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SPECIAL REPORT

The effect of nociceptin on Ca^{2+} channel current and intracellular Ca^{2+} in the SH-SY5Y human neuroblastoma cell line

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The human neuroblastoma cell line SH-SY5Y expresses the 'orphan' opioid receptor (ORL1). We have demonstrated that nociceptin, the putative endogenous ligand for ORL1, produces a concentration-dependent inhibition of the N-type calcium channel current in these cells (IC_{50} 42 nM). In addition, in the presence of carbachol, nociceptin increased the intracellular concentration of Ca^{2+} (EC_{50} 60 nM). Both effects of nociceptin were blocked by pertussis toxin pretreatment but not by the opioid antagonists CTAP (1 μM), naltrindole (1 μM) and naloxone (10 μM).

Keywords: Nociceptin; ORL1; orphanin FQ; SH-SY5Y cells; calcium currents; calcium release

Introduction Nociceptin (Meunier *et al.*, 1995), also called orphanin FQ (Reinscheid *et al.*, 1995), is a putative endogenous ligand for the 'orphan' opioid receptor (ORL1) (Mollereau *et al.*, 1994). Nociceptin is a 17 amino acid peptide (Phe - Gly - Gly - Phe - Thr - Gly - Ala - Arg - Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln) which has some homology to the dynorphin family of peptides, but lacks the N terminal tyrosine essential for activity at μ, δ and κ opioid receptors. When heterologously expressed, the ORL1 receptor has a very low affinity for prototypic opioid receptor ligands. ORL1 has been identified in the human neuroblastoma cell line SH-SY5Y (Cheng *et al.*, 1995), which also expresses both μ and δ opioid receptors. We have previously demonstrated that μ and δ opioid receptor activation inhibits voltage-dependent Ca^{2+} currents (Seward *et al.*, 1990; 1991) and mobilizes intracellular Ca^{2+} (Connor & Henderson, 1996) in SH-SY5Y cells. In this study we examine the responses evoked by nociceptin in SH-SY5Y cells.

Methods The methods used for cell culture, electrophysiological recording and measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) have been described previously (Seward *et al.*, 1991; Connor & Henderson, 1996). Cells used for electrophysiological recording were differentiated by exposure to the retinoic acid analogue, 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-napthalenyl)carboxamido]benzoic acid (1 μM) for at least 6 days. Whole cell patch clamp recordings were made at room temperature from single cells continually superperfused (2–3 ml min^{-1}) with a solution containing (mM): TEACl 130, $BaCl_2$ 10, CsCl 5, HEPES 10, glucose 10, sucrose 40, pH 7.3. Recording pipettes were filled with (mM): CsCl 120, MgATP 5, Na_2GTP 0.5, BAPTA 10 and HEPES 10, pH 7.3. Ca^{2+} channel currents were elicited by stepping the membrane potential from a holding potential of −90 mV to +10 mV for 40 ms every 15 s. $[Ca^{2+}]_i$ was measured at 37°C in confluent monolayers of undifferentiated SH-SY5Y cells using the fluorescent Ca^{2+}-sensitive dye fura 2. Cells were continually perfused with a solution containing (mM): NaCl 140, KCl 2, $CaCl_2$ 2.5, $MgCl_2$ 1, HEPES 10, glucose 10, sucrose 40 and bovine serum albumin 0.05%, pH 7.3. In both types of experiment drugs were added to the bathing solution in known concentrations. Data are presented as mean ± standard error of the mean (s.e.mean).

Drugs and chemicals Bestatin, carbamylcholine chloride (carbachol), fura 2-AM, naloxone hydrochloride, pertussis toxin (PTX) and DL-thiorphan were all obtained from Sigma. 4-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-napthalenyl)-carboxamido]benzoic acid was obtained from Tocris Cookson, CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Pen-Thr-NH_2) was a kind gift from Dr Victor Hruby and nociceptin was synthesized in the Molecular Recognition Centre at the University of Bristol.

