washout of DADLE, as reported previously (Morikawa et al., 1998).

inhibition of total I_{Ba} by 1 μM nociceptin and 100 nM DADLE in the presence of nifedipine ($28 \pm 7\%$ and $28 \pm 5\%$, respectively) was comparable to that in control solution ($27 \pm 8\%$ and $27 \pm 4\%$, respectively). ω-Conotoxin GVIA (1 μM) irreversibly reduced I_{Ba} by $66 \pm 7\%$ in 5 cells. In these five cells, 1 μM nociceptin and 100 nM DADLE inhibited I_{Ba} by $19 \pm 1\%$ and $45 \pm 5\%$, respectively, before application of ω-conotoxin GVIA, whereas the responses to nociceptin and DADLE were completely abolished after treatment with ω-conotoxin GVIA (Fig. 3). Furthermore, the responses to maximally effective concentrations of nociceptin (1 μM) and DADLE (100 nM) were not additive in 10 cells tested; both drugs elicited $\geq 20\%$ inhibition (Fig. 4). These results indicate that the nociceptin receptor and the δ-opioid receptor are coupled to the same population of N-type Ca^{2+} channels.

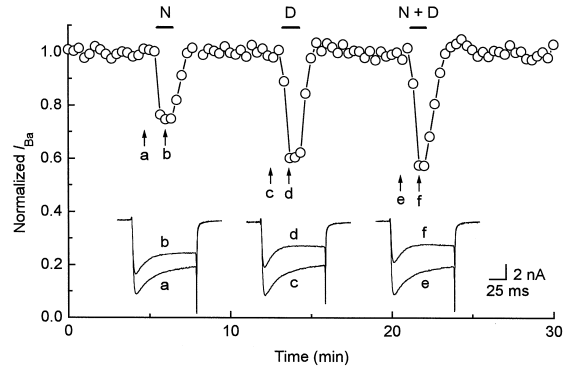

Fig. 4. Non-additivity of the effects of nociceptin and DADLE. Normalized I_{Ba} amplitude is plotted vs. time. Current amplitudes were normalized to the control amplitude after correction for rundown of I_{Ba} (see Section 2 for detail). Nociceptin (1 μM; N), DADLE (100 nM; D), and both in combination (N + D) were perfused at the times indicated by the horizontal bars. Current traces at the times indicated by the arrows are shown in the inset. In this cell, nociceptin, DADLE, and both in combination inhibited I_{Ba} by 25%, 40% and 43%, respectively.

3.5. Voltage dependence of inhibition

Inhibition of the N-type Ca^{2+} channel mediated by pertussis toxin-sensitive G proteins is known to have a voltage-dependent component, characterized by the relief of inhibition by depolarizing conditioning prepulses (prepulse facilitation; Dolphin, 1996). To assess the voltage dependence of the inhibition mediated by the nociceptin receptor, I_{Ba} was evoked with or without a depolarizing prepulse in the presence of 1 μM nociceptin. In 5 out of 10 nociceptin-responsive cells tested, I_{Ba} inhibited by nociceptin was facilitated by the prepulse and exhibited slowed activation kinetics, another hallmark of voltage-dependent G protein inhibition (Fig. 5A). In the remaining five cells, the nociceptin-induced inhibition was not relieved by the prepulse and was not associated with slowing of the activation kinetics of I_{Ba} (Fig. 5B). The DADLE-induced inhibition was relieved by the prepulse in 8 out of 10 cells tested. Furthermore, we found two cells in which the prepulse facilitated I_{Ba} in the presence of DADLE but not in the presence of nociceptin. Thus, the nociceptin receptor-mediated inhibition appears to have a smaller voltage-dependent component than the δ-opioid receptor-mediated inhibition.

3.6. Desensitization of nociceptin-induced inhibition of Ca^{2+} channel currents

DADLE-induced inhibition of I_{Ba} declines (or desensitizes) by more than 50% in minutes in NG108-15 cells (Morikawa et al., 1998). Continuous application of 1 μM nociceptin also resulted in desensitization, from an initial peak inhibition of $27 \pm 2\%$ to a sustained inhibition of $11 \pm 2\%$ ($n = 15$; Fig. 6). The magnitude and time constant of desensitization were $62 \pm 6\%$ and 5.3 ± 0.5 min, respectively ($n = 15$).