Results *Inhibition of Ca^{2+} channel currents* Under our culture conditions the Ca^{2+} channel current in SH-SY5Y cells is almost entirely N-type (Kennedy & Henderson, 1992). Nociceptin inhibited the Ca^{2+} channel current in a concentration-dependent manner (Figure 1a and c). When exposed to a maximally effective concentration of nociceptin (3 μM) the Ca^{2+} channel current was inhibited by 36 ± 6% (n = 6). The IC_{50} for nociceptin was 42 ± 13 nM. The inhibition of Ca^{2+} channel current by nociceptin was rapid in onset (maximal inhibition was achieved within 2 min of the drug entering the recording chamber), was maintained for the duration of the drug application (5 min) and was reversible within 5 min of washout of the drug. Naloxone did not affect the inhibition of the Ca^{2+} channel current by nociceptin. In 5 cells nociceptin (100 nM) inhibited the Ca^{2+} channel current by 20 ± 2%. When nociceptin was reapplied in the continued presence of naloxone (1 μM or 10 μM) the inhibition of the Ca^{2+} channel current was 24 ± 4% (in 1 μM, n = 4) and 25 ± 6% (in 10 μM, n = 3). Nociceptin inhibited the Ca^{2+} channel current through PTX-sensitive G-proteins. In cells pretreated for 16 h with PTX (200 ng ml^{-1}) application of nociceptin (300 nM) did not inhibit the Ca^{2+} channel current yet in cells not treated with PTX, nociceptin (300 nM) inhibited the Ca^{2+} channel current by 41 ± 9% (n = 3).

Elevation of intracellular Ca^{2+} Application of nociceptin (30–300 nM) alone to undifferentiated SH-SY5Y cells never altered the $[Ca^{2+}]_i$ of the cells (n = 6). When the cholinergic agonist carbachol (1 μM) was applied to the cells there was a rapid increase in $[Ca^{2+}]_i$ which declined to a plateau of elevated $[Ca^{2+}]_i$ that persisted as long as carbachol was present (Connor & Henderson, 1996). Nociceptin applied in the continued presence of carbachol evoked a further, rapid elevation of $[Ca^{2+}]_i$ above that caused by carbachol alone (Figure 1b). The response to nociceptin was not sustained but declined rapidly, even in the continued presence of the drug. The elevation of $[Ca^{2+}]_i$ was dependent on the concentration of nociceptin

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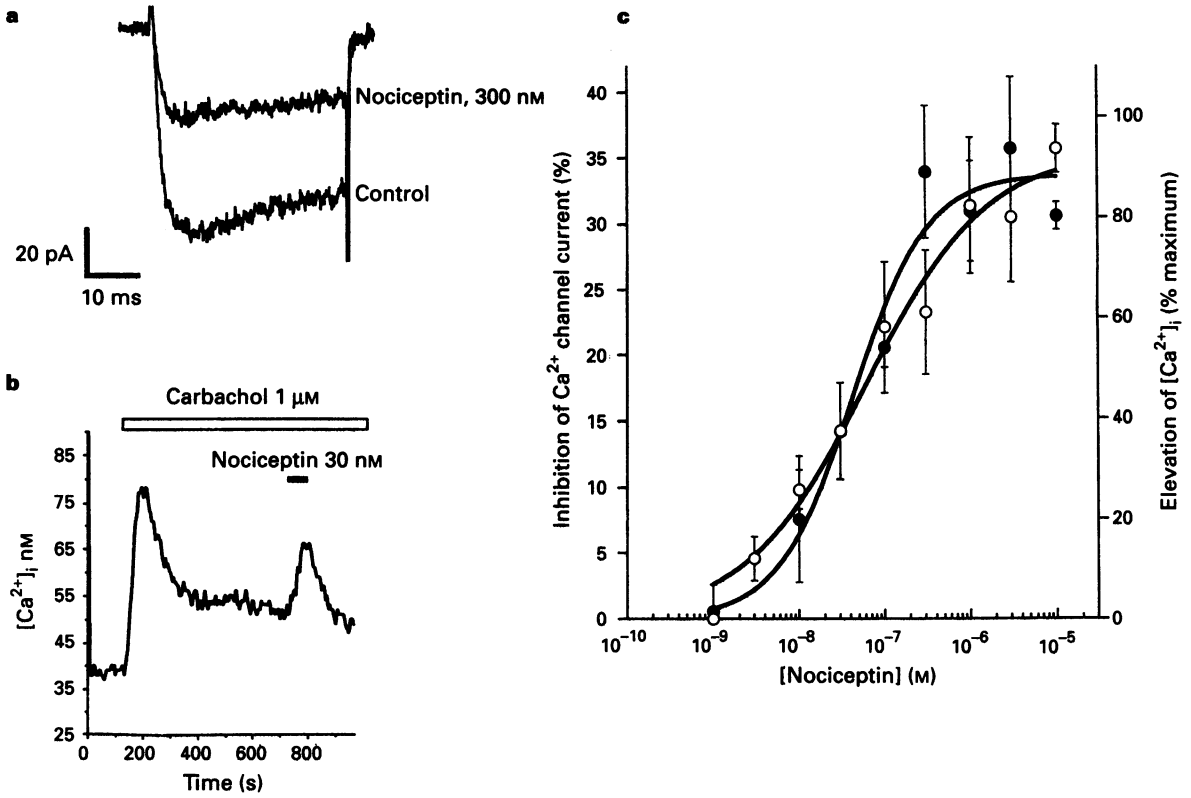