We further examined whether this nociceptin-induced desensitization was homologous or whether nociceptin could elicit heterologous desensitization of δ-opioid receptor signalling. To this end, 100 nM DADLE was applied before and immediately after 1 μM nociceptin induced steady-state desensitization (Fig. 6). The magnitude of the nociceptin-induced desensitization was $62 \pm 6\%$ in these experiments ($n = 14$), whereas the response to DADLE

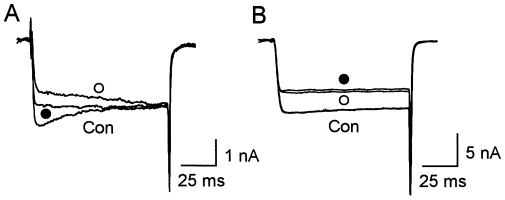

Fig. 5. Voltage dependence of nociceptin-induced I_{Ba} inhibition. Shown are I_{Ba} records in control solution (Con) and in the presence of 1 μM nociceptin with (●) or without (○) a 100-ms prepulse to +80 mV.

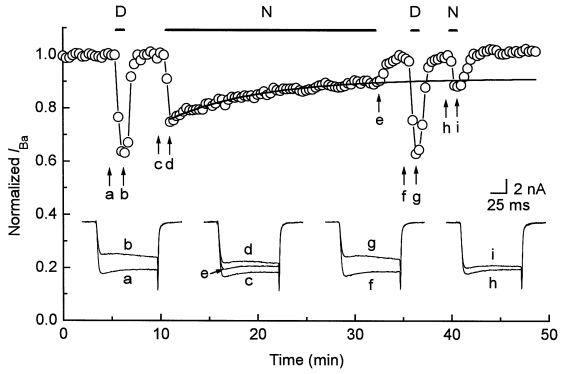

Fig. 6. Desensitization of nociceptin-induced I_{Ba} inhibition. Normalized I_{Ba} amplitude is plotted vs. time. The smooth line represents a fit to a single exponential function. Current traces at the times indicated by the arrows are shown in the inset.

following nociceptin-induced desensitization was comparable to the control response ($96 \pm 3\%$ of control, $n = 14$). We confirmed that nociceptin-induced desensitization was not reversed at the time of the second application of DADLE, since the response to nociceptin shortly after the second application of DADLE still remained largely desensitized (Fig. 6). Therefore, nociceptin was unable to cross-desensitize the δ-opioid receptor response.

4. Discussion

We detected the expression of nociceptin receptor mRNA in NG108-15 cells by RNA blot hybridization analysis, using the rat nociceptin receptor (ROR-C) cDNA probe. This strongly suggests that ROR-C or its mouse counterpart represents the high-affinity binding site for nociceptin in NG108-15 cells reported recently (Ma et al., 1997).

Nociceptin rapidly and potently inhibited Ca^{2+} channel currents in NG108-15 cells, with an EC_{50} of 29 nM. The potency of nociceptin observed here is comparable to the EC_{50} values reported for inhibition of Ca^{2+} channel currents in SH-SY5Y cells (42 nM; Connor et al., 1996a) and hippocampal pyramidal neurones (100 nM; Knoflach et al., 1996), for increasing membrane K^+ conductance in dorsal raphe nucleus neurones (12 nM; Vaughan and Christie, 1996), locus coeruleus neurones (90 nM; Connor et al., 1996b) and periaqueductal gray neurones (39 nM; Vaughan et al., 1997), and for MAPK activation in Chinese hamster ovary (CHO) cells expressing the cloned nociceptin receptor (11 nM; Fukuda et al., 1997). However, nociceptin inhibited adenylate cyclase activity more than one order of magnitude more potently in NG108-15 cells ($EC_{50} = 0.7$ nM; Ma et al., 1997) and CHO cells expressing the cloned nociceptin receptor ($EC_{50} = 1$ nM; Meunier et al., 1995; Reinscheid et al., 1995). These differences in potency might be due to differences in the transducing G proteins and/or the effectors involved (Kenakin and Morgan, 1989).