Figure 1 Nociceptin inhibited the voltage-sensitive Ca^{2+} channel current and elevated $[Ca^{2+}]_i$ in SH-SY5Y cells. (a) Superimposed Ca^{2+} channel currents recorded from a single cell in the absence and presence of nociceptin (300 nM) (see Methods for further details). (b) Nociceptin (30 nM) elevated $[Ca^{2+}]_i$ in the presence of carbachol (1 μM). (c) Concentration-response curves for the nociceptin inhibition of Ca^{2+} channel currents (●) and elevation of $[Ca^{2+}]_i$ (○). Data were pooled from a number of experiments ($n=3$ to 9 for both Ca^{2+} channel currents and the elevation of $[Ca^{2+}]_i$). In each experiment the elevation of $[Ca^{2+}]_i$ was expressed as a percentage of the maximum response. Curves were obtained by fitting the data to the Hill Equation by use of Kaleidagraph (Abelbeck Software).

(Figure 1c) and was reproducible on a given population of cells. The EC_{50} for the nociceptin elevation of $[Ca^{2+}]_i$ was 60 ± 22 nM. Addition of the peptidase inhibitors bestatin (20 μM) and thiorphan (2 μM) to the bathing medium did not affect the potency of nociceptin to elevate $[Ca^{2+}]_i$. The elevation of $[Ca^{2+}]_i$ by nociceptin in the presence of carbachol was abolished by pretreatment of the SH-SY5Y cells with PTX (200 ng ml^{-1}, 16 h; $n=4$).

To test the ability of a range of opioid antagonists to block the elevations of $[Ca^{2+}]_i$ caused by nociceptin, cells were challenged repeatedly with nociceptin (30 nM, 60 s) at 15 min intervals and then the opioid antagonists applied for at least 5 min before the second and third challenges. In control conditions the second and third nociceptin challenges caused elevations of $[Ca^{2+}]_i$ that were $113 \pm 4\%$ ($n=7$) and $105 \pm 3\%$ ($n=7$) respectively of the first challenge. The μ selective opioid antagonist CTAP (1 μM; $n=4$), the δ opioid selective antagonist naltrindole (1 μM; $n=4$) and the less selective opioid antagonist naloxone (10 μM; $n=7$) all failed to reduce the responses to nociceptin (30 nM).

Discussion The principal findings of this study are that nociceptin inhibits voltage-dependent N-type Ca^{2+} channels and, in the presence of carbachol, elevates $[Ca^{2+}]_i$ in SH-SY5Y cells. Nociceptin has a similar potency to produce these two effects and its potency was unaffected by peptidase inhibitors. Nociceptin appeared to be acting through a receptor coupled to Gi or Go proteins because the responses were blocked by PTX pretreatment. The inability of the μ opioid receptor-selective antagonist CTAP, the δ opioid receptor-selective antagonist naltrindole and the non-selective opioid antagonist naloxone to inhibit either the nociceptin modulation of the voltage-dependent Ca^{2+} channel currents or the nociceptin elevation of $[Ca^{2+}]_i$ demonstrates that nociceptin is not acting through μ or δ opioid receptors which are also present on SH-SY5Y cells. The lack of a specific antagonist at the ORL1 receptor precludes definitive identification that the responses of nociceptin are mediated through that receptor.

Receptors of the Gi- and Go-coupled superfamily, which includes α_2-adrenoceptors, μ, δ and κ opioid, $GABA_B$, D_2-dopamine and $5\text{-}HT_{1A}$ all couple to multiple effectors. The best characterized of these are inhibition of adenylyl cyclase (Childers, 1993), inhibition of Ca^{2+} channel current and activation of the inwardly rectifying potassium conductance (North, 1993). Nociceptin in now known to inhibit adenylyl cyclase (Meunier *et al.*, 1995) and to inhibit N-type Ca^{2+} channel curent (this paper); it remains to be seen if it will also activate the inwardly rectifying potassium conductance which is not expressed in SH-SY5Y cells. Recently, receptors in the Gi- and Go-coupled superfamily have been observed to release Ca^{2+} from intracellular stores in SH-SY5Y cells but only in the presence of ongoing stimulation of muscarinic, Gq-coupled receptors (Connor & Henderson, 1996). Such an elevation of $[Ca^{2+}]_i$ can also be evoked by nociceptin.

This study demonstrates that the receptors for nociceptin (presumably ORL1) couple to multiple effectors in the human neuroblastoma cell line SH-SY5Y. This cell line thus provides a valuable model system for studying the pharmacology of this novel receptor.

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