All subtypes of Ca^{2+} channels, including the L-, N- and P/Q-types, are affected by nociceptin in hippocampal neurones (Knoflach et al., 1996). However, nociceptin inhibited exclusively the N-type Ca^{2+} channel in NG108-15 cells, although NG108-15 cells express the L-type Ca^{2+} channel as well (Morikawa et al., 1995). It may be that the L-type Ca^{2+} channel expressed in NG108-15 cells is different from that in hippocampal neurones.

The nociceptin receptor and the δ-opioid receptor appeared to converge on the same population of N-type Ca^{2+} channels via pertussis toxin-sensitive G proteins. However, the maximal inhibition produced by nociceptin (24%) was considerably smaller than that produced by DADLE (37%). Furthermore, nociceptin-induced inhibition had a smaller voltage-dependent component than DADLE-induced inhibition. We also found that while virtually all cells (77 out of 80 cells) responded to DADLE, nociceptin inhibited Ca^{2+} channel currents in only 72% of cells tested (71 out of 98 cells). These results may reflect the lower expression of the nociceptin receptor (155 fmol/mg protein; Ma et al., 1997) compared to that of the δ-opioid receptor (510 fmol/mg protein; Morikawa et al., 1995) in NG108-15 cells. Alternatively, these two receptors might use different modulatory pathways for N-type Ca^{2+} channel inhibition, as has been shown for the α_2-adrenoceptor and the $GABA_B$ receptor in chick dorsal root ganglion neurones (Diversé-Pierluissi et al., 1995).

Prolonged application of nociceptin invariably caused desensitization whenever a measurable inhibition was observed. The response to 1 μM nociceptin was desensitized by 62% with a time constant of 5.3 min. The magnitude and rate of nociceptin-induced desensitization were comparable to those for the desensitization of DADLE-induced Ca^{2+} channel inhibition in NG108-15 cells, namely 57% and 4.4 min, respectively (Morikawa et al., 1998). Nociceptin-induced desensitization was homologous because nociceptin was unable to cross-desensitize the DADLE response. This is consistent with the report that nociceptin induces homologous desensitization of adenylate cyclase inhibition in NG108-15 cells (Ma et al., 1997) and of K^+ conductance activation in locus coeruleus neurones (Connor et al., 1996b).

Although nociceptin was originally reported to have a pronociceptive action (Meunier et al., 1995; Reinscheid et al., 1995), recent evidence demonstrates that nociceptin exerts antinociceptive actions in the dorsal horn of the spinal cord and medulla (Stanfa et al., 1996; Faber et al., 1996; Wang et al., 1996; Liebel et al., 1997). Furthermore, nociceptin is known to decrease transmitter release in the central and peripheral nervous systems (Giuliani and Maggi, 1996; Murphy et al., 1996; Nicol et al., 1996; Helyes et al., 1997). The Ca^{2+} channel inhibition described here may well be the underlying cellular mechanism for these inhibitory effects of nociceptin. The antinociceptive effect of spinally administered nociceptin has been shown to be short-lasting (10–20 min; Tian et al.,

1997), which could be due to the rapid desensitization of nociceptin-induced cellular responses. In the light of the recent demonstration that nociceptin is relatively resistant to enzymatic degradation (Montiel et al., 1997), which implies that endogenously released nociceptin may persist in the extracellular space for a long time, desensitization could have a role in tuning the physiological actions of nociceptin.

In conclusion, we have demonstrated that nociceptin inhibits the N-type Ca^{2+} channel current in a pertussis toxin-sensitive manner through activation of the nociceptin receptor endogenously expressed in NG108-15 cells. We have further shown that this nociceptin-induced Ca^{2+} channel inhibition undergoes rapid and homologous desensitization.

